Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 3126536 C 2023/07/25

(11)(21) **3 126 536**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(22) **Date de dépôt/Filing Date:** 2015/10/14

(41) **Mise à la disp. pub./Open to Public Insp.:** 2016/04/21

(45) **Date de délivrance/Issue Date:** 2023/07/25

(62) **Demande originale/Original Application:** 2 964 317

(30) **Priorité/Priority:** 2014/10/14 (US62/063,936)

(51) **Cl.Int./Int.Cl.** ***C12N 9/78*** (2006.01),
A61K 35/12 (2015.01), ***A61K 35/14*** (2015.01),
A61K 35/17 (2015.01), ***A61K 38/47*** (2006.01),
A61K 47/50 (2017.01), ***A61K 47/54*** (2017.01),
A61K 47/56 (2017.01), ***A61K 47/60*** (2017.01),
A61P 35/00 (2006.01), ***C07K 16/28*** (2006.01),
C12N 15/55 (2006.01), ***C12N 15/86*** (2006.01),
C12N 5/10 (2006.01), ***C12N 7/01*** (2006.01),
C12N 9/26 (2006.01), ***C12N 9/96*** (2006.01)

(72) **Inventeurs/Inventors:**
THANOS, CHRISTOPHER D., US;
WANG, LIN, US;
SHEPARD, H. MICHAEL, US

(73) **Propriétaire/Owner:**

(54) **Titre : COMPOSITIONS D'ADENOSINE DEAMINASE-2, VARIANTS ET METHODES D'UTILISATION**
(54) **Title: COMPOSITIONS OF ADENOSINE DEAMINASE-2 (ADA2), VARIANTS THEREOF AND METHODS OF USING SAME**

```
Human          MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGKLVLNTKEELAN 60
Chimpanzee     MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGKLVLNTKEELAN 60
               ************************************************************

Human          ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Chimpanzee     ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
               ************************************************************

Human          MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD 180
Chimpanzee     MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRTSEKCSKWILLEDYRKRVQNVTEFDD 180
               **********************************.*************************

Human          SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Chimpanzee     SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
               ************************************************************

Human          LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
Chimpanzee     LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
               ************************************************************

Human          IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
Chimpanzee     IAESIRTAMGLRTKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKVGVKLPYFFHAGET 360
               ****** ***** ******************************** *************

Human          DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
Chimpanzee     DWQGTSIDRNILDALMLNTSRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
               ******************:***************************************

Human          LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Chimpanzee     LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
               ************************************************************

Human          KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Chimpanzee     KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
               ********************************
```

(57) **Abrégé/Abstract:**
Provided herein are variant adenosine deaminase 2 (ADA2) proteins. Also provided are ADA2 conjugates and compositions containing an ADA2 protein or conjugate. Also provided are methods and uses of the ADA2 proteins or conjugates for treating diseases and conditions, such as a tumor or cancer, and in particular any disease or condition associated with elevated adenosine or other associated marker.

CA 3126536 C 2023/07/25
(11)(21) 3 126 536
(13) C

(73) **Propriétaires(suite)/Owners(continued):**HALOZYME, INC., US
(74) **Agent:** SMART & BIGGAR LP

Abstract

Provided herein are variant adenosine deaminase 2 (ADA2) proteins. Also provided are ADA2 conjugates and compositions containing an ADA2 protein or conjugate. Also provided are methods and uses of the ADA2 proteins or conjugates for treating diseases and conditions, such as a tumor or cancer, and in particular any disease or condition associated with elevated adenosine or other associated marker.

Date Reçue/Date Received 2021-07-28

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 3
CONTENANT LES PAGES 1 À 196

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 3
CONTAINING PAGES 1 TO 196

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

COMPOSITIONS OF ADENOSINE DEAMINASE-2 (ADA2), VARIANTS THEREOF AND METHODS OF USING SAME

RELATED APPLICATIONS

This application is a division of application 2964317 filed October 14, 2015. Benefit of priority is claimed to U.S. provisional application Serial No. 62/063,936, filed October 14, 2014, to Christopher Thanos, Lin Wang and H. Michael Shepard, entitled COMPOSITIONS OF ADENOSINE DEAMINASE-2 (ADA2), VARIANTS THEREOF AND METHODS OF USING SAME.

FIELD OF THE INVENTION

Provided are variant adenosine deaminase 2 (ADA2) proteins. Also provided are ADA2 conjugates and compositions containing an ADA2 protein or ADA2 conjugate. Also provided are methods and uses of the ADA2 proteins or conjugates for treating diseases and conditions, such as a tumor or cancer, and in particular any disease or condition associated with elevated adenosine or other associated marker.

BACKGROUND

Adenosine is a well-known effector of immune function. In T-cells, adenosine decreases T-cell receptor induced activation of NF-κB, and inhibits IL-2, IL-4, and IFN-γ. Adenosine decreases T-cell cytotoxicity, increases T-cell anergy, and increases T-cell differentiation to Fop3+ or Lag-3+ regulatory (T-reg) T-cells. On NK cells, adenosine is known to decrease IFN-γ production, and suppress NK cell cytoxicity. Adenosine is known to block neutrophil adhesion and extravasation, decrease phagocytosis, and attenuate levels of superoxide and nitric oxide. Adenosine also decreases the expression of TNF-α, IL-12, and MIP-1α on macrophages, attenuates MHC Class II expression, and increases levels of IL-10 and IL-6. In addition, adenosine decreases phagocytosis and superoxide and nitric oxide levels on macrophages. Through these immune-related activities, and others, aberrant or accumulated levels of adenosine is associated with a number of diseases and conditions, including those in which the

Date Reçue/Date Received 2021-07-28

adenosine-mediated immunosuppressive effects play a role. Hence, there is a need for treatments of such diseases and conditions.

SUMMARY

Provided herein are variant Adenosine Deaminase 2 (ADA2) proteins or catalytically active portions thereof that contain a modification(s) in the sequence of amino acids of an unmodified ADA2 protein or a catalytically active portion thereof. In some embodiments, the unmodified ADA2 protein can include the sequence of amino acids set forth in SEQ ID NO:5, or a sequence of amino acids that can exhibit at least 85% sequence identity to the sequence of amino acids set forth in SEQ ID NO:5, or is a catalytically active portion thereof; the amino acid modification(s) are selected from among amino acid replacement(s), deletion(s) and insertion(s); and the variant ADA2, when in dimer form, can exhibit one or more properties selected from among increased adenosine deaminase activity, reduced heparin binding, longer serum half-life, altered pH optimum, increased thermal stability, altered receptor binding and hyperglycosylation compared to the corresponding dimer form of the unmodified ADA2 protein. A variety of amino acid modifications, including replacements, deletions and insertions are provided. It is understood that the discreet modifications that confer a particular activity or property can be combined; as in proteins effects of mutation or modifications generally are additive. Any of the variant ADA2 or catalytically active portion thereof provided herein that contains modifications, including replacements, deletions and insertions, and nucleic acids encoding the variant ADA2 or catalytically active portion thereof, can be used in any of the methods, compositions, conjugates, modified forms, vectors, cells, combinations, uses and compositions for use, and combinations for use, provided herein.

In some embodiments, the variant ADA2 protein or catalytically active portion thereof, when in dimer form, exhibits increased adenosine deaminase activity or increased adenosine deaminase activity and reduced heparin binding.

In some embodiments, the unmodified ADA2 protein is a homodimer, and the monomer form comprises the sequence of amino acid residues set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 is a catalytically active portion of the variant ADA2 protein as provided herein, wherein the unmodified ADA2 protein is a homodimer of corresponding catalytically active portions of the polypeptide whose

Date Reçue/Date Received 2021-07-28

sequence is set forth in of SEQ ID NO:5, wherein corresponding portions are determined by alignment.

In some embodiment, the ADA2 protein or catalytically active portion thereof does not contain a modification selected from among an amino acid replacement corresponding to H7R, G18A, G18R, G18V, I64T, A80D, H83Q, V90A, C108G, A120V, H121R, W133G, R125C, R140Q, K141R, R142W, P164L, P222L, W235S, H306R, E330G, W333G, V365L, Y424C, F464S or a deletion corresponding to R8-K14del→-- , with numbering with reference to amino acid residues set forth in SEQ ID NO:5.

In some embodiments, the unmodified ADA2 protein can include a sequence of amino acids that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of amino acids set forth in SEQ ID NO:5 or is a catalytically active portion thereof. For example, the unmodified ADA2 protein has at least 95% sequence identity with the sequence of amino acids set forth in SEQ ID NO:5 or with the corresponding catalytically active portion thereof. For example, the unmodified ADA2 protein includes the sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383 or is a catalytically active portion thereof, or the unmodified ADA2 protein has a sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 and 380-383 or is a catalytically active portion thereof. In particular embodiments, the unmodified ADA2 protein includes the sequence of amino acids set forth in SEQ ID NO:5 or is a catalytically active portion thereof.

In some embodiments, the catalytically active portion of the ADA2 protein can be an ADA2 protein that lacks all or a portion of the putative receptor binding (PRB) domain. For example, the catalytically active portion of the ADA2 protein can include the sequence of amino acids set forth in SEQ ID NOS:548-550. In some embodiments, the catalytically active portion of the unmodified ADA2 protein has the sequence set forth as residues 77-473 of the protein set forth in SEQ ID NO:5.

In some embodiments, the variant ADA2 protein can include up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70 or more amino

Date Reçue/Date Received 2021-07-28

acid modifications compared to the unmodified ADA2 protein. In some embodiments the variant ADA2 protein includes up to 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid modifications. In some embodiments, the variant ADA2 protein does not contain the sequence of amino acids set forth in any of SEQ ID NOS:1, 5, 68, 286-302, 326-342 or 374-383 or catalytically active fragment thereof. In some embodiments, the primary amino acid sequence of the ADA2 protein variant is not the sequence of amino acids set forth in any of SEQ ID NOS:1, 5, 68, 286-302, 326-342 or 374-383.

In some embodiments, the variant ADA2 protein, when in dimer form, exhibits adenosine deaminase activity to convert adenosine to inosine. In some embodiments herein, the variant ADA2 protein, when in dimer form, can exhibit a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 5×10^3 $M^{-1}s^{-1}$, 6×10^3 $M^{-1}s^{-1}$, 7×10^3 $M^{-1}s^{-1}$, 8×10^3 $M^{-1}s^{-1}$, 9×10^3 $M^{-1}s^{-1}$, 1×10^4 $M^{-1}s^{-1}$, 2×10^4 $M^{-1}s^{-1}$, 3×10^4 $M^{-1}s^{-1}$, 4×10^4 $M^{-1}s^{-1}$, 5×10^4 $M^{-1}s^{-1}$, 6×10^4 $M^{-1}s^{-1}$, 7×10^4 $M^{-1}s^{-1}$, 8×10^4 $M^{-1}s^{-1}$, 9×10^4 $M^{-1}s^{-1}$, 1×10^5 $M^{-1}s^{-1}$, 2×10^5 $M^{-1}s^{-1}$, 3×10^5 $M^{-1}s^{-1}$, 4×10^5 $M^{-1}s^{-1}$, 5×10^5 $M^{-1}s^{-1}$ or greater.

In some embodiments, the variant ADA2 protein, when in dimer form, can exhibit a thermal stability with a melting temperature (Tm) of at least 58°C. For example, the Tm of the ADA2 protein is at least 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 68°C, 69°C, 70°C, 71°C, 72°C or greater.

In some embodiments, the variant ADA2 protein can contain a modification(s) that is an amino acid replacement(s); and the variant ADA2 protein includes one or more amino acid replacement(s) at an amino acid position corresponding to amino acid residue 11, 13, 20, 22, 26, 86, 179, 217, 219, 221, 258, 262, 264, 266, 267, 277, 283, 296, 309, 317, 321, 352, 366, 371, 372, 373, 374, 403, 404, 405, 406, 441, 444, 452, 461, 469 or 470, with reference to amino acid positions set forth in SEQ ID NO:5. For example, the amino acid replacement(s) are positions corresponding to amino acid residue 11, 20, 219, 221, 262, 264, 366, 371, 372 or 452, with reference to amino acid positions set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 protein can include one or more amino acid replacement(s) selected from among K11A, K11D, K11E, K13A, K13D, K13E, R20A, R20D, R20E, R20N, V22S, K26A, K26D, K26E, D86A, D86C, D86E, D86F, D86G, D86H, D86I, D86K, D86L, D86M, D86N, D86P, D86Q, D86R, D86S, D86T,

Date Reçue/Date Received 2021-07-28

D86V, D86W, D86Y, E179A, E179C, E179D, E179F, E179G, E179H, E179I, E179K, E179L, E179M, E179N, E179P, E179Q, E179R, E179S, E179T, E179V, E179W, E179Y, R217A, R217D, R217E, R219A, R219C, R219D, R219E, R219F, R219G, R219H, R219I, R219K, R219L, R219M, R219N, R219P, R219Q, R219S, R219T, R219V, R219W, R219Y, L221A, L221C, L221D, L221E, L221F, L221G, L221H, L221I, L221K, L221M, L221N, L221P, L221Q, L221R, L221S, L221T, L221V, L221W, L221Y, K258A, K258D, K258E, S262A, S262C, S262D, S262E, S262F, S262G, S262H, S262I, S262K, S262L, S262M, S262N, S262P, S262Q, S262R, S262T, S262V, S262W, S262Y, H264A, H264C, H264D, H264E, H264F, H264G, H264I, H264K, H264L, H264M, H264N, H264P, H264Q, H264R, H264S, H264T, H264V, H264W, H264Y, S266A, S266C, S266D, S266E, S266F, S266G, S266H, S266I, S266K, S266L, S266M, S266N, S266P, S266Q, S266R, S266T, S266V, S266W, S266Y, K267A, K267C, K267D, K267E, K267F, K267G, K267H, K267I, K267L, K267M, K267N, K267P, K267Q, K267R, K267S, K267T, K267V, K267W, K267Y, R277A, R277D, R277E, R283A, R283D, R283E, V296A, V296C, V296D, V296E, V296F, V296G, V296H, V296I, V296K, V296L, V296M, V296N, V296P, V296Q, V296R, V296S, V296T, V296W, V296Y, K309A, K309D, K309E, K317A, K317D, K317E, K321A, K321D, K321E, R352A, R352D, R352E, R366A, R366D, R366E, K371A, K371D, K371E, K371N, K372A, K372D, K372E, K372N, D373S, I374S, T403N, G404N, H405S, P406S, R441A, R441D, R441E, K444A, K444D, K444E, K452A, K452D, K452E, K461A, K461D, K461E, K469A, K469D, K469E, K470A, K470D, K470E, with reference to amino acid positions set forth in SEQ ID NO:5. For example, the variant ADA2 protein contains one or more amino acid replacement(s) selected from among replacements corresponding to H264A; H264Q; H264N; H264G; R219K; R219Q; R219N; R219A; L221A; L221V; L221G; E179D; E179A; E179S; E179T; E179V; E179G; S262A; S262V; S262M; S262N; D86A; D86C; D86E; D86F; D86G; D86H; D86I; D86K; D86L; D86M; D86N; D86P; D86Q; D86R; D86S; D86T; D86V; D86W; D86Y; E179C; E179F; E179H; E179I; E179K; E179L; E179M; E179N; E179P; E179Q; E179R; E179W; E179Y; R219C; R219D; R219E; R219F; R219G; R219H; R219I; R219L; R219M; R219P; R219S; R219T; R219V; R219W; R219Y; L221C; L221D; L221E; L221F; L221H; L221I; L221K; L221M; L221N; L221P; L221Q; L221R; L221S; L221T; L221W; L221Y;

Date Reçue/Date Received 2021-07-28

S262C; S262D; S262E; S262F; S262G; S262H; S262I; S262K; S262L; S262P; S262Q; S262R; S262T; S262W; S262Y; H264C; H264D; H264E; H264F; H264I; H264K; H264L; H264M; H264P; H264R; H264S; H264T; H264V; H264W; H264Y; S266A; S266C; S266D; S266E; S266F; S266G; S266H; S266I; S266K; S266L; S266M; S266N; S266P; S266Q; S266R; S266T; S266V; S266W; S266Y; K267A; K267C; K267D; K267E; K267F; K267G; K267H; K267I; K267L; K267M; K267N; K267P; K267Q; K267R; K267S; K267T; K267V; K267W; K267Y; V296A; V296C; V296D; V296E; V296F; V296G; V296H; V296I; V296K; V296L; V296M; V296N; V296P; V296Q; V296R; V296S; V296T; V296W; and V296Y.

In some embodiments, the variant ADA2 protein contains an amino acid replacement at one or both of positions corresponding to amino acid residue 219 and 262, with reference to amino acid positions set forth in SEQ ID NO:5. For Example, the variant ADA2 protein or catalytically active portion thereof contains the replacement corresponding to S262N or S262Q. In some embodiments, the variant ADA2 contains the replacement corresponding to S262N. In some embodiments, the variant ADA2 contains the replacement corresponding to R219K, R219Q, R219N or R219A. In other embodiments, the variant ADA2 contains the replacement corresponding to R219Q or the replacements R219Q/R20E. In other embodiments, the variant ADA2 contains the replacement corresponding to R219Q/S262N. For example, the variant ADA2 protein or catalytically active portion thereof contains modification(s) selected from among any of R219Q/S262N/---→N1/---→A2/---→S3, R219Q/S262N/R20N/V22S, R219Q/S262N/K371N/D373S, R219Q/S262N/K372N/I374S, R219Q/S262N/T403N/H405S, R219Q/S262N/G404N/P406S, R219Q/S262N/C105-T147del→$(Gly)_{15}$, R219Q/S262N/C105-T147del→$(Gly)_{10}$, R219Q/S262N/C105-T147del→$(Gly)_7$, R219Q/S262N/C105-T147del→$(Gly)_5$, R219Q/S262N/C105-T147del→$(Gly)_3$, R219Q/S262N/R125N/P126A, R219Q/S262N/S127N/K129S, R219Q/S262N/P126N/E128T, R219Q/S262N/R112N/I114T, R219Q/S262N/I134N/L135C/L136T, R219Q/S262N/I134N/L135S/L136T, R219Q/S262N/R142N/Q144S, R219Q/S262N/E137N/Y139T, R219Q/S262N/P111N/G113S, R219Q/S262N/F119S, R219Q/S262N/F119K, R219Q/S262N/Y224R, R219Q/S262N/Y224N, R219Q/S262N/Y191S,

R219Q/S262N/Y191D, R219Q/S262N/F183K, R219Q/S262N/Y191D/Y224R, R219Q/S262N/F109S, R219Q/S262N/F109A, R219Q/S262N/R118D, R219Q/S262N/R118A, R219Q/S262N/Y139T, R219Q/S262N/Y139A, R219Q/S262N/W133S, R219Q/S262N/W133T, R219Q/S262N/P124A, R219Q/S262N/P124S, R219Q/S262N/V99-Q144del→$(GGGGS)_1$, R219Q/S262N/V99-Q144del→$(GGGGS)_2$, R219Q/S262N/V99-Q144del→$(GGGGS)_3$, R219Q/S262N/C105-T147del→$(GGGGS)_1$, R219Q/S262N/C105-T147del→$(GGGGS)_2$, R219Q/S262N/C105-T147del→$(GGGGS)_3$, R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_1$, R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_2$, R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_3$, R219Q/S262N/K371D/C105-T147del→$(GGGGS)_1$, R219Q/S262N/K371D/C105-T147del→$(GGGGS)_2$, R219Q/S262N/K371D/C105-T147del→$(GGGGS)_3$, R219Q/S262N/C105-T147del→(Gly)n, R219Q/S262N/K11A, R219Q/S262N/K11D, R219Q/S262N/K11E, R219Q/S262N/K13A, R219Q/S262N/K13D, R219Q/S262N/V99-Q144del→(GGGGS)n, R219Q/S262N/C105-T147del→(GGGGS)n, R219Q/S262N/N98-N156del, R219Q/S262N/C105-E148del, R219Q/S262N/C105-T147del, R219Q/S262N/V99-Q144del, R219Q/S262N/K371D/C105-T147del→(Gly)n, R219Q/S262N/K371D/C105-T147del→$(Gly)_{15}$, R219Q/S262N/K371D/C105-T147del→$(Gly)_{10}$, R219Q/S262N/K371D/C105-T147del→$(Gly)_7$, R219Q/S262N/K371D/C105-T147del→$(Gly)_5$, R219Q/S262N/K371D/C105-T147del→$(Gly)_3$, R219Q/S262N/K371D/V99-Q144del→(GGGGS)n, R219Q/S262N/K371D/C105-T147del→(GGGGS)n, R219Q/S262N/K371D/N98-N156del, R219Q/S262N/K371D/C105-E148del, R219Q/S262N/K371D/C105-T147del, R219Q/S262N/K371D/V99-Q144del, R219Q/S262N/K13E, R219Q/S262N/K371A, R219Q/S262N/K372A, R219Q/S262N/K372D, R219Q/S262N/K372E, R219Q/S262N/K452A, R219Q/S262N/K452D, R219Q/S262N/K452E, R219Q/S262N/R20A, R219Q/S262N/R20D, R219Q/S262N/R366A, R219Q/S262N/R366D, R219Q/S262N/R366E, R219Q/S262N/H264A, R219Q/S262N/H264Q, R219Q/S262N/H264N, R219Q/S262N/H264G, R219K/S262N, R219N/S262N, R219A/S262N, R219Q/S262N/L221A, R219Q/S262N/L221V, R219Q/S262N/L221G,

R219Q/S262N/E179D, R219Q/S262N/E179A, R219Q/S262N/E179S, R219Q/S262N/E179T, R219Q/S262N/E179V, R219Q/S262N/E179G, R219Q/S262A, R219Q/S262V, R219Q/S262M, R219Q/S262N/K11A/R20A, R219Q/S262N/K11A/R20A/K371A, R219Q/S262N/R20A/K371A, R219Q/S262N/K11A/K371A, R219Q/S262N/K26A, R219Q/S262N/K26D, R219Q/S262N/K26E, R219Q/S262N/R217A, R219Q/S262N/R217D, R219Q/S262N/R217E, R219Q/S262N/K258A, R219Q/S262N/K258D, R219Q/S262N/K258E, R219Q/S262N/R277A, R219Q/S262N/R277D, R219Q/S262N/R277E, R219Q/S262N/R283A, R219Q/S262N/R283D, R219Q/S262N/R283E, R219Q/S262N/K309A, R219Q/S262N/K309D, R219Q/S262N/K309E, R219Q/S262N/K317A, R219Q/S262N/K317D, R219Q/S262N/K317E, R219Q/S262N/K321A, R219Q/S262N/K321D, R219Q/S262N/K321E, R219Q/S262N/R352A, R219Q/S262N/R352D, R219Q/S262N/R352E, R219Q/S262N/R441A, R219Q/S262N/R441D, R219Q/S262N/R441E, R219Q/S262N/K444A, R219Q/S262N/K444D, R219Q/S262N/K444E, R219Q/S262N/K461A, R219Q/S262N/K461D, R219Q/S262N/K461E, R219Q/S262N/K469A, R219Q/S262N/K469D, R219Q/S262N/K469E, R219Q/S262N/K470A, R219Q/S262N/K470D, R219Q/S262N/K470E, R219Q/S262N/D86A, R219Q/S262N/D86C, R219Q/S262N/D86E, R219Q/S262N/D86F, R219Q/S262N/D86G, R219Q/S262N/D86H, R219Q/S262N/D86I, R219Q/S262N/D86K, R219Q/S262N/D86L, R219Q/S262N/D86M, R219Q/S262N/D86N, R219Q/S262N/D86P, R219Q/S262N/D86Q, R219Q/S262N/D86R, R219Q/S262N/D86S, R219Q/S262N/D86T, R219Q/S262N/D86V, R219Q/S262N/D86W, R219Q/S262N/D86Y, R219Q/S262N/E179C, R219Q/S262N/E179F, R219Q/S262N/E179H, R219Q/S262N/E179I, R219Q/S262N/E179K, R219Q/S262N/E179L, R219Q/S262N/E179M, R219Q/S262N/E179N, R219Q/S262N/E179P,, R219Q/S262N/E179Q, R219Q/S262N/E179R, R219Q/S262N/E179W, R219Q/S262N/E179Y, R219C/S262N, R219D/S262N, R219E/S262N, R219F/S262N, R219G/S262N, R219H/S262N, R219I/S262N, R219L/S262N, R219M/S262N, R219P/S262N, R219S/S262N, R219T/S262N, R219V/S262N, R219W/S262N, R219Y/S262N,

R219Q/S262N/L221C, R219Q/S262N/L221D, R219Q/S262N/L221E, R219Q/S262N/L221F,R219Q/S262N/L221H,R219Q/S262N/L221I, R219Q/S262N/L221K, R219Q/S262N/L221M, R219Q/S262N/L221N, R219Q/S262N/L221P, R219Q/S262N/L221Q, R219Q/S262N/L221R, R219Q/S262N/L221S, R219Q/S262N/L221T, R219Q/S262N/L221W, R219Q/S262N/L221Y, R219Q/S262C, R219Q/S262D, R219Q/S262E, R219Q/S262F, R219Q/S262G, R219Q/S262H, R219Q/S262I, R219Q/S262K, R219Q/S262L, R219Q/S262P, R219Q/S262Q, R219Q/S262R, R219Q/S262T, R219Q/S262W, R219Q/S262Y, R219Q/S262N/H264C, R219Q/S262N/H264D, R219Q/S262N/H264E, R219Q/S262N/H264F, R219Q/S262N/H264I, R219Q/S262N/H264K, R219Q/S262N/H264L, R219Q/S262N/H264M, R219Q/S262N/H264P, R219Q/S262N/H264R, R219Q/S262N/H264S, R219Q/S262N/H264T, R219Q/S262N/H264V, R219Q/S262N/H264W, R219Q/S262N/H264Y, R219Q/S262N/S266A, R219Q/S262N/S266C, R219Q/S262N/S266D, R219Q/S262N/S266E, R219Q/S262N/S266F, R219Q/S262N/S266G, R219Q/S262N/S266H, R219Q/S262N/S266I, R219Q/S262N/S266K, R219Q/S262N/S266L, R219Q/S262N/S266M, R219Q/S262N/S266N, R219Q/S262N/S266P, R219Q/S262N/S266Q, R219Q/S262N/S266R, R219Q/S262N/S266T, R219Q/S262N/S266V, R219Q/S262N/S266W, R219Q/S262N/S266Y, R219Q/S262N/K267A, R219Q/S262N/K267C, R219Q/S262N/K267D, R219Q/S262N/K267E, R219Q/S262N/K267F, R219Q/S262N/K267G, R219Q/S262N/K267H, R219Q/S262N/K267I, R219Q/S262N/K267L, R219Q/S262N/K267M, R219Q/S262N/K267N, R219Q/S262N/K267P, R219Q/S262N/K267Q, R219Q/S262N/K267R, R219Q/S262N/K267S, R219Q/S262N/K267T, R219Q/S262N/K267V, R219Q/S262N/K267W, R219Q/S262N/K267Y, R219Q/S262N/V296A, R219Q/S262N/V296C, R219Q/S262N/V296D, R219Q/S262N/V296E, R219Q/S262N/V296F, R219Q/S262N/V296G, R219Q/S262N/V296H, R219Q/S262N/V296I, R219Q/S262N/V296K, R219Q/S262N/V296L, R219Q/S262N/V296M, R219Q/S262N/V296N, R219Q/S262N/V296P, R219Q/S262N/V296Q, R219Q/S262N/V296R,, R219Q/S262N/V296S, R219Q/S262N/V296T, R219Q/S262N/V296W and

R219Q/S262N/V296Y. In some embodiments, the variant ADA2 protein comprises the modifications selected from among R219Q/K11A/R20A, R219Q/K11A/R20A/K371A, R219Q/R20A/K371A, 219Q/K11A/K371A, S262N/K11A/R20A, S262N/K11A/R20A/K371A, S262N/R20A/K371A, S262N/K11A/K371A, R219Q/C105-T147del→(Gly)n, R219Q/V99-Q144del→(GGGGS)n, R219Q/C105-T147del→(GGGGS)n, R219Q/N98-N156del, R219Q/C105-E148del, R219Q/C105-T147del, R219Q/V99-Q144del, S262N/C105-T147del→(Gly)n, S262N/V99-Q144del→(GGGGS)n, S262N/C105-T147del→(GGGGS)n, S262N/N98-N156del, S262N/C105-E148del, S262N/C105-T147del and S262N/V99-Q144del.

In some embodiments, the variant ADA2 protein, when in dimer form, can exhibit increased adenosine deaminase activity. For example, the variant ADA2 protein, when in dimer form, can exhibit at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 225%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800% or more activity of the corresponding dimer form of the unmodified ADA2 protein, wherein adenosine deaminase activity is assessed under the same conditions. In some embodiments, the variant ADA2 protein, when in dimer form, can exhibit a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2.0-fold, 2.2-fold, 2.5-fold, 3.0-fold, 3.5-fold, 4-fold, 4.5-fold, 5.0-fold, 6.0-fold, 7.0-fold, 8.0-fold, 9.0-fold, 10.0-fold or more compared to the catalytic efficiency (k_{cat}/K_M) of the corresponding dimer form of the unmodified ADA2 protein, wherein catalytic efficiency of adenosine deaminase activity is assessed under the same conditions. For example, the variant ADA2 protein, when in dimer form, can exhibit a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 2×10^4 M^{-1} s^{-1}, 3×10^4 M^{-1} s^{-1}, 4×10^4 M^{-1} s^{-1}, 5×10^4 M^{-1} s^{-1}, 6×10^4 M^{-1} s^{-1}, 7×10^4 M^{-1} s^{-1}, 8×10^4 M^{-1} s^{-1}, 9×10^4 M^{-1} s^{-1}, 1×10^5 M^{-1} s^{-1}, 2×10^5 M^{-1} s^{-1}, 3×10^5 M^{-1} s^{-1}, 4×10^5 M^{-1} s^{-1}, 5×10^5 M^{-1} s^{-1} or greater.

In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains the modifications selected from among K371D/V99-Q144del→$(GGGGS)_1$, K371D/V99-Q144del→$(GGGGS)_2$,

K371D/V99-Q144del→$(GGGGS)_3$, K371D/C105-T147del→$(GGGGS)_1$, K371D/C105-T147del→$(GGGGS)_2$, K371D/C105-T147del→$(GGGGS)_3$, R219Q/S262N/---→N1/---→A2/---→S3 , K371D/C105-T147del→(Gly)n, K371D/C105-T147del→$(Gly)_{15}$, K371D/C105-T147del→$(Gly)_{10}$, K371D/C105-T147del→$(Gly)_7$, K371D/C105-T147del→$(Gly)_5$, K371D/C105-T147del→$(Gly)_3$, K371D/V99-Q144del→(GGGGS)n, K371D/C105-T147del→(GGGGS)n, K371D/N98-N156del, K371D/C105-E148del, K371D/C105-T147del and K371D/V99-Q144del. In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains modifications selected from among R125N/P126A, S127N/K129S, P126N/E128T, R112N/I114T, I134N/L135C/L136T, I134N/L135S/L136T, R142N/Q144S, E137N/Y139T, P111N/G113S, F119S, F119K, Y224R, Y224N, Y191S, Y191D, F183K, Y191D/Y224R, F109S, F109A, R118D, R118A, Y139T, Y139A, W133S, W133T, P124A, P124S, V99-Q144del, V99-Q144del→(GGGGS)n, C105-T147del→(GGGGS)n, V99-Q144del→$(GGGGS)_1$, V99-Q144del→$(GGGGS)_2$, V99-Q144del→$(GGGGS)_3$, C105-T147del→$(GGGGS)_1$, C105-T147del→$(GGGGS)_2$, and C105-T147del→$(GGGGS)_3$.

In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains modifications selected from among R125N/P126A, S127N/K129S, P126N/E128T, R112N/I114T, I134N/L135C/L136T, I134N/L135S/L136T, R142N/Q144S, E137N/Y139T, P111N/G113S, F119S, F119K, Y224R, Y224N, Y191S, Y191D, F183K, Y191D/Y224R, F109S, F109A, R118D, R118A, Y139T, Y139A, W133S, W133T, P124A, P124S, V99-Q144del, V99-Q144del→(GGGGS)n, C105-T147del→(GGGGS)n, V99-Q144del→$(GGGGS)_1$, V99-Q144del→$(GGGGS)_2$, V99-Q144del→$(GGGGS)_3$, C105-T147del→$(GGGGS)_1$, C105-T147del→$(GGGGS)_2$ and C105-T147del→$(GGGGS)_3$.

For example, among such variant ADA2 proteins are any that include one or more amino acid replacement(s) at an amino acid position corresponding to amino acid residue 11, 20, 219, 221, 262, 264, 366, 371, 372 or 452, with reference to amino acid positions set forth in SEQ ID NO:5. For example, the variant ADA2 protein can include one or more amino acid replacement(s) selected from among K11A, K11E, R20A, R20E, R219K, R219Q, L221A, L221V, L221G, S262N, H264Q, H264G, R366E, K371A, K371D, K371E, K372D, K372E, K452D and K452E, with reference

to amino acid positions set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 protein can include amino acid replacements selected from among K11A/R20A, K11A/R20A/K371A, R20A/K371A, K11A/K371A, S262N/K371D, S262N/K371E, S262N/R20E, S262N/R20E/K371D, S262N/R20E/K371E, R219Q/K371E, R219Q/K371D, R219Q/R20E, R219Q/K371E/R20E, R219Q/K371D/R20E, R219Q/S262N/K371E, R219Q/S262N/K371D, R219Q/S262N/R20E, R219Q/S262N/K371E/R20E, R219Q/S262N/K371D/R20E and R219Q/S262N, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments, the variant ADA2 protein, when in dimer form, can exhibit reduced heparin binding. For example, the variant ADA2 protein, when in dimer form, can exhibit no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% of the heparin binding of the corresponding dimer form of the unmodified ADA2 protein, wherein heparin binding is assessed under the same conditions.

For example, among such the variant ADA2 proteins are any that include one or more amino acid replacement(s) at an amino acid position corresponding to amino acid residue 20, 262, 366, 371, 372, or 452, with reference to amino acid positions set forth in SEQ ID NO:5. For example, the variant ADA2 protein can include one or more amino acid replacement(s) selected from among R20A, R20D, R20E, S262N, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E and K452E, with reference to amino acid positions set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 protein can include amino acid replacements selected from among K11A/R20A, K11A/R20A/K371A, R20A/K371A, K11A/K371A, S262N/K371D, S262N/K371E, S262N/R20E, S262N/R20E/K371D and S262N/R20E/K371E, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains one or more amino acid replacement(s) corresponding to K11A; K11D; K11E; K13A; K13D; K13E; K371A; K371D; K371E; K372A; K372D; K372E; K452A; K452D; K452E; R20A; R20D; R20E; R366A; R366D; R366E; K26A; K26D; K26E; R217A; R217D; R217E; K258A; K258D; K258E; R277A;

R277D; R277E; R283A; R283D; R283E; K309A; K309D; K309E; K317A; K317D; K317E; K321A; K321D; K321E; R352A; R352D; R352E; R441A; R441D; R441E; K444A; K444D; K444E; K461A; K461D; K461E; K469A; K469D; K469E; K470A; K470D; and K470E.

In some embodiments, the variant ADA2 protein, when in dimer form, can exhibit a longer serum half-life ($t_{1/2}$). For example, the variant ADA2, when in dimer form, can exhibit a half-life that is at least or at least about 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 225%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800% or more longer than the half-life of the corresponding dimer form of the unmodified ADA2 protein, wherein half-life is assessed under the same conditions.

In some embodiments, the variant ADA2 protein, when in dimer form, can exhibit increased thermal stability. For example, the variant ADA2 protein, when in dimer form, can exhibit thermal stability with a melting temperature (Tm) that is increased at least or at least about 0.5°C, 1.0°C, 2.0°C, 3.0°C, 4.0°C, 5.0°C, 6.0°C, 7.0°C, 8.0°C, 9.0°C, 10.0°C or more compared to the Tm of the corresponding dimer form of the unmodified ADA2 protein, wherein Tm is assessed under the same conditions. For example, the variant ADA2 protein can have a melting temperature (Tm) of at least or at least about 67.6°C, 67.8°C, 68.0°C, 68.2°C, 68.4°C, 68.6°C, 68.8°C, 69.0°C, 69.2°C, 69.4°C, 69.6°C, 69.8°C, 70.0°C, 70.2°C, 70.4°C, 70.6°C, 70.8°C, 71.0°C, 71.2°C, 71.4°C, 71.6°C, 71.8°C or higher.

In examples of any of the variant ADA2 proteins provided herein, the adenosine deaminase activity of the variant ADA2 protein can be assessed or exhibited at or about pH 6.5 ± 0.2. In some examples, the variant ADA2 protein, when in dimer form, can exhibit an altered pH optimum for adenosine deaminase activity. For example, the variant ADA2 protein, when in dimer form, can exhibit a pH optimum for adenosine deaminase activity that is at a higher pH compared to the pH optimum of the corresponding dimer form of the unmodified ADA2 protein. For example, the variant ADA2 protein, when in dimer form, can have a pH optimum with a pH that is at least or at least about 6.6, 6.7, 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5 or higher. In other examples, the variant ADA2 protein, when in dimer form, can exhibit a pH optimum for adenosine deaminase activity that is at a lower pH compared to the

pH optimum of the corresponding dimer form of the unmodified ADA2 protein. For example, the variant ADA2 protein, when in dimer form, can have a pH optimum with a pH that is less than or less than about 6.5, 6.4, 6.3, 6.3, 6.2, 6.1, 6.0 or less.

In some embodiments, the variant ADA2 protein can include a modification of one or more amino acids in the putative receptor binding (PRB) domain, wherein the modification is an amino acid deletion, insertion or replacement. In any of such examples, the variant ADA2 protein does not contain a modification that is an amino acid replacement corresponding to amino acid replacement C108G, A120V, H121R, R125C, R140Q, K141R or R142W, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments, the variant ADA2 protein or catalytically active portion thereof lacks all or a portion of the putative receptor binding (PRB) domain or has a modification of the PRB, whereby any receptor binding or growth factor activity is reduced or eliminated or other activity of ADA2 other than deaminase activity is reduced or eliminated or interaction with the ADA domain is reduced or eliminated and the PRB domain corresponds to residues 98 to 156 set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 lacks residues 105-148 or 105 to 147 or 99 to 144, with reference to amino acid positions set forth in SEQ ID NO:5. In some examples, the variant ADA2 protein or catalytically active portion thereof contains the sequence of amino acids set forth in any of SEQ ID NOS:548-550 and 579. In some embodiments, variant ADA2 protein or catalytically active portion thereof contains a deletion of all or a portion of the PRB domain and optionally an insertion of peptide linker.

In some examples, the variant ADA2 protein can have a deletion of one or more contiguous amino acid residues corresponding to any one or more contiguous amino acid residues between or between about amino acid residues 98 and 156 or amino acid residues 105 and 148, inclusive, with reference to amino acid positions set forth in SEQ ID NO:5. In any of such examples, the variant of the ADA2 polypeptide can further include substitution of the deleted region with a peptide linker. For example, the peptide linker can be selected from among (Gly)n (SEQ ID NO:368), where n is 2 to 20; (GGGGS)n (SEQ ID NO:343), where n is 1 to 6; (SSSSG)n (SEQ ID NO:344), where n is 1 to 6; (AlaAlaProAla)n (SEQ ID NO:350), where n is 1 to 6;

Date Reçue/Date Received 2021-07-28

GKSSGSGSESKS (SEQ ID NO:345); GGSTSGSGKSSEGKG (SEQ ID NO:346); GSTSGSGKSSSEGSGSTKG (SEQ ID NO:347); GSTSGSGKPGSGEGSTKG (SEQ ID NO:348); and EGKSSGSGSESKEF (SEQ ID NO:349). For example, the peptide linker can be selected from among GGG (SEQ ID NO:369); GGGGG (SEQ ID NO:360); GGGGGGG (SEQ ID NO:370); GGGGGGGGGG (SEQ ID NO:371); and GGGGGGGGGGGGGGG (SEQ ID NO:372).

In some embodiments, the variant ADA2 can contain the variant ADA2 polypeptide that can include a modification in the PRB domain that corresponds to C105-T147del→$(Gly)_n$, where n is 2 to 20, such as C105-T147del→$(Gly)_{15}$, C105-T147del→$(Gly)_{10}$, C105-T147del→$(Gly)_7$, C105-T147del→$(Gly)_5$ or C105-T147del→$(Gly)_3$, with reference to amino acid positions set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 can include modification in the PRB domain that corresponds to C105-T147del→(Gly)n, where n = 2 to 20; C105-T147del→$(Gly)_{15}$; C105-T147del→$(Gly)_{10}$; C105-T147del→$(Gly)_7$; C105-T147del→$(Gly)_5$; C105-T147del→$(Gly)_3$; N98-N156del; C105-E148del; C105-T147del; V99-Q144del; V99-Q144del→(GGGGS)n, where n = 1 to 5; C105-T147del→(GGGGS)n, where n = 1 to 5; V99-Q144del→$(GGGGS)_1$; V99-Q144del→$(GGGGS)_2$; V99-Q144del→$(GGGGS)_3$; C105-T147del→$(GGGGS)_1$; C105-T147del→$(GGGGS)_2$; and C105-T147del→$(GGGGS)_3$, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains one or more amino acid replacement(s) selected from among replacements corresponding to F119S; F119K; Y224R; Y224N; Y191S; Y191D; F183K; Y191D/Y224R; F109S; F109A; R118D; R118A; Y139T; Y139A; W133S; W133T; P124A; and P124S, with reference to amino acid positions set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains amino acid replacements selected from among replacements corresponding to R219Q/S262N/F119S; R219Q/S262N/F119K; R219Q/S262N/Y224R; R219Q/S262N/Y224N; R219Q/S262N/Y191S; R219Q/S262N/Y191D; R219Q/S262N/F183K; R219Q/S262N/Y191D/Y224R; R219Q/S262N/F109S; R219Q/S262N/F109A; R219Q/S262N/R118D; R219Q/S262N/R118A; R219Q/S262N/Y139T; R219Q/S262N/Y139A; R219Q/S262N/W133S;

R219Q/S262N/W133T; R219Q/S262N/P124A; and R219Q/S262N/P124S. In some embodiments, the variant ADA2 contains modifications selected from among K371D/V99-Q144del→$(GGGGS)_1$; K371D/V99-Q144del→$(GGGGS)_2$; K371D/V99-Q144del→$(GGGGS)_3$; K371D/C105-T147del→$(GGGGS)_1$; K371D/C105-T147del→$(GGGGS)_2$; K371D/C105-T147del→$(GGGGS)_3$; R219Q/S262N/C105-T147del→$(Gly)_{15}$; R219Q/S262N/C105-T147del→$(Gly)_{10}$; R219Q/S262N/C105-T147del→$(Gly)_7$; R219Q/S262N/C105-T147del→$(Gly)_5$; R219Q/S262N/C105-T147del→$(Gly)_3$; R219Q/S262N/V99-Q144del→$(GGGGS)_1$; R219Q/S262N/V99-Q144del→$(GGGGS)_2$; R219Q/S262N/V99-Q144del→$(GGGGS)_3$; R219Q/S262N/C105-T147del→$(GGGGS)_1$; R219Q/S262N/C105-T147del→$(GGGGS)_2$; R219Q/S262N/C105-T147del→$(GGGGS)_3$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_1$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_2$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_3$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_1$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_2$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_3$; K371D/C105-T147del→(Gly)n, where n = 2 to 20; K371D/C105-T147del→$(Gly)_{15}$; K371D/C105-T147del→$(Gly)_{10}$; K371D/C105-T147del→$(Gly)_7$; K371D/C105-T147del→$(Gly)_5$; K371D/C105-T147del→$(Gly)_3$; K371D/V99-Q144del→(GGGGS)n, where n = 1 to 5; K371D/C105-T147del→(GGGGS)n, where n = 1 to 5; K371D/N98-N156del; K371D/C105-E148del; K371D/C105-T147del; K371D/V99-Q144del; R219Q/S262N/C105-T147del→(Gly)n, where n = 2 to 20; R219Q/S262N/V99-Q144del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/C105-T147del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/N98-N156del; R219Q/S262N/C105-E148del; R219Q/S262N/C105-T147del; R219Q/S262N/V99-Q144del; R219Q/S262N/K371D/C105-T147del→(Gly)n, where n = 2 to 20; R219Q/S262N/K371D/C105-T147del→$(Gly)_{15}$; R219Q/S262N/K371D/C105-T147del→$(Gly)_{10}$; R219Q/S262N/K371D/C105-T147del→$(Gly)_7$; R219Q/S262N/K371D/C105-T147del→$(Gly)_5$; R219Q/S262N/K371D/C105-T147del→$(Gly)_3$; R219Q/S262N/K371D/V99-Q144del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/K371D/C105-T147del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/K371D/N98-N156del; R219Q/S262N/K371D/C105-E148del; R219Q/S262N/K371D/C105-T147del;

R219Q/S262N/K371D/V99-Q144del; R219Q/C105-T147del→(Gly)n, where n = 2 to 20; R219Q/V99-Q144del→(GGGGS)n, where n = 1 to 5; R219Q/C105-T147del→(GGGGS)n, where n = 1 to 5; R219Q/N98-N156del; R219Q/C105-E148del; R219Q/C105-T147del; R219Q/V99-Q144del; S262N/C105-T147del→(Gly)n, where n = 2 to 20; S262N/V99-Q144del→(GGGGS)n, where n = 1 to 5; S262N/C105-T147del→(GGGGS)n, where n = 1 to 5; S262N/N98-N156del; and S262N/C105-E148del; S262N/C105-T147del; and S262N/V99-Q144del.

In some embodiments of a variant ADA2 protein, including examples containing a modified PRB domain, the variant ADA2 protein, when in dimer form, can exhibit reduced binding to any one or more adenosine receptor (ADR) selected from among A_1, A_{2A}, A_{2B} and A_3 compared to binding of the unmodified ADA2 protein to the same receptor when assessed under the same conditions. For example, the variant ADA2 protein has a binding that is reduced at least or at least about 0.5-fold, 1-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or more.

In some embodiments of the variant ADA2 provided herein, the variant ADA2 can be glycosylated, for example, at a native or a non-native glycosylation site. In some embodiments, the variant ADA2 protein can include a modification(s) that alters glycosylation by introduction of a non-native glycosylation site, whereby, when expressed in a cell capable of glycosylation, the variant ADA2 protein is hyperglycosylated compared to the unmodified ADA2 polypeptide. For example, the non-native glycosylation site is introduced by amino acid replacement(s) or insertion of one, two or three amino acids. For example, the modifications are selected from among modifications corresponding to ---→N1/---→A2/---→S3, R20N/V22S, K371N/D373S, K372N/I374S, T403N/H405S and G404N/P406S, with reference to amino acid positions set forth in SEQ ID NO:5. In some embodiments, the variant ADA2 or catalytically active portion thereof contains modifications corresponding to R219Q/S262N/---→N1/---→A2/---→S3 ; R219Q/S262N/R20N/V22S; R219Q/S262N/K371N/D373S; R219Q/S262N/K372N/I374S; R219Q/S262N/T403N/H405S; or R219Q/S262N/G404N/P406S. In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains a modification in the putative receptor binding domain (PRB) corresponding

to one or more of the modifications selected from among: R125N/P126A; S127N/K129S; P126N/E128T; R112N/I114T; I134N/L135C/L136T; I134N/L135S/L136T; R142N/Q144S; E137N/Y139T; and P111N/G113S. In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains amino acid replacements corresponding to R219Q/S262N/R125N/P126A; R219Q/S262N/S127N/K129S; R219Q/S262N/P126N/E128T; R219Q/S262N/R112N/I114T; R219Q/S262N/I134N/L135C/L136T; R219Q/S262N/I134N/L135S/L136T; R219Q/S262N/R142N/Q144S; R219Q/S262N/E137N/Y139T; or R219Q/S262N/P111N/G113S.

In some embodiments, the variant ADA2 protein can be a human ADA2. In some embodiments, the variant ADA2 protein can be isolated or purified.

In some embodiments, the variant ADA2 protein can contain a polypeptide that exhibits at least 65% sequence identity to SEQ ID NO:5 or a catalytically active portion thereof. For example, the variant ADA2 protein can contain a polypeptide that exhibits at least 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:5 or a catalytically active portion thereof. For example, the variant ADA2 protein contains a polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS:13-63 or 71-285 or a catalytically active portion thereof. In some embodiments, the variant ADA2 protein or catalytically active portion thereof contains the sequence of amino acids set forth in any of SEQ ID NOS: 551-579 or 581-993 or a catalytically active portion thereof.

In some embodiments, the variant ADA2 protein or a catalytically active portion thereof can contain amino acid replacements selected from among replacements corresponding to K11A/R20A; K11A/R20A/K371A; R20A/K371A; K11A/K371A; S262N/K371D; S262N/K371E; S262N/R20E; S262N/R20E/K371D; S262N/R20E/K371E; R219Q/K371E; R219Q/K371D; R219Q/R20E; R219Q/K371E/R20E; R219Q/K371D/R20E; R219Q/S262N/K371E; R219Q/S262N/K371D; R219Q/S262N/R20E; R219Q/S262N/K371E/R20E; R219Q/S262N/K371D/R20E; R219Q/S262N; R219Q/S262N/K11A; R219Q/S262N/K11D; R219Q/S262N/K11E; R219Q/S262N/K13A; R219Q/S262N/K13D; R219Q/S262N/K13E; R219Q/S262N/K371A;

Date Reçue/Date Received 2021-07-28

R219Q/S262N/K372A; R219Q/S262N/K372D; R219Q/S262N/K372E; R219Q/S262N/K452A; R219Q/S262N/K452D; R219Q/S262N/K452E; R219Q/S262N/R20A; R219Q/S262N/R20D; R219Q/S262N/R366A; R219Q/S262N/R366D; R219Q/S262N/R366E; R219Q/S262N/H264A; R219Q/S262N/H264Q; R219Q/S262N/H264N; R219Q/S262N/H264G; R219K/S262N; R219N/S262N; R219A/S262N; R219Q/S262N/L221A; R219Q/S262N/L221V; R219Q/S262N/L221G; R219Q/S262N/E179D; R219Q/S262N/E179A; R219Q/S262N/E179S; R219Q/S262N/E179T; R219Q/S262N/E179V; R219Q/S262N/E179G; R219Q/S262A; R219Q/S262V; R219Q/S262M; R219Q/S262N/K11A/R20A; R219Q/S262N/K11A/R20A/K371A; R219Q/S262N/R20A/K371A; R219Q/S262N/K11A/K371A; R219Q/S262N/K26A; R219Q/S262N/K26D; R219Q/S262N/K26E; R219Q/S262N/R217A; R219Q/S262N/R217D; R219Q/S262N/R217E; R219Q/S262N/K258A; R219Q/S262N/K258D; R219Q/S262N/K258E; R219Q/S262N/R277A; R219Q/S262N/R277D; R219Q/S262N/R277E; R219Q/S262N/R283A; R219Q/S262N/R283D; R219Q/S262N/R283E; R219Q/S262N/K309A; R219Q/S262N/K309D; R219Q/S262N/K309E; R219Q/S262N/K317A; R219Q/S262N/K317D; R219Q/S262N/K317E; R219Q/S262N/K321A; R219Q/S262N/K321D; R219Q/S262N/K321E; R219Q/S262N/R352A; R219Q/S262N/R352D; R219Q/S262N/R352E; R219Q/S262N/R441A; R219Q/S262N/R441D; R219Q/S262N/R441E; R219Q/S262N/K444A; R219Q/S262N/K444D; R219Q/S262N/K444E; R219Q/S262N/K461A; R219Q/S262N/K461D; R219Q/S262N/K461E; R219Q/S262N/K469A; R219Q/S262N/K469D; R219Q/S262N/K469E; R219Q/S262N/K470A; R219Q/S262N/K470D; R219Q/S262N/K470E; R219Q/S262N/D86A; R219Q/S262N/D86C; R219Q/S262N/D86E; R219Q/S262N/D86F; R219Q/S262N/D86G; R219Q/S262N/D86H; R219Q/S262N/D86I; R219Q/S262N/D86K; R219Q/S262N/D86L; R219Q/S262N/D86M; R219Q/S262N/D86N; R219Q/S262N/D86P; R219Q/S262N/D86Q; R219Q/S262N/D86R; R219Q/S262N/D86S; R219Q/S262N/D86T; R219Q/S262N/D86V; R219Q/S262N/D86W; R219Q/S262N/D86Y; R219Q/S262N/E179C; R219Q/S262N/E179F; R219Q/S262N/E179H;

R219Q/S262N/E179I; R219Q/S262N/E179K; R219Q/S262N/E179L; R219Q/S262N/E179M; R219Q/S262N/E179N; R219Q/S262N/E179P; R219Q/S262N/E179Q; R219Q/S262N/E179R; R219Q/S262N/E179W; R219Q/S262N/E179Y; R219C/S262N; R219D/S262N; R219E/S262N; R219F/S262N; R219G/S262N; R219H/S262N; R219I/S262N; R219L/S262N; R219M/S262N; R219P/S262N; R219S/S262N; R219T/S262N; R219V/S262N; R219W/S262N; R219Y/S262N; R219Q/S262N/L221C; R219Q/S262N/L221D; R219Q/S262N/L221E; R219Q/S262N/L221F; R219Q/S262N/L221H; R219Q/S262N/L221I; R219Q/S262N/L221K; R219Q/S262N/L221M; R219Q/S262N/L221N; R219Q/S262N/L221P; R219Q/S262N/L221Q; R219Q/S262N/L221R; R219Q/S262N/L221S; R219Q/S262N/L221T; R219Q/S262N/L221W; R219Q/S262N/L221Y; R219Q/S262C; R219Q/S262D; R219Q/S262E; R219Q/S262F; R219Q/S262G; R219Q/S262H; R219Q/S262I; R219Q/S262K; R219Q/S262L; R219Q/S262P; R219Q/S262Q; R219Q/S262R; R219Q/S262T; R219Q/S262W; R219Q/S262Y; R219Q/S262N/H264C; R219Q/S262N/H264D; R219Q/S262N/H264E; R219Q/S262N/H264F; R219Q/S262N/H264I; R219Q/S262N/H264K; R219Q/S262N/H264L; R219Q/S262N/H264M; R219Q/S262N/H264P; R219Q/S262N/H264R; R219Q/S262N/H264S; R219Q/S262N/H264T; R219Q/S262N/H264V; R219Q/S262N/H264W; R219Q/S262N/H264Y; R219Q/S262N/S266A; R219Q/S262N/S266C; R219Q/S262N/S266D; R219Q/S262N/S266E; R219Q/S262N/S266F; R219Q/S262N/S266G; R219Q/S262N/S266H; R219Q/S262N/S266I; R219Q/S262N/S266K; R219Q/S262N/S266L; R219Q/S262N/S266M; R219Q/S262N/S266N; R219Q/S262N/S266P; R219Q/S262N/S266Q; R219Q/S262N/S266R; R219Q/S262N/S266T; R219Q/S262N/S266V; R219Q/S262N/S266W; R219Q/S262N/S266Y; R219Q/S262N/K267A; R219Q/S262N/K267C; R219Q/S262N/K267D; R219Q/S262N/K267E; R219Q/S262N/K267F; R219Q/S262N/K267G; R219Q/S262N/K267H; R219Q/S262N/K267I; R219Q/S262N/K267L; R219Q/S262N/K267M; R219Q/S262N/K267N; R219Q/S262N/K267P; R219Q/S262N/K267Q; R219Q/S262N/K267R; R219Q/S262N/K267S; R219Q/S262N/K267T; R219Q/S262N/K267V; R219Q/S262N/K267W;

R219Q/S262N/K267Y; R219Q/S262N/V296A; R219Q/S262N/V296C; R219Q/S262N/V296D; R219Q/S262N/V296E; R219Q/S262N/V296F; R219Q/S262N/V296G; R219Q/S262N/V296H; R219Q/S262N/V296I; R219Q/S262N/V296K; R219Q/S262N/V296L; R219Q/S262N/V296M; R219Q/S262N/V296N; R219Q/S262N/V296P; R219Q/S262N/V296Q; R219Q/S262N/V296R; R219Q/S262N/V296S; R219Q/S262N/V296T; R219Q/S262N/V296W; R219Q/S262N/V296Y; R219Q/K11A/R20A; R219Q/K11A/R20A/K371A; R219Q/R20A/K371A; R219Q/K11A/K371A; S262N/K11A/R20A; S262N/K11A/R20A/K371A; S262N/R20A/K371A; and S262N/K11A/K371A, with reference to SEQ ID NO:5.

The variant ADA2 protein can be a monomer or a dimer. In particular, among variant ADA2 proteins provided herein are dimers of a variant ADA2 protein, that can include any of the variant ADA2 proteins provided. In some examples, the dimer can be a homodimer. In other examples, the dimer can be a heterodimer.

Provided are nucleic acid molecules encoding the variant ADA2 proteins provided herein. Also provided herein is a vector that includes the nucleic acid encoding any of the variant ADA2 proteins provided herein. The vector can be a eukaryotic or a prokaryotic vector, such as a mammalian vector or a viral vector. For example, the vector is an adenovirus vector, an adeno-associated-virus vector, a retrovirus vector, a herpes virus vector, a lentivirus vector, a poxvirus vector, or a cytomegalovirus vector. In some embodiments, the vector is an oncolytic vector. In some embodiments, the vector can also encode a soluble hyaluronidase. Also provided herein are cells containing any of the vectors provided herein. The cell can be a eukaryotic cell, such as a mammalian cell. If human, the cells are isolated or are provided as a cell culture. For example, the cell is a mammalian cell, such as a Chinese Hamster Ovary (CHO) cell. In some embodiments, the cell can expresses the variant ADA2 protein, such as a dimer. Also provided herein are variant ADA2 proteins, such as variant ADA2 dimers, that are produced by the cell provided herein. In some embodiments, the cell is an isolated cell or a cell culture, such as a eukaryotic cell, a non-human cell, a mammalian cell, or a cell that is not a human stem cell. In some embodiments, the cell is an immune cell, such as a T cell, a tumor-infiltrating

Date Reçue/Date Received 2021-07-28

lymphocyte (TIL), a cytotoxic T lymphocyte (CTL), a natural killer (NK) cell or a lymphokine-activated killer (LAK) cell. In some embodiments, the cell is a T cell that encodes and expresses chimeric antigen receptor (CAR) and the variant ADA2 protein or dimer. In some examples, the CAR is specific for a tumor cell antigen, and the tumor antigen is selected from among HER2, CD19, HERV-K, CD20, CD22, ROR1, mesothelin, CD33/IL3Ra, c-Met, PSMA, Glycolipid F77, EGFRvIII, GD-2, NY- ESO-1 TCR, MAGE A3 TCR and GD2 and combinations thereof.

Provided herein are conjugates include a variant ADA2 protein or a catalytically active portion of any ADA2 protein provided herein, such as a variant ADA2 dimer provided in any examples herein, linked directly or indirectly via a linker to a heterologous moiety, such as a toxin or therapeutic drug.

Also provided herein are conjugates that include an ADA2 protein linked directly or indirectly via a linker to a heterologous moiety. In any of the conjugates, the ADA2 protein can be a monomer or a dimer. In some examples, the dimer is a homodimer; in other it is a heterodimer. In any of the conjugates in the examples herein, the heterologous moiety is conjugated to one or both subunits in the dimer. The heterologous moiety, for example, can be selected from among a peptide, small molecule, nucleic acid, carbohydrate and polymer.

In some embodiments of the conjugates provided herein, the heterologous moiety is a half-life extending moiety. For example, the half-life extending moiety is selected from among biocompatible fatty acids and derivatives thereof, hydroxy alkyl starch (HAS), polyethylene glycol (PEG), Poly $(Gly_x\text{- }Ser_y)_n$, homo-amino-acid polymers (HAP), hyaluronic acid (HA), heparosan polymers (HEP), phosphorylcholine-based polymers (PC polymer), Fleximers, dextran, polysialic acids (PSA), Fc domain, Transferrin, Albumin, elastin-like peptides, XTEN sequences, albumin binding peptides, a CTP peptide, and any combination thereof.

In some examples, the half-life extending moiety is a PEG and the ADA2 protein is PEGylated. For example, the PEG can be selected from among methoxy-polyethylene glycols (mPEG), PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole (CDI-PEG), branched PEGs and polyethylene oxide (PEO). In some examples, the PEG has a molecular weight of from or from about 1 kDa to about 100 kDa. The PEG can be linear or branched. In some embodiments of

conjugates provided herein, the PEG moieties result from reaction with a PEG reagent selected from among mPEG-Succinimidyl Propionates (mPEG-SPA), mPEG Succinimidyl Carboxymethyl Ester (mPEG-SCM), mPEG-Succinimidyl Butanoates (mPEG-SBA), mPEG2-N-Hydroxylsuccinimide, mPEG-succinimidyl butanoate (mPEG-SBA), mPEG-succinimidyl α-methylbutanoate (mPEG-SMB), mPEG-butyrldehyde, PEG-p-nitrophenyl-carbonate and PEG-propionaldehyde. For example, the PEG moieities result from reaction with a PEG reagent selected from among mPEG-SCM (20kDa), mPEG-SCM (30kDa), mPEG-SBA (5kDa), mPEG-SBA (20kDa), mPEG-SBA (30kDa), mPEG-SMB (20kDa), mPEG-SMB (30kDa), mPEG-butyrldehyde (30kDa), mPEG-SPA (20kDa), mPEG-SPA (30kDa), mPEG2-NHS (10kDa branched), mPEG2-NHS (20kDa branched), mPEG2-NHS (40kDa branched), mPEG2-NHS (60kDa branched), PEG-NHS-biotin (5kDa biotinylated), PEG-p-nitrophenyl-carbonate (30kDa) and PEG-propionaldehyde (30kDa).

In embodiments of the conjugates provided herein, the ADA2 protein can contain the sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383, a sequence that exhibits at least 85% sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383 or a catalytically form thereof. For example, the ADA2 protein can contain a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383 or a catalytically active portion thereof. For example, the ADA2 protein can contain a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of amino acids set forth in SEQ ID NOS:5 or a catalytically active portion thereof. In another example, the ADA2 protein can include a polypeptide having the sequence of amino acids set forth in SEQ ID NO:5 or a catalytically active portion thereof.

In embodiments of conjugates provided herein, the ADA2 protein is a variant ADA2 protein that contains a sequence of amino acids that includes a modification(s) in the sequence of amino acids of an unmodified ADA2 protein or a catalytically active portion thereof, where the unmodified ADA2 protein contains the sequence of

amino acids set forth in SEQ ID NO:5, or a sequence of amino acids that exhibits at least 85% sequence identity to the sequence of amino acids set forth in SEQ ID NO:5, or is a catalytically active portion thereof. In any of such examples, the amino acid modification(s) are selected from among amino acid replacement(s), deletion(s) and insertion(s); and the variant ADA2 protein, when in dimer form, can exhibit adenosine deaminase activity to convert adenosine to inosine. In any of the conjugates provided herein, the ADA2 protein, when in dimer form, can exhibit a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 5×10^3 M^{-1} s^{-1}, 6×10^3 M^{-1} s^{-1}, 7×10^3 M^{-1} s^{-1}, 8×10^3 M^{-1} s^{-1}, 9×10^3 M^{-1} s^{-1}, 1×10^4 M^{-1} s^{-1}, 2×10^4 M^{-1} s^{-1}, 3×10^4 M^{-1} s^{-1}, 4×10^4 M^{-1} s^{-1}, 5×10^4 M^{-1} s^{-1}, 6×10^4 M^{-1} s^{-1}, 7×10^4 M^{-1} s^{-1}, 8×10^4 M^{-1} s^{-1}, 9×10^4 M^{-1} s^{-1}, 1×10^5 M^{-1} s^{-1}, 2×10^5 M^{-1} s^{-1}, 3×10^5 M^{-1} s^{-1}, 4×10^5 M^{-1} s^{-1}, 5×10^5 M^{-1} s^{-1} or greater.

In any of the embodiments of conjugates provided herein, the modification(s) of the ADA2 protein can be an amino acid replacement(s); and the variant ADA2 protein can include one or more amino acid replacement(s) at an amino acid position corresponding to amino acid residue 11, 13, 20, 22, 26, 86, 179, 217, 219, 221, 258, 262, 264, 266, 267, 277, 283, 296, 309, 317, 321, 352, 366, 371, 372, 373, 374, 403, 404, 405, 406, 441, 444, 452, 461, 469 or 470, with reference to amino acid positions set forth in SEQ ID NO:5. For example, in some embodiments of conjugates provided herein, the variant ADA2 protein can include one or more amino acid replacement(s) selected from among K11A, K11D, K11E, K13A, K13D, K13E, R20A, R20D, R20E, R20N, V22S, K26A, K26D, K26E, D86A, D86C, D86E, D86F, D86G, D86H, D86I, D86K, D86L, D86M, D86N, D86P, D86Q, D86R, D86S, D86T, D86V, D86W, D86Y, E179A, E179C, E179D, E179F, E179G, E179H, E179I, E179K, E179L, E179M, E179N, E179P, E179Q, E179R, E179S, E179T, E179V, E179W, E179Y, R217A, R217D, R217E, R219A, R219C, R219D, R219E, R219F, R219G, R219H, R219I, R219K, R219L, R219M, R219N, R219P, R219Q, R219S, R219T, R219V, R219W, R219Y, L221A, L221C, L221D, L221E, L221F, L221G, L221H, L221I, L221K, L221M, L221N, L221P, L221Q, L221R, L221S, L221T, L221V, L221W, L221Y, K258A, K258D, K258E, S262A, S262C, S262D, S262E, S262F, S262G, S262H, S262I, S262K, S262L, S262M, S262N, S262P, S262Q, S262R, S262T, S262V, S262W, S262Y, H264A, H264C, H264D, H264E, H264F, H264G, H264I, H264K,

Date Reçue/Date Received 2021-07-28

H264L, H264M, H264N, H264P, H264Q, H264R, H264S, H264T, H264V, H264W, H264Y, S266A, S266C, S266D, S266E, S266F, S266G, S266H, S266I, S266K, S266L, S266M, S266N, S266P, S266Q, S266R, S266T, S266V, S266W, S266Y, K267A, K267C, K267D, K267E, K267F, K267G, K267H, K267I, K267L, K267M, K267N, K267P, K267Q, K267R, K267S, K267T, K267V, K267W, K267Y, R277A, R277D, R277E, R283A, R283D, R283E, V296A, V296C, V296D, V296E, V296F, V296G, V296H, V296I, V296K, V296L, V296M, V296N, V296P, V296Q, V296R, V296S, V296T, V296W, V296Y, K309A, K309D, K309E, K317A, K317D, K317E, K321A, K321D, K321E, R352A, R352D, R352E, R366A, R366D, R366E, K371A, K371D, K371E, K371N, K372A, K372D, K372E, K372N, D373S, I374S, T403N, G404N, H405S, P406S, R441A, R441D, R441E, K444A, K444D, K444E, K452A, K452D, K452E, K461A, K461D, K461E, K469A, K469D, K469E, K470A, K470D, K470E, with reference to amino acid positions set forth in SEQ ID NO:5. For example, the variant ADA2 protien can include one or more amino acid replacement(s) selected from among K11A, K11E, R20A, R20D, R20E, R219K, R219Q, L221A, L221V, L221G, S262N, H264Q, H264G, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E, K452D and K452E, with reference to amino acid positions set forth in SEQ ID NO:5. In some examples, the variant ADA2 protein can include amino acid replacements selected from among K11A/R20A, K11A/R20A/K371A, R20A/K371A, K11A/K371A, S262N/K371D, S262N/K371E, S262N/R20E, S262N/R20E/K371D, S262N/R20E/K371E, R219Q/K371E, R219Q/K371D, R219Q/R20E, R219Q/K371E/R20E, R219Q/K371D/R20E, R219Q/S262N/K371E, R219Q/S262N/K371D, R219Q/S262N/R20E, R219Q/S262N/K371E/R20E, R219Q/S262N/K371D/R20E and R219Q/S262N, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of conjugates provided herein, the variant ADA2 protein can include a modification of one or more amino acids in the putative receptor binding (PRB) domain that is an amino acid deletion, insertion or replacement. For example, in some embodiments of conjugates provided herein, the variant ADA2 protein can include deletion of one or more contiguous amino acid residues corresponding to any one or more contiguous amino acid residues between or between about amino acid residues 98 and 156 or amino acid residues 105 and 148, inclusive, with reference to

Date Reçue/Date Received 2021-07-28

amino acid positions set forth in SEQ ID NO:5. In some examples, the variant ADA2 protein in the conjugate can further include substitution of the deleted region with a peptide linker. For example, the peptide linker can be selected from among (Gly)n (SEQ ID NO:368), where n is 2 to 20; (GGGGS)n (SEQ ID NO:343), where n is 1 to 6; (SSSSG)n (SEQ ID NO:344), where n is 1 to 6; (AlaAlaProAla)n (SEQ ID NO:350), where n is 1 to 6; GKSSGSGSESKS (SEQ ID NO:345); GGSTSGSGKSSEGKG (SEQ ID NO:346); GSTSGSGKSSSEGSGSTKG (SEQ ID NO:347); GSTSGSGKPGSGEGSTKG (SEQ ID NO:348); and EGKSSGSGSESKEF (SEQ ID NO:349). In some examples, the peptide linker is selected from among GGG (SEQ ID NO:369); GGGGG (SEQ ID NO:360); GGGGGGG (SEQ ID NO:370); GGGGGGGGGG (SEQ ID NO:371); and GGGGGGGGGGGGGGG (SEQ ID NO:372). For example, the modification in the PRB domain can correspond to C105-T147del→$(Gly)_n$, where n is 2 to 20, such as, C105-T147del→$(Gly)_{15}$, C105-T148del→$(Gly)_{10}$, C105-T147del→$(Gly)_7$, C105-T147del→$(Gly)_5$ or C105-T147del→$(Gly)_3$, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of conjugates provided herein, the ADA2 protein in the conjugate can be glycosylated at one or more native or non-native glycosylation site. For example, in some embodiments of conjugates provided herein containing a variant ADA2 protein, the variant ADA2 protein in the conjugate can include a modification(s) that alters glycosylation by introduction of a non-native glycosylation site. The non-native glycosylation site can be introduced by creating the canonical glycosylation sequence (NXT/S, where X is not Pro for N-linked carbohydrates, S/T for O-linked) by introducing amino acid replacement(s), insertions or deletions of one, two or three amino acids. For example, the modifications that alter glycosylation are selected from among modifications corresponding to ---→N1/---→A2/---→S3, R20N/V22S, K371N/D373S, K372N/I374S, T403N/H405S and G404N/P406S, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of conjugates provided herein, the variant ADA2 protein in the conjugate can have the sequence of amino acid set forth in any of SEQ ID NOS:13-63 or 71-285 or a catalytically active portion thereof.

In some embodiments of conjugates containing an ADA2 or variant ADA2 protein provided herein, the conjugate retains the adenosine deaminase activity

Date Reçue/Date Received 2021-07-28

compared to the ADA2 protein that is not conjugated. For example, the conjugate can exhibit from or from about 50% to 500%, 50% to 200%, 50% to 100%, 50% to 80%, 80% to 500%, 80% to 200%, 80% to 100%, 100% to 500% or 100% to 200%, each inclusive, of the adenosine deaminase activity of the ADA2 protein that is not conjugated, such as at least 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 300%, 400%, 500% or more the adenosine deaminase activity of the ADA2 protein that is not conjugated. In some embodiments of conjugates provided herein, the ADA2 in the conjugate can exhibit a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 5×10^3 M^{-1} s^{-1}, 6×10^3 M^{-1} s^{-1}, 7×10^3 M^{-1} s^{-1}, 8×10^3 M^{-1} s^{-1}, 9×10^3 M^{-1} s^{-1}, 1×10^4 M^{-1} s^{-1}, 2×10^4 M^{-1} s^{-1}, 3×10^4 M^{-1} s^{-1}, 4×10^4 M^{-1} s^{-1}, 5×10^4 M^{-1} s^{-1}, 6×10^4 M^{-1} s^{-1}, 7×10^4 M^{-1} s^{-1}, 8×10^4 M^{-1} s^{-1}, 9×10^4 M^{-1} s^{-1}, 1×10^5 M^{-1} s^{-1}, 2×10^5 M^{-1} s^{-1}, 3×10^5 M^{-1} s^{-1}, 4×10^5 M^{-1} s^{-1}, 5×10^5 M^{-1} s^{-1} or greater.

Provided herein are combinations containing any of the variant ADA2 proteins or a catalytically active portion thereof provided herein, any variant ADA2 dimer provided herein or any conjugate of any of the examples provided herein, and a therapeutic agent. Also provided herein are combinations containing any ADA2 protein; and a therapeutic agent. In any examples of the combination provided herein, the ADA2 protein can be a monomer or a dimer. For example, the ADA2 protein can be a dimer, such as a homodimer.

In some embodiments of combinations provided herein, the therapeutic agent can be selected from among an antibody, cytotoxic agent, chemotherapeutic agents, cytokine, growth inhibitory agent, anti-hormonal agent, kinase inhibitor, anti-angiogenic agent, cardioprotectant, immunostimulatory agent, immunosuppressive agent, immune checkpoint inhibitor, antibiotic and angiogenesis inhibitor. For example, the therapeutic agent can be an anti-cancer agent. In some embodiments of combinations provided herein, the anti-cancer agent can be an anti-cancer antibody, a chemotherapeutic agent, a radioimmunotherapeutic, an anti-angiogenic agent or an immune checkpoint inhibitor.

For example, the anti-cancer agent can be an immune checkpoint inhibitor; and the target of the immune checkpoint inhibitor can be selected from among CTLA4, PD-1, and PD-L1.In some embodiments of combinations provided herein,

Date Reçue/Date Received 2021-07-28

the immune checkpoint inhibitor can be an antibody, a fusion protein, an aptamer, or an immune checkpoint protein-binding fragment thereof. For example, the immune checkpoint inhibitor is an anti-immune checkpoint protein antibody or antigen-binding fragment thereof. In particular examples, the immune checkpoint inhibitor is selected from among: an anti-CTLA4 antibody, derivative thereof, or antigen-binding fragment thereof; an anti-PD-L1 antibody, derivative thereof, or antigen-binding fragment thereof; and an anti-PD-1 antibody, derivative thereof, or antigen-binding fragment thereof. For example, the immune checkpoint inhibitor can be selected from among: Ipilimumab, a derivative thereof, or an antigen-binding fragment thereof; Tremelimumab, a derivative thereof, or an antigen-binding fragment thereof; Nivolumab, a derivative thereof, or an antigen-binding fragment thereof; and Pidilizumab, a derivative thereof, or an antigen-binding fragment thereof.

In some embodiments of combinations provided herein, the therapeutic agent can be an anti-hyaluronan agent. For example, the anti-hyaluronan agent can be a soluble hyaluronidase. In some embodiments of combinations provided herein, the soluble hyaluronidase can exhibit hyaluronidase activity at neutral pH. In particular, the soluble hyaluronidase can be selected from among bovine, ovine or a C-terminal truncated human PH20 that lacks all or a portion of the glycosylphosphatidylinositol (GPI) anchor attachment sequence. For example, the soluble hyaluronidase is a C-terminally truncated human PH20 that lacks all or a portion of the GPI anchor attachment sequence, such as those set forth in any of SEQ ID NOS:481-488, 493-514, or 526-532, or that has a sequence of amino acids that has at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to a sequence of amino acids set forth in any of SEQ ID NOS:481-488, 493-514, or 526-532 and is soluble and retains hyaluronidase activity. In some embodiments of combinations provided herein, the anti-hyaluronan agent or soluble hyaluronidase can be conjugated to a polymer, such as a PEG moiety.

In some embodiments of combinations provided herein, the ADA2 protein can include a polypeptide having the sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383, a sequence that can exhibit at least 85% sequence identity to the sequence of amino acids set forth in SEQ ID NOS:5, 326-334, 340, 375 or 380-383 or a catalytically active form thereof. For example, the ADA2

Date Reçue/Date Received 2021-07-28

protein can include a protein having a sequence of amino acids that can exhibit at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of amino acids set forth in SEQ ID NOS:5, 326-334, 340, 375 or 380-383 or a catalytically active portion thereof. In particular examples, the ADA2 protein can contain a sequence of amino acids that can exhibit at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of amino acids set forth in SEQ ID NO:5. For example, the ADA2 protein can contain the sequence of amino acids set forth in SEQ ID NO:5.

In some embodiments of combinations provided herein, the ADA2 protein is a variant ADA2 protein having a sequence of amino acids that includes a modification(s) in the sequence of amino acids of an unmodified ADA2 polypeptide or a catalytically active portion thereof. In any of such examples, the unmodified ADA2 protein can include the sequence of amino acids set forth in SEQ ID NO:5, or a sequence of amino acids that can exhibit at least 85% sequence identity to the sequence of amino acids set forth in SEQ ID NO:5, or is a catalytically active portion thereof; the amino acid modification(s) are selected from among amino acid replacement(s), deletion(s) and insertion(s); and the variant ADA2 protein, when in dimer form, can exhibit adenosine deaminase activity to convert adenosine to inosine.

In some embodiments of combinations provided herein, the ADA2 protein, when in dimer form, can exhibit a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 5×10^3 M^{-1} s^{-1}, 6×10^3 M^{-1} s^{-1}, 7×10^3 M^{-1} s^{-1}, 8×10^3 M^{-1} s^{-1}, 9×10^3 M^{-1} s^{-1}, 1×10^4 M^{-1} s^{-1}, 2×10^4 M^{-1} s^{-1}, 3×10^4 M^{-1} s^{-1}, 4×10^4 M^{-1} s^{-1}, 5×10^4 M^{-1} s^{-1}, 6×10^4 M^{-1} s^{-1}, 7×10^4 M^{-1} s^{-1}, 8×10^4 M^{-1} s^{-1}, 9×10^4 M^{-1} s^{-1}, 1×10^5 M^{-1} s^{-1}, 2×10^5 M^{-1} s^{-1}, 3×10^5 M^{-1} s^{-1}, 4×10^5 M^{-1} s^{-1}, 5×10^5 M^{-1} s^{-1} or greater.

In some embodiments of combinations provided herein, the modification(s) in the sequence of amino acids of an unmodified ADA2 protein can include an amino acid replacement(s); and the variant ADA2 protein can include one or more amino acid replacement(s) at an amino acid position corresponding to amino acid residue 11, 13, 20, 22, 26, 86, 179, 217, 219, 221, 258, 262, 264, 266, 267, 277, 283, 296, 309, 317, 321, 352, 366, 371, 372, 373, 374, 403, 404, 405, 406, 441, 444, 452, 461, 469 or 470, with reference to amino acid positions set forth in SEQ ID NO:5. For example,

Date Reçue/Date Received 2021-07-28

the variant ADA2 protein can include one or more amino acid replacement(s) selected from among K11A, K11D, K11E, K13A, K13D, K13E, R20A, R20D, R20E, R20N, V22S, K26A, K26D, K26E, D86A, D86C, D86E, D86F, D86G, D86H, D86I, D86K, D86L, D86M, D86N, D86P, D86Q, D86R, D86S, D86T, D86V, D86W, D86Y, E179A, E179C, E179D, E179F, E179G, E179H, E179I, E179K, E179L, E179M, E179N, E179P, E179Q, E179R, E179S, E179T, E179V, E179W, E179Y, R217A, R217D, R217E, R219A, R219C, R219D, R219E, R219F, R219G, R219H, R219I, R219K, R219L, R219M, R219N, R219P, R219Q, R219S, R219T, R219V, R219W, R219Y, L221A, L221C, L221D, L221E, L221F, L221G, L221H, L221I, L221K, L221M, L221N, L221P, L221Q, L221R, L221S, L221T, L221V, L221W, L221Y, K258A, K258D, K258E, S262A, S262C, S262D, S262E, S262F, S262G, S262H, S262I, S262K, S262L, S262M, S262N, S262P, S262Q, S262R, S262T, S262V, S262W, S262Y, H264A, H264C, H264D, H264E, H264F, H264G, H264I, H264K, H264L, H264M, H264N, H264P, H264Q, H264R, H264S, H264T, H264V, H264W, H264Y, S266A, S266C, S266D, S266E, S266F, S266G, S266H, S266I, S266K, S266L, S266M, S266N, S266P, S266Q, S266R, S266T, S266V, S266W, S266Y, K267A, K267C, K267D, K267E, K267F, K267G, K267H, K267I, K267L, K267M, K267N, K267P, K267Q, K267R, K267S, K267T, K267V, K267W, K267Y, R277A, R277D, R277E, R283A, R283D, R283E, V296A, V296C, V296D, V296E, V296F, V296G, V296H, V296I, V296K, V296L, V296M, V296N, V296P, V296Q, V296R, V296S, V296T, V296W, V296Y, K309A, K309D, K309E, K317A, K317D, K317E, K321A, K321D, K321E, R352A, R352D, R352E, R366A, R366D, R366E, K371A, K371D, K371E, K371N, K372A, K372D, K372E, K372N, D373S, I374S, T403N, G404N, H405S, P406S, R441A, R441D, R441E, K444A, K444D, K444E, K452A, K452D, K452E, K461A, K461D, K461E, K469A, K469D, K469E, K470A, K470D, and K470E, with reference to amino acid positions set forth in SEQ ID NO:5. In particular examples, the variant ADA2 protein can include one or more amino acid replacement(s) selected from among K11A, K11E, R20A, R20D, R20E, R219K, R219Q, L221A, L221V, L221G, S262N, H264Q, H264G, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E, K452D and K452E, with reference to amino acid positions set forth in SEQ ID NO:5. In some examples of the combinations provided herein, the variant ADA2 protein can include amino acid

Date Reçue/Date Received 2021-07-28

replacements selected from among K11A/R20A, K11A/R20A/K371A, R20A/K371A, K11A/K371A, S262N/K371D, S262N/K371E, S262N/R20E, S262N/R20E/K371D, S262N/R20E/K371E, R219Q/K371E, R219Q/K371D, R219Q/R20E, R219Q/K371E/R20E, R219Q/K371D/R20E, R219Q/S262N/K371E, R219Q/S262N/K371D, R219Q/S262N/R20E, R219Q/S262N/K371E/R20E, R219Q/S262N/K371D/R20E and R219Q/S262N, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of combinations provided herein, the variant ADA2 protein can include a modification of one or more amino acids in the putative receptor binding (PRB) domain, wherein the modification is an amino acid deletion, insertion or replacement. For example, the variant ADA2 can include deletion of one or more contiguous amino acid residues corresponding to any one or more contiguous amino acid residues between or between about amino acid residues 98 and 156 or amino acid residues 105 and 148, inclusive, with reference to amino acid positions set forth in SEQ ID NO:5. In some embodiments, the variant of ADA2 polypeptide can further include substitution of the deleted region with a peptide linker. For example, the peptide linker can be selected from among (Gly)n (SEQ ID NO:368), where n is 2 to 20; (GGGGS)n (SEQ ID NO:343), where n is 1 to 6; (SSSSG)n (SEQ ID NO:344), where n is 1 to 6; (AlaAlaProAla)n (SEQ ID NO:350), where n is 1 to 6; GKSSGSGSESKS (SEQ ID NO:345); GGSTSGSGKSSEGKG (SEQ ID NO:346); GSTSGSGKSSSEGSGSTKG (SEQ ID NO:347); GSTSGSGKPGSGEGSTKG (SEQ ID NO:348); and EGKSSGSGSESKEF (SEQ ID NO:349). In particular examples, the peptide linker is selected from among GGG (SEQ ID NO:369); GGGGG (SEQ ID NO:360); GGGGGGG (SEQ ID NO:370); GGGGGGGGGG (SEQ ID NO:371); and GGGGGGGGGGGGGGG (SEQ ID NO:372). In some embodiments of combinations provided herein, the modification in the PRB domain of the variant ADA2 polypeptide corresponds to C105-T147del→$(Gly)_n$, where n is 2 to 20, such as C105-T147del→$(Gly)_{15}$, C105-T147del→$(Gly)_{10}$, C105-T147del→$(Gly)_7$, C105-T147del→$(Gly)_5$ or C105-T147del→$(Gly)_3$, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of combinations provided herein, the ADA2 protein in the combination can be glycosylated at one or more native or non-native glycosylation

Date Reçue/Date Received 2021-07-28

site. For example, in some embodiments of combinations provided herein containing a variant ADA2 protein, the variant ADA2 protein in the combination include a modification(s) that alters glycosylation by introduction of a non-native glycosylation site. The non-native glycosylation site is introduced by amino acid replacement(s) or insertion of one, two or three amino acids. For example, the modifications that alter hyperglycosylation can be selected from among modifications corresponding to --→N1/--→A2/--→S3, R20N/V22S, K371N/D373S, K372N/I374S, T403N/H405S and G404N/P406S, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of combinations provided herein, the variant ADA2 polypeptide has the sequence of amino acids set forth in any of SEQ ID NOS:13-63 or 71-285 or a catalytically active portion thereof.

Provided herein are pharmaceutical compositions that can include any of the variant ADA2 proteins or a catalytically active portion thereof provided herein, any variant ADA2 dimer provided herein or any conjugate provided herein, in a pharmaceutically acceptable vehicle. In some embodiments, the pharmaceutical composition can be formulated for local or systemic administration. For example, the pharmaceutical composition is formulated for intravenous administration.

Provided herein are methods of treating a tumor or cancer in a subject, that can include administering to the subject any of the variant ADA2 proteins or a catalytically active portion thereof provided herein, any variant ADA2 dimer provided herein, any conjugate provided herein, or any combination provided herein. Also provided are medical uses or pharmaceutical compositions for use of any of the variant ADA2 proteins or a catalytically active portion thereof provided herein, any variant ADA2 dimer provided herein or any conjugate provided herein for treating a tumor or a cancer in a subject. Also provided are combinations for use of any of the combinations provided herein for use in treating a tumor or cancer.

Also provided herein are methods of treating a tumor or cancer in a subject that can include administering to the subject any ADA2 protein. Also provided are medical uses of an ADA2 protein or pharmaceutical composition for use containing an ADA2 protein for treating a tumor or a cancer. Also provided are combinations for use containing an ADA2 protein and a therapeutic agent for treating a tumor or cancer.

Date Reçue/Date Received 2021-07-28

In some embodiments of methods, uses, pharmaceutical compositions for use or uses provided herein, the tumor can be a solid tumor or a metastatic tumor. In particular examples, the tumor can be a carcinoma, gliomas, sarcoma, adenocarcinoma, adenosarcoma, or adenoma. In some embodiments, the tumor can be a tumor of the breast, heart, lung, small intestine, colon, spleen, kidney, bladder, head and neck, ovary, prostate, brain, pancreas, skin, bone, bone marrow, blood, thymus, uterus, testicles, cervix or liver.

In some embodiments of methods provided herein, the subject can be selected for treatment based on elevated levels of plasma adenosine, tumor-associated expression of adenosine receptor (ADR) or tumor-associated expression of a nucleotidase. In particular examples, the ADR is A2A or A2B. In particular examples, the nucleotidase is CD39 or CD73. In some embodiments of methods provided herein, the elevated level is at least 0.5-fold, 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 15-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 150-fold, 200-fold, 500-fold, 1000-fold or more, compared to the predetermined level or predetermined amount or control sample.

In some embodiments of methods provided herein, the method of treating a tumor or cancer in a subject can further include administration of one or more anticancer agents or treatments. For example, the anti-cancer agent can be selected from among an anti-cancer antibody, a chemotherapeutic agent, a radioimmunotherapeutic, an anti-angiogenic agent and an immune checkpoint inhibitor.

Provided herein are methods of treating a disease or condition in a subject, that can include administering to the subject any of the variant ADA2 proteins or a catalytically active portion thereof provided herein, any variant ADA2 dimer provided herein, any conjugate provided herein, or any combination provided herein for treating a disease or condition that is a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy or Severe Combined Immunodeficiency (SCID). Also provided are medical uses or pharmaceutical compositions for use of any of the variant ADA2 proteins or a catalytically active portion thereof provided herein, any variant ADA2 dimer provided herein or any

conjugate provided herein for treating a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy or Severe Combined Immunodeficiency (SCID) in a subject. Also provided are combinations for use of any of the combinations provided herein for use in treating a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy or Severe Combined Immunodeficiency (SCID). Also provided herein are methods of treating a disease or condition in a subject, that can include administering to the subject any ADA2 protein, for treating a disease or condition that is a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy or Severe Combined Immunodeficiency (SCID). Also provided are medical uses of an ADA2 protein or pharmaceutical compositions for use containing an ADA2 protein for treating a disease or condition that is a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy or Severe Combined Immunodeficiency (SCID). Also provided are combinations for use containing an ADA2 protein and a therapeutic agent for treating a disease or condition that is a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy or Severe Combined Immunodeficiency (SCID)

In some embodiments of methods, uses, pharmaceutical compositions for use or uses provided herein, the ADA2 protein can be a monomer or a dimer. For example, the ADA2 protein can be a dimer, in particular, a homodimer. In some embodiments of methods, uses, pharmaceutical compositions for use or combinations for use provided herein, the ADA2 protein can contain the sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383, a sequence that can exhibit at least 85% sequence identity to the sequence of amino acids set forth in SEQ ID NOS:5, 326-334, 340, 375 or 380-383 or a catalytically active form thereof. For example, the ADA2 protein can contain a sequence of amino acids that can exhibit at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of amino acids set forth in SEQ ID NOS:5, 326-334, 340, 375 or 380-383 or a catalytically active portion thereof. In particular examples, the ADA2 protein can contain a sequence of amino acids that can exhibit at least 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the sequence of amino acids set forth in SEQ ID

Date Reçue/Date Received 2021-07-28

NOS:5 or a catalytically active portion thereof. For example, the ADA2 protein can contain the sequence of amino acids set forth in SEQ ID NO:5.

In some embodiments of methods, uses, pharmaceutical compositions for use, or combinations for use provided herein, the ADA2 protein is a variant ADA2 protein that includes modification(s) in the sequence of amino acids of an unmodified ADA2 polypeptide or a catalytically active portion thereof. In any of such examples, the unmodified ADA2 protein can include the sequence of amino acids set forth in SEQ ID NO:5, or a sequence of amino acids that can exhibit at least 85% sequence identity to the sequence of amino acids set forth in SEQ ID NO:5, or is a catalytically active portion thereof; the amino acid modification(s) are selected from among amino acid replacement(s), deletion(s) and insertion(s); and the variant ADA2 protein, when in dimer form, can exhibit adenosine deaminase activity to convert adenosine to inosine.

In some embodiments of methods, uses, pharmaceutical compositions for use or combinations for use provided herein, the ADA2 protein, when in dimer form, can exhibit a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 5 x 10^3 M^{-1} s^{-1}, 6 x 10^3 M^{-1} s^{-1}, 7 x 10^3 M^{-1} s^{-1}, 8 x 10^3 M^{-1} s^{-1}, 9 x 10^3 M^{-1} s^{-1}, 1 x 10^4 M^{-1} s^{-1}, 2 x 10^4 M^{-1} s^{-1}, 3 x 10^4 M^{-1} s^{-1}, 4 x 10^4 M^{-1} s^{-1}, 5 x 10^4 M^{-1} s^{-1}, 6 x 10^4 M^{-1} s^{-1}, 7 x 10^4 M^{-1} s^{-1}, 8 x 10^4 M^{-1} s^{-1}, 9 x 10^4 M^{-1} s^{-1}, 1 x 10^5 M^{-1} s^{-1}, 2 x 10^5 M^{-1} s^{-1}, 3 x 10^5 M^{-1} s^{-1}, 4 x 10^5 M^{-1} s^{-1}, 5 x 10^5 M^{-1} s^{-1} or greater.

In some embodiments of methods, uses, pharmaceutical compositions for use, or combinations for use provided herein, the modification(s) in the sequence of amino acids of an unmodified ADA2 polypeptide can include an amino acid replacement(s); and the variant ADA2 protein can include one or more amino acid replacement(s) at an amino acid position corresponding to amino acid residue 11, 13, 20, 22, 26, 86, 179, 217, 219, 221, 258, 262, 264, 266, 267, 277, 283, 296, 309, 317, 321, 352, 366, 371, 372, 373, 374, 403, 404, 405, 406, 441, 444, 452, 461, 469 or 470, with reference to amino acid positions set forth in SEQ ID NO:5. For example, the variant ADA2 protein can include one or more amino acid replacement selected from among K11A, K11D, K11E, K13A, K13D, K13E, R20A, R20D, R20E, R20N, V22S, K26A, K26D, K26E, D86A, D86C, D86E, D86F, D86G, D86H, D86I, D86K, D86L, D86M, D86N, D86P, D86Q, D86R, D86S, D86T, D86V, D86W, D86Y, E179A, E179C, E179D, E179F, E179G, E179H, E179I, E179K, E179L, E179M, E179N, E179P, E179Q,

E179R, E179S, E179T, E179V, E179W, E179Y, R217A, R217D, R217E, R219A, R219C, R219D, R219E, R219F, R219G, R219H, R219I, R219K, R219L, R219M, R219N, R219P, R219Q, R219S, R219T, R219V, R219W, R219Y, L221A, L221C, L221D, L221E, L221F, L221G, L221H, L221I, L221K, L221M, L221N, L221P, L221Q, L221R, L221S, L221T, L221V, L221W, L221Y, K258A, K258D, K258E, S262A, S262C, S262D, S262E, S262F, S262G, S262H, S262I, S262K, S262L, S262M, S262N, S262P, S262Q, S262R, S262T, S262V, S262W, S262Y, H264A, H264C, H264D, H264E, H264F, H264G, H264I, H264K, H264L, H264M, H264N, H264P, H264Q, H264R, H654S, H264T, H264V, H264W, H264Y, S266A, S266C, S266D, S266E, S266F, S266G, S266H, S266I, S266K, S266L, S266M, S266N, S266P, S266Q, S266R, S266T, S266V, S266W, S266Y, K267A, K267C, K267D, K267E, K267F, K267G, K267H, K267I, K267L, K267M, K267N, K267P, K267Q, K267R, K267S, K267T, K267V, K267W, K267Y, R277A, R277D, R277E, R283A, R283D, R283E, V296A, V296C, V296D, V296E, V296F, V296G, V296H, V296I, V296K, V296L, V296M, V296N, V296P, V296Q, V296R, V296S, V296T, V296W, V296Y, K309A, K309D, K309E, K317A, K317D, K317E, K321A, K321D, K321E, R352A, R352D, R352E, R366A, R366D, R366E, K371A, K371D, K371E, K371N, K372A, K372D, K372E, K372N, D373S, I374S, T403N, G404N, H405S, P406S, R441A, R441D, R441E, K444A, K444D, K444E, K452A, K452D, K452E, K461A, K461D, K461E, K469A, K469D, K469E, K470A, K470D, and K470E, with reference to amino acid positions set forth in SEQ ID NO:5.

In particular examples, the variant ADA2 protein can include one or more amino acid replacement(s) selected from among K11A, K11E, R20A, R20D, R20E, R219K, R219Q, L221A, L221V, L221G, S262N, H264Q, H264G, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E, K452D and K452E, with reference to amino acid positions set forth in SEQ ID NO:5. In some embodiments of methods, uses, pharmaceutical compositions for use or combinations for use provided herein, the variant ADA2 protein can include amino acid replacements selected from among K11A/R20A, K11A/R20A/K371A, R20A/K371A, K11A/K371A, S262N/K371D, S262N/K371E, S262N/R20E, S262N/R20E/K371D, S262N/R20E/K371E, R219Q/K371E, R219Q/K371D, R219Q/R20E, R219Q/K371E/R20E, R219Q/K371D/R20E, R219Q/S262N/K371E,

Date Reçue/Date Received 2021-07-28

R219Q/S262N/K371D, R219Q/S262N/R20E, R219Q/S262N/K371E/R20E, R219Q/S262N/K371D/R20E and R219Q/S262N, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of methods, uses, pharmaceutical compositions for use or combinations for use provided herein, the variant ADA2 protein can include a modification of one or more amino acids in the putative receptor binding (PRB) domain, such as an amino acid deletion, insertion or replacement. For example, the variant ADA2 can include deletion of one or more contiguous amino acid residues corresponding to any one or more contiguous amino acid residues between or between about amino acid residues 98 and 156 or amino acid residues 105 and 148, inclusive, with reference to amino acid positions set forth in SEQ ID NO:5. In any of such examples of methods, uses, pharmaceutical compositions for use or combinations for use provided herein, the variant of ADA2 protein can further include substitution of the deleted region with a peptide linker. For example, the peptide linker is selected from among (Gly)n (SEQ ID NO:368), where n is 2 to 20; (GGGGS)n (SEQ ID NO:343), where n is 1 to 6; (SSSSG)n (SEQ ID NO:344), where n is 1 to 6; (AlaAlaProAla)n (SEQ ID NO:350), where n is 1 to 6; GKSSGSGSESKS (SEQ ID NO:345); GGSTSGSGKSSEGKG (SEQ ID NO:346); GSTSGSGKSSSEGSGSTKG (SEQ ID NO:347); GSTSGSGKPGSGEGSTKG (SEQ ID NO:348); and EGKSSGSGSESKEF (SEQ ID NO:349). In particular examples the peptide linker is selected from among GGG (SEQ ID NO:369); GGGGG (SEQ ID NO:360); GGGGGGG (SEQ ID NO:370); GGGGGGGGGG (SEQ ID NO:371); and GGGGGGGGGGGGGGG (SEQ ID NO:372).

For example, the modification in the PRB domain of the ADA2 protein corresponds to C105-T147del→$(Gly)_n$, where n is 2 to 20, such as C105-T147del→$(Gly)_{15}$, C105-T147del→$(Gly)_{10}$, C105-T147del→$(Gly)_7$, C105-T147del→$(Gly)_5$ or C105-T147del→$(Gly)_3$, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of methods, uses, pharmaceutical compositions for use or combinations for use provided herein, the ADA2 protein can be glycosylated at one or more native or non-native glycosylation sites. For example, in some embodiments provided herein containing a variant ADA2 protein, the variant ADA2 protein includes a modification(s) that alters glycosylation by introduction of a non-native

Date Reçue/Date Received 2021-07-28

glycosylation site. For example, the non-native glycosylation site is introduced by amino acid replacement(s) or insertion of one, two or three amino acids. In particular examples, the modifications that alter glycosylation are selected from among modifications corresponding to ---→N1/---→A2/---→S3, R20N/V22S, K371N/D373S, K372N/I374S, T403N/H405S and G404N/P406S, with reference to amino acid positions set forth in SEQ ID NO:5.

In some embodiments of methods, uses, pharmaceutical compositions for use or combinations for use provided herein, the variant ADA2 can include a polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS:13-63 or 71-285 or a catalytically active portion thereof.

In some embodiments of methods, uses, pharmaceutical compositions for use or uses provided herein, the subject can be a mammal, in particular a human. In some embodiments of methods provided herein, the pharmaceutical composition can be administered parenterally, locally, or systemically. For example, the pharmaceutical composition can be administered intranasally, intramuscularly, intradermally, intraperitoneally, intravenously, subcutaneously, orally, or by pulmonary administration.

In some embodiments, in the variant ADA2 protein or catalytically active portion thereof provided herein, the variant ADA2 protein in the methods, compositions, conjugates, modified forms, vectors, cells, combinations, uses and compositions for use, and the nucleic acids encoding the variant ADA2 provided herein and vectors that include the nucleic acids, the modifications can be from any one or more of the following amino acid replacement(s), insertion(s), deletion(s), and any combination thereof. The modification(s) listed below are with reference to mature numbering, as set forth in the amino acid positions set forth in SEQ ID NO:5. Exemplary of ADA2 variants provided herein are the following; it is understood that the different types of mutants (amino acid modifications) can be combined to exploit the properties of each type of mutation. It is understood by those of skill in the art that, in general, the effects of mutations in proteins are at least additive, and can be synergistic.

Date Reçue/Date Received 2021-07-28

1. **Heparin binding mutants**

The following modifications can confer reduced heparin binding. Binding to heparin can deplete circulating levels of administered ADA2. Thus, the following ADA2 variants can increase the bioavailability and pharmacokinetics of the administered ADA2:

K11A; K11D; K11E; K13A; K13D; K13E; K371A; K371D; K371E; K372A; K372D; K372E; K452A; K452D; K452E; R20A; R20D; R20E; R366A; R366D; R366E; K26A; K26D; K26E; R217A; R217D; R217E; K258A; K258D; K258E; R277A; R277D; R277E; R283A; R283D; R283E; K309A; K309D; K309E; K317A; K317D; K317E; K321A; K321D; K321E; R352A; R352D; R352E; R441A; R441D; R441E; K444A; K444D; K444E; K461A; K461D; K461E; K469A; K469D; K469E; K470A; K470D; and K470E.

Examples of heparin binding mutants containing these replacements.

K11A (SEQ ID NO:13); K11D (SEQ ID NO:14); K11E (SEQ ID NO:15); K13A (SEQ ID NO:16); K13D (SEQ ID NO:17); K13E (SEQ ID NO:18); K371A (SEQ ID NO:19); K371D (SEQ ID NO:20); K371E (SEQ ID NO:21); K372A (SEQ ID NO:22); K372D (SEQ ID NO:23); K372E (SEQ ID NO:24); K452A (SEQ ID NO:25); K452D (SEQ ID NO:26); K452E (SEQ ID NO:27); R20A (SEQ ID NO:28); R20D (SEQ ID NO:29); R20E (SEQ ID NO:30); R366A (SEQ ID NO:31); R366D (SEQ ID NO:32); R366E (SEQ ID NO:33); K26A (SEQ ID NO:71); K26D (SEQ ID NO:72); K26E (SEQ ID NO:73); R217A (SEQ ID NO:74); R217D (SEQ ID NO:75); R217E (SEQ ID NO:76); K258A (SEQ ID NO:77); K258D (SEQ ID NO:78); K258E (SEQ ID NO:79); R277A (SEQ ID NO:80); R277D (SEQ ID NO:81); R277E (SEQ ID NO:82); R283A (SEQ ID NO:83); R283D (SEQ ID NO:84); R283E (SEQ ID NO:85); K309A (SEQ ID NO:86); K309D (SEQ ID NO:87); K309E (SEQ ID NO:88); K317A (SEQ ID NO:89); K317D (SEQ ID NO:90); K317E (SEQ ID NO:91); K321A (SEQ ID NO:92); K321D (SEQ ID NO:93); K321E (SEQ ID NO:94); R352A (SEQ ID NO:95); R352D (SEQ ID NO:96); R352E (SEQ ID NO:97); R441A (SEQ ID NO:98); R441D (SEQ ID NO:99); R441E (SEQ ID NO:100); K444A (SEQ ID NO:101); K444D (SEQ ID NO:102); K444E (SEQ ID NO:103); K461A (SEQ ID NO:104); K461D (SEQ ID NO:105); K461E (SEQ ID NO:106); K469A (SEQ ID NO:107); K469D (SEQ ID NO:108); K469E (SEQ ID

NO:109); K470A (SEQ ID NO:110); K470D (SEQ ID NO:111); and K470E (SEQ ID NO:112).

2. Active site mutants

The following modifications can confer increased catalytic efficiency. The modifications are in select residues of the active site, and can effect improved catalytic efficiency (k_{cat}/K_m) for adenosine. Binding to heparin can deplete circulating levels of administered ADA2. Thus, the following ADA2 variants can confer increased adenosine deaminase activity:

H264A; H264Q; H264N; H264G; R219K; R219Q; R219N; R219A; L221A; L221V; L221G; E179D; E179A; E179S; E179T; E179V; E179G; S262A; S262V; S262M; S262N; D86A; D86C; D86E; D86F; D86G; D86H; D86I; D86K; D86L; D86M; D86N; D86P; D86Q; D86R; D86S; D86T; D86V; D86W; D86Y; E179C; E179F; E179H; E179I; E179K; E179L; E179M; E179N; E179P; E179Q; E179R; E179W; E179Y; R219C; R219D; R219E; R219F; R219G; R219H; R219I; R219L; R219M; R219P; R219S; R219T; R219V; R219W; R219Y; L221C; L221D; L221E; L221F; L221H; L221I; L221K; L221M; L221N; L221P; L221Q; L221R; L221S; L221T; L221W; L221Y; S262C; S262D; S262E; S262F; S262G; S262H; S262I; S262K; S262L; S262P; S262Q; S262R; S262T; S262W; S262Y; H264C; H264D; H264E; H264F; H264I; H264K; H264L; H264M; H264P; H264R; H264S; H264T; H264V; H264W; H264Y; S266A; S266C; S266D; S266E; S266F; S266G; S266H; S266I; S266K; S266L; S266M; S266N; S266P; S266Q; S266R; S266T; S266V; S266W; S266Y; K267A; K267C; K267D; K267E; K267F; K267G; K267H; K267I; K267L; K267M; K267N; K267P; K267Q; K267R; K267S; K267T; K267V; K267W; K267Y; V296A; V296C; V296D; V296E; V296F; V296G; V296H; V296I; V296K; V296L; V296M; V296N; V296P; V296Q; V296R; V296S; V296T; V296W; and V296Y.

Examples of Active site mutants containing these replacements:

H264A (SEQ ID NO:34); H264Q (SEQ ID NO:35); H264N (SEQ ID NO:36); H264G (SEQ ID NO:37); R219K (SEQ ID NO:38); R219Q (SEQ ID NO:39); R219N (SEQ ID NO:40); R219A (SEQ ID NO:41); L221A (SEQ ID NO:42); L221V (SEQ ID NO:43); L221G (SEQ ID NO:44); E179D (SEQ ID NO:45); E179A (SEQ ID NO:46); E179S (SEQ ID NO:47); E179T (SEQ ID NO:48); E179V (SEQ ID NO:49); E179G

(SEQ ID NO:50); S262A (SEQ ID NO:51); S262V (SEQ ID NO:52); S262M (SEQ ID NO:53); S262N (SEQ ID NO:54); D86A (SEQ ID NO:113); D86C (SEQ ID NO:114); D86E (SEQ ID NO:115); D86F (SEQ ID NO:116); D86G (SEQ ID NO:117); D86H (SEQ ID NO:118); D86I (SEQ ID NO:119); D86K (SEQ ID NO:120); D86L (SEQ ID NO:121); D86M (SEQ ID NO:122); D86N (SEQ ID NO:123); D86P (SEQ ID NO:124); D86Q (SEQ ID NO:125); D86R (SEQ ID NO:126); D86S (SEQ ID NO:127); D86T (SEQ ID NO:128); D86V (SEQ ID NO:129); D86W (SEQ ID NO:130); D86Y (SEQ ID NO:131); E179C (SEQ ID NO:132); E179F (SEQ ID NO:133); E179H (SEQ ID NO:134); E179I (SEQ ID NO:135); E179K (SEQ ID NO:136); E179L (SEQ ID NO:137); E179M (SEQ ID NO:138); E179N (SEQ ID NO:139); E179P (SEQ ID NO:140); E179Q (SEQ ID NO:141); E179R (SEQ ID NO:142); E179W (SEQ ID NO:143); E179Y (SEQ ID NO:144); R219C (SEQ ID NO:145); R219D (SEQ ID NO:146); R219E (SEQ ID NO:147); R219F (SEQ ID NO:148); R219G (SEQ ID NO:149); R219H (SEQ ID NO:150); R219I (SEQ ID NO:151); R219L (SEQ ID NO:152); R219M (SEQ ID NO:153); R219P (SEQ ID NO:154); R219S (SEQ ID NO:155); R219T (SEQ ID NO:156); R219V (SEQ ID NO:157); R219W (SEQ ID NO:158); R219Y (SEQ ID NO:159); L221C (SEQ ID NO:160); L221D (SEQ ID NO:161); L221E (SEQ ID NO:162); L221F (SEQ ID NO:163); L221H (SEQ ID NO:164); L221I (SEQ ID NO:165); L221K (SEQ ID NO:166); L221M (SEQ ID NO:167); L221N (SEQ ID NO:168); L221P (SEQ ID NO:169); L221Q (SEQ ID NO:170); L221R (SEQ ID NO:171); L221S (SEQ ID NO:172); L221T (SEQ ID NO:173); L221W (SEQ ID NO:174); L221Y (SEQ ID NO:175); S262C (SEQ ID NO:176); S262D (SEQ ID NO:177); S262E (SEQ ID NO:178); S262F (SEQ ID NO:179); S262G (SEQ ID NO:180); S262H (SEQ ID NO:181); S262I (SEQ ID NO:182); S262K (SEQ ID NO:183); S262L (SEQ ID NO:184); S262P (SEQ ID NO:185); S262Q (SEQ ID NO:186); S262R (SEQ ID NO:187); S262T (SEQ ID NO:188); S262W (SEQ ID NO:189); S262Y (SEQ ID NO:190); H264C (SEQ ID NO:191); H264D (SEQ ID NO:192); H264E (SEQ ID NO:193); H264F (SEQ ID NO:194); H264I (SEQ ID NO:195); H264K (SEQ ID NO:196); H264L (SEQ ID NO:197); H264M (SEQ ID NO:198); H264P (SEQ ID NO:199); H264R (SEQ ID NO:200); H264S (SEQ ID NO:201); H264T (SEQ ID NO:202); H264V (SEQ ID NO:203); H264W (SEQ ID

NO:204); H264Y (SEQ ID NO:205); S266A (SEQ ID NO:206); S266C (SEQ ID NO:207); S266D (SEQ ID NO:208); S266E (SEQ ID NO:209); S266F (SEQ ID NO:210); S266G (SEQ ID NO:211); S266H (SEQ ID NO:212); S266I (SEQ ID NO:213); S266K (SEQ ID NO:214); S266L (SEQ ID NO:215); S266M (SEQ ID NO:216); S266N (SEQ ID NO:217); S266P (SEQ ID NO:218); S266Q (SEQ ID NO:219); S266R (SEQ ID NO:220); S266T (SEQ ID NO:221); S266V (SEQ ID NO:222); S266W (SEQ ID NO:223); S266Y (SEQ ID NO:224); K267A (SEQ ID NO:225); K267C (SEQ ID NO:226); K267D (SEQ ID NO:227); K267E (SEQ ID NO:228); K267F (SEQ ID NO:229); K267G (SEQ ID NO:230); K267H (SEQ ID NO:231); K267I (SEQ ID NO:232); K267L (SEQ ID NO:233); K267M (SEQ ID NO:234); K267N (SEQ ID NO:235); K267P (SEQ ID NO:236); K267Q (SEQ ID NO:237); K267R (SEQ ID NO:238); K267S (SEQ ID NO:239); K267T (SEQ ID NO:240); K267V (SEQ ID NO:241); K267W (SEQ ID NO:242); K267Y (SEQ ID NO:243); V296A (SEQ ID NO:244); V296C (SEQ ID NO:245); V296D (SEQ ID NO:246); V296E (SEQ ID NO:247); V296F (SEQ ID NO:248); V296G (SEQ ID NO:249); V296H (SEQ ID NO:250); V296I (SEQ ID NO:251); V296K (SEQ ID NO:252); V296L (SEQ ID NO:253); V296M (SEQ ID NO:254); V296N (SEQ ID NO:255); V296P (SEQ ID NO:256); V296Q (SEQ ID NO:257); V296R (SEQ ID NO:258); V296S (SEQ ID NO:259); V296T (SEQ ID NO:260); V296W (SEQ ID NO:261); and V296Y (SEQ ID NO:262).

3. Hyperglycosylation mutants

The following modifications introduce a non-native glycosylation site in ADA2. Introduction of non-native glycosylation sites, such as N-linked glycosylation sites, can confer an increase in stability and pharmacokinetic profiles. Thus, the following ADA2 variants can effect hyperglycosylation of ADA2, and increase the stability and pharmacokinetic profiles of the administered ADA2:

---→N1/---→A2/---→S3 ; R20N/V22S; K371N/D373S; K372N/I374S; T403N/H405S; and G404N/P406S.

Examples of Hyperglycosylation mutants containing these replacements: ---→N1/---→A2/---→S3 (SEQ ID NO:274); R20N/V22S (SEQ ID NO:275); K371N/D373S (SEQ ID NO:276); K372N/I374S (SEQ ID NO:277); T403N/H405S (SEQ ID NO:278); and G404N/P406S (SEQ ID NO:279).

4. PRB deletion and replacement mutants

The following variants contain a modified PRB domain. The modifications of the PRB domain can include deletion of all or a portion of the PRB domain (i.e. deletion of one or more residues of the PRB domain), insertion of one or more amino acid residues into the PRB domain, amino acid replacement of one or more residues of the PRB domain or a combination thereof. Deletion and/or substitution of the PRB domain can confer altered activity, e.g., reduction in binding to a receptor and/or the activity mediated by the receptor.

C105-T147del→(Gly)n, where n = 2 to 20; C105-T147del→$(Gly)_{15}$; C105-T147del→(Gly)10; C105-T147del→$(Gly)_7$; C105-T147del→$(Gly)_5$; C105-T147del→$(Gly)_3$; N98-N156del; C105-E148del; C105-T147del; V99-Q144del; V99-Q144del→(GGGGS)n, where n = 1 to 5; C105-T147del→(GGGGS)n, where n = 1 to 5; V99-Q144del→$(GGGGS)_1$; V99-Q144del→$(GGGGS)_2$; V99-Q144del→$(GGGGS)_3$; C105-T147del→$(GGGGS)_1$; C105-T147del→$(GGGGS)_2$; and C105-T147del→$(GGGGS)_3$.

Examples of PRB deletion and replacement mutants containing these replacements:

C105-T147del→(Gly)n (SEQ ID NO:280); C105-T147del→$(Gly)_{15}$ (SEQ ID NO:281); C105-T147del→$(Gly)_{10}$ (SEQ ID NO:282); C105-T147del→$(Gly)_7$ (SEQ ID NO:283); C105-T147del→$(Gly)_5$ (SEQ ID NO:284); C105-T147del→$(Gly)_3$ (SEQ ID NO:285); N98-N156del (SEQ ID NO:548); C105-E148del (SEQ ID NO:549); C105-T147del (SEQ ID NO:550); V99-Q144del (SEQ ID NO:579); V99-Q144del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:581); C105-T147del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:582); V99-Q144del→$(GGGGS)_1$ (SEQ ID NO:583); V99-Q144del→$(GGGGS)_2$ (SEQ ID NO:584); V99-Q144del→$(GGGGS)_3$ (SEQ ID NO:585); C105-T147del→$(GGGGS)_1$ (SEQ ID NO:586); C105-T147del→$(GGGGS)_2$ (SEQ ID NO:587); and C105-T147del→$(GGGGS)_3$ (SEQ ID NO:588)

5. PRB hyperglycosylation mutants

The following modifications can introduce a non-native glycosylation site in the PRB domain. Introduction of non-native glycosylation sites, such as N-linked glycosylation sites, in the PRB domain can confer an increase in stability and

Date Reçue/Date Received 2021-07-28

pharmacokinetic profiles and/or other activities, e.g., reduction in binding to a receptor. Thus, the following ADA2 variants can effect hyperglycosylation of the ADA2 in the PRB domain, reduce receptor binding, and increase the stability and pharmacokinetic profiles of the administered ADA2:

R125N/P126A; S127N/K129S; P126N/E128T; R112N/I114T; I134N/L135C/L136T; I134N/L135S/L136T; R142N/Q144S; E137N/Y139T; and P111N/G113S.

Examples of PRB hyperglycosylation mutants containing these replacements:

R125N/P126A (SEQ ID NO:552); S127N/K129S (SEQ ID NO:553); P126N/E128T (SEQ ID NO:554); R112N/I114T (SEQ ID NO:555); I134N/L135C/L136T (SEQ ID NO:556); I134N/L135S/L136T (SEQ ID NO:557); R142N/Q144S (SEQ ID NO:558); E137N/Y139T (SEQ ID NO:559); and P111N/G113S (SEQ ID NO:560).

6. PRB-ADA domain interaction mutants

The following modifications can confer altered interaction between the PRB domain and the rest of ADA2 (e.g., the adenosine deaminase (ADA) domain). Altering the interaction between the PRB domain and the rest of ADA2, such as the ADA domain, can confer an activity, e.g., an increase in the adenosine deaminse activity and a reduction in receptor binding:

F119S; F119K; Y224R; Y224N; Y191S; Y191D; F183K; Y191D/Y224R; F109S; F109A; R118D; R118A; Y139T; Y139A; W133S; W133T; P124A; and P124S.

Examples of PRB-ADA domain interaction mutants containing these replacements:

F119S (SEQ ID NO:561); F119K (SEQ ID NO:562); Y224R (SEQ ID NO:563); Y224N (SEQ ID NO:564); Y191S (SEQ ID NO:565); Y191D (SEQ ID NO:566); F183K (SEQ ID NO:567); Y191D/Y224R (SEQ ID NO:568); F109S (SEQ ID NO:569); F109A (SEQ ID NO:570); R118D (SEQ ID NO:571); R118A (SEQ ID NO:572); Y139T (SEQ ID NO:573); Y139A (SEQ ID NO:574); W133S (SEQ ID NO:575); W133T (SEQ ID NO:576); P124A (SEQ ID NO:577); and P124S (SEQ ID NO:578).

Date Reçue/Date Received 2021-07-28

7. Combinations of mutations with hyperglycosylation mutants

The following variants combine modifications that effect improved catalytic efficiency (k_{cat}/K_m) for adenosine, such as R219Q and/or S262N, with modifications that introduce non-native glycosylation sites:

R219Q/S262N/--→N1/--→A2/--→S3 ; R219Q/S262N/R20N/V22S; R219Q/S262N/K371N/D373S; R219Q/S262N/K372N/I374S; R219Q/S262N/T403N/H405S; and R219Q/S262N/G404N/P406S.

Combination with hyperglycosylation mutants containing these replacements:

R219Q/S262N/--→N1/--→A2/--→S3 (SEQ ID NO:596); R219Q/S262N/R20N/V22S (SEQ ID NO:597); R219Q/S262N/K371N/D373S (SEQ ID NO:598); R219Q/S262N/K372N/I374S (SEQ ID NO:599); R219Q/S262N/T403N/H405S (SEQ ID NO:600); and R219Q/S262N/G404N/P406S (SEQ ID NO:601).

8. Combinations of muations with PRB hyperglycosylation mutants

The following variants combine modifications that effect improved catalytic efficiency (k_{cat}/K_m) for adenosine, such as R219Q and/or S262N, with modifications that introduce non-native glycosylation sites in the PRB domain:

R219Q/S262N/R125N/P126A; R219Q/S262N/S127N/K129S; R219Q/S262N/P126N/E128T; R219Q/S262N/R112N/I114T; R219Q/S262N/I134N/L135C/L136T; R219Q/S262N/I134N/L135S/L136T; R219Q/S262N/R142N/Q144S; R219Q/S262N/E137N/Y139T; and R219Q/S262N/P111N/G113S.

Examples of combinations with PRB hyperglycosylation mutants containing these replacements:

R219Q/S262N/R125N/P126A (SEQ ID NO:607); R219Q/S262N/S127N/K129S (SEQ ID NO:608); R219Q/S262N/P126N/E128T (SEQ ID NO:609); R219Q/S262N/R112N/I114T (SEQ ID NO:610); R219Q/S262N/I134N/L135C/L136T (SEQ ID NO:611); R219Q/S262N/I134N/L135S/L136T (SEQ ID NO:612); R219Q/S262N/R142N/Q144S (SEQ ID NO:613); R219Q/S262N/E137N/Y139T (SEQ ID NO:614); and R219Q/S262N/P111N/G113S (SEQ ID NO:615).

9. Combinations with PRB-ADA domain interaction mutants

The following variants combine modifications that effect improved catalytic efficiency (k_{cat}/K_m) for adenosine, such as R219Q and/or S262N, with modifications that alter the interaction between the PRB domain and the rest of ADA2 (e.g., the adenosine deaminase (ADA) domain):

R219Q/S262N/F119S; R219Q/S262N/F119K; R219Q/S262N/Y224R; R219Q/S262N/Y224N; R219Q/S262N/Y191S; R219Q/S262N/Y191D; R219Q/S262N/F183K; R219Q/S262N/Y191D/Y224R; R219Q/S262N/F109S; R219Q/S262N/F109A; R219Q/S262N/R118D; R219Q/S262N/R118A; R219Q/S262N/Y139T; R219Q/S262N/Y139A; R219Q/S262N/W133S; R219Q/S262N/W133T; R219Q/S262N/P124A; and R219Q/S262N/P124S.

Combinations with PRB-ADA domain interaction mutants containing these replacements:

R219Q/S262N/F119S (SEQ ID NO:616); R219Q/S262N/F119K (SEQ ID NO:617); R219Q/S262N/Y224R (SEQ ID NO:618); R219Q/S262N/Y224N (SEQ ID NO:619); R219Q/S262N/Y191S (SEQ ID NO:620); R219Q/S262N/Y191D (SEQ ID NO:621); R219Q/S262N/F183K (SEQ ID NO:622); R219Q/S262N/Y191D/Y224R (SEQ ID NO:623); R219Q/S262N/F109S (SEQ ID NO:624); R219Q/S262N/F109A (SEQ ID NO:625); R219Q/S262N/R118D (SEQ ID NO:626); R219Q/S262N/R118A (SEQ ID NO:627); R219Q/S262N/Y139T (SEQ ID NO:628); R219Q/S262N/Y139A (SEQ ID NO:629); R219Q/S262N/W133S (SEQ ID NO:630); R219Q/S262N/W133T (SEQ ID NO:631); R219Q/S262N/P124A (SEQ ID NO:632); and R219Q/S262N/P124S (SEQ ID NO:633).

10. Combinations with PRB deletion mutants

The following variants combine modifications that effect improved catalytic efficiency (k_{cat}/K_m) for adenosine, such as R219Q and/or S262N, and/or modifications that confer reduced heparin binding, such as K371D, with modifications, e.g., deletions, insertions, substitutions, and/or replacements, in the PRB domain:

K371D/V99-Q144del→$(GGGGS)_1$; K371D/V99-Q144del→$(GGGGS)_2$; K371D/V99-Q144del→$(GGGGS)_3$; K371D/C105-T147del→$(GGGGS)_1$; K371D/C105-T147del→$(GGGGS)_2$; K371D/C105-T147del→$(GGGGS)_3$; R219Q/S262N/C105-T147del→$(Gly)_{15}$; R219Q/S262N/C105-T147del→$(Gly)_{10}$;

R219Q/S262N/C105-T147del→$(Gly)_7$; R219Q/S262N/C105-T147del→$(Gly)_5$; R219Q/S262N/C105-T147del→$(Gly)_3$; R219Q/S262N/V99-Q144del→$(GGGGS)_1$; R219Q/S262N/V99-Q144del→$(GGGGS)_2$; R219Q/S262N/V99-Q144del→$(GGGGS)_3$; R219Q/S262N/C105-T147del→$(GGGGS)_1$; R219Q/S262N/C105-T147del→$(GGGGS)_2$; R219Q/S262N/C105-T147del→$(GGGGS)_3$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_1$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_2$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_3$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_1$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_2$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_3$; K371D/C105-T147del→(Gly)n, where n = 2 to 20; K371D/C105-T147del→$(Gly)_{15}$; K371D/C105-T147del→$(Gly)_{10}$; K371D/C105-T147del→$(Gly)_7$; K371D/C105-T147del→$(Gly)_5$; K371D/C105-T147del→$(Gly)_3$; K371D/V99-Q144del→(GGGGS)n, where n = 1 to 5; K371D/C105-T147del→(GGGGS)n, where n = 1 to 5; K371D/N98-N156del; K371D/C105-E148del; K371D/C105-T147del; K371D/V99-Q144del; R219Q/S262N/C105-T147del→(Gly)n, where n = 2 to 20; R219Q/S262N/V99-Q144del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/C105-T147del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/N98-N156del; R219Q/S262N/C105-E148del; R219Q/S262N/C105-T147del; R219Q/S262N/V99-Q144del; R219Q/S262N/K371D/C105-T147del→(Gly)n, where n = 2 to 20; R219Q/S262N/K371D/C105-T147del→$(Gly)_{15}$; R219Q/S262N/K371D/C105-T147del→$(Gly)_{10}$; R219Q/S262N/K371D/C105-T147del→$(Gly)_7$; R219Q/S262N/K371D/C105-T147del→$(Gly)_5$; R219Q/S262N/K371D/C105-T147del→$(Gly)_3$; R219Q/S262N/K371D/V99-Q144del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/K371D/C105-T147del→(GGGGS)n, where n = 1 to 5; R219Q/S262N/K371D/N98-N156del; R219Q/S262N/K371D/C105-E148del; R219Q/S262N/K371D/C105-T147del; R219Q/S262N/K371D/V99-Q144del; R219Q/C105-T147del→(Gly)n, where n = 2 to 20; R219Q/V99-Q144del→(GGGGS)n, where n = 1 to 5; R219Q/C105-T147del→(GGGGS)n, where n = 1 to 5; R219Q/N98-N156del; R219Q/C105-E148del; R219Q/C105-T147del; R219Q/V99-Q144del; S262N/C105-T147del→(Gly)n, where n = 2 to 20; S262N/V99-Q144del→(GGGGS)n, where n = 1

to 5; S262N/C105-T147del→(GGGGS)n, where n = 1 to 5; S262N/N98-N156del; and S262N/C105-E148del; S262N/C105-T147del; and S262N/V99-Q144del.

Exampes of combination with PRB deletion mutants containing these replacements:

K371D/V99-Q144del→$(GGGGS)_1$ (SEQ ID NO:589); K371D/V99-Q144del→$(GGGGS)_2$ (SEQ ID NO:590); K371D/V99-Q144del→$(GGGGS)_3$ (SEQ ID NO:591); K371D/C105-T147del→$(GGGGS)_1$ (SEQ ID NO:592); K371D/C105-T147del→$(GGGGS)_2$ (SEQ ID NO:593); K371D/C105-T147del→$(GGGGS)_3$ (SEQ ID NO:594); R219Q/S262N/C105-T147del→$(Gly)_{15}$ (SEQ ID NO:602); R219Q/S262N/C105-T147del→$(Gly)_{10}$ (SEQ ID NO:603); R219Q/S262N/C105-T147del→$(Gly)_7$ (SEQ ID NO:604); R219Q/S262N/C105-T147del→$(Gly)_5$ (SEQ ID NO:605); R219Q/S262N/C105-T147del→$(Gly)_3$ (SEQ ID NO:606); R219Q/S262N/V99-Q144del→$(GGGGS)_1$ (SEQ ID NO:634); R219Q/S262N/V99-Q144del→$(GGGGS)_2$ (SEQ ID NO:635); R219Q/S262N/V99-Q144del→$(GGGGS)_3$ (SEQ ID NO:636); R219Q/S262N/C105-T147del→$(GGGGS)_1$ (SEQ ID NO:637); R219Q/S262N/C105-T147del→$(GGGGS)_2$ (SEQ ID NO:638); R219Q/S262N/C105-T147del→$(GGGGS)_3$ (SEQ ID NO:639); R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_1$ (SEQ ID NO:640); R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_2$ (SEQ ID NO:641); R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_3$ (SEQ ID NO:642); R219Q/S262N/K371D/C105-T147del→$(GGGGS)_1$ (SEQ ID NO:643); R219Q/S262N/K371D/C105-T147del→$(GGGGS)_2$ (SEQ ID NO:644); R219Q/S262N/K371D/C105-T147del→$(GGGGS)_3$ (SEQ ID NO:645); K371D/C105-T147del→(Gly)n, where n = 2 to 20 (SEQ ID NO:646); K371D/C105-T147del→$(Gly)_{15}$ (SEQ ID NO:647); K371D/C105-T147del→$(Gly)_{10}$ (SEQ ID NO:648); K371D/C105-T147del→$(Gly)_7$ (SEQ ID NO:649); K371D/C105-T147del→$(Gly)_5$ (SEQ ID NO:650); K371D/C105-T147del→$(Gly)_3$ (SEQ ID NO:651); K371D/V99-Q144del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:652); K371D/C105-T147del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:653); K371D/N98-N156del (SEQ ID NO:654); K371D/C105-E148del (SEQ ID NO:655); K371D/C105-T147del (SEQ ID NO:656); K371D/V99-Q144del (SEQ ID NO:657); R219Q/S262N/C105-T147del→(Gly)n, where n = 2 to 20 (SEQ ID NO:658); R219Q/S262N/V99-Q144del→(GGGGS)n, where n = 1 to 5 (SEQ ID

NO:664); R219Q/S262N/C105-T147del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:665); R219Q/S262N/N98-N156del (SEQ ID NO:666); R219Q/S262N/C105-E148del (SEQ ID NO:667); R219Q/S262N/C105-T147del (SEQ ID NO:668); R219Q/S262N/V99-Q144del (SEQ ID NO:669); R219Q/S262N/K371D/C105-T147del→(Gly)n, where n = 2 to 20 (SEQ ID NO:670); R219Q/S262N/K371D/C105-T147del→$(Gly)_{15}$ (SEQ ID NO:671); R219Q/S262N/K371D/C105-T147del→$(Gly)_{10}$ (SEQ ID NO:672); R219Q/S262N/K371D/C105-T147del→$(Gly)_7$ (SEQ ID NO:673); R219Q/S262N/K371D/C105-T147del→$(Gly)_5$ (SEQ ID NO:674); R219Q/S262N/K371D/C105-T147del→$(Gly)_3$ (SEQ ID NO:675); R219Q/S262N/K371D/V99-Q144del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:676); R219Q/S262N/K371D/C105-T147del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:677); R219Q/S262N/K371D/N98-N156del (SEQ ID NO:678); R219Q/S262N/K371D/C105-E148del (SEQ ID NO:679); R219Q/S262N/K371D/C105-T147del (SEQ ID NO:680); R219Q/S262N/K371D/V99-Q144del (SEQ ID NO:681); R219Q/C105-T147del→(Gly)n, where n = 2 to 20 (SEQ ID NO:918); R219Q/V99-Q144del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:919); R219Q/C105-T147del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:920); R219Q/N98-N156del (SEQ ID NO:921); R219Q/C105-E148del (SEQ ID NO:922); R219Q/C105-T147del (SEQ ID NO:923); R219Q/V99-Q144del (SEQ ID NO:924); S262N/C105-T147del→(Gly)n, where n = 2 to 20 (SEQ ID NO:925); S262N/V99-Q144del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:926); S262N/C105-T147del→(GGGGS)n, where n = 1 to 5 (SEQ ID NO:927); S262N/N98-N156del (SEQ ID NO:928); S262N/C105-E148del (SEQ ID NO:929); S262N/C105-T147del (SEQ ID NO:930); and S262N/V99-Q144del (SEQ ID NO:931).

11. Other combination mutants

The following variants combine various modifications, such as modifications that effect improved catalytic efficiency (k_{cat}/K_m) for adenosine, such as R219Q and/or S262N, modifications that confer reduced heparin binding, such as K371D, and other modifications:

K11A/R20A; K11A/R20A/K371A; R20A/K371A; K11A/K371A; S262N/K371D; S262N/K371E; S262N/R20E; S262N/R20E/K371D;

S262N/R20E/K371E; R219Q/K371E; R219Q/K371D; R219Q/R20E; R219Q/K371E/R20E; R219Q/K371D/R20E; R219Q/S262N/K371E; R219Q/S262N/K371D; R219Q/S262N/R20E; R219Q/S262N/K371E/R20E; R219Q/S262N/K371D/R20E; R219Q/S262N; R219Q/S262N/K11A; R219Q/S262N/K11D; R219Q/S262N/K11E; R219Q/S262N/K13A; R219Q/S262N/K13D; R219Q/S262N/K13E; R219Q/S262N/K371A; R219Q/S262N/K372A; R219Q/S262N/K372D; R219Q/S262N/K372E; R219Q/S262N/K452A; R219Q/S262N/K452D; R219Q/S262N/K452E; R219Q/S262N/R20A; R219Q/S262N/R20D; R219Q/S262N/R366A; R219Q/S262N/R366D; R219Q/S262N/R366E; R219Q/S262N/H264A; R219Q/S262N/H264Q; R219Q/S262N/H264N; R219Q/S262N/H264G; R219K/S262N; R219N/S262N; R219A/S262N; R219Q/S262N/L221A; R219Q/S262N/L221V; R219Q/S262N/L221G; R219Q/S262N/E179D; R219Q/S262N/E179A; R219Q/S262N/E179S; R219Q/S262N/E179T; R219Q/S262N/E179V; R219Q/S262N/E179G; R219Q/S262A; R219Q/S262V; R219Q/S262M; R219Q/S262N/K11A/R20A; R219Q/S262N/K11A/R20A/K371A; R219Q/S262N/R20A/K371A; R219Q/S262N/K11A/K371A; R219Q/S262N/K26A; R219Q/S262N/K26D; R219Q/S262N/K26E; R219Q/S262N/R217A; R219Q/S262N/R217D; R219Q/S262N/R217E; R219Q/S262N/K258A; R219Q/S262N/K258D; R219Q/S262N/K258E; R219Q/S262N/R277A; R219Q/S262N/R277D; R219Q/S262N/R277E; R219Q/S262N/R283A; R219Q/S262N/R283D; R219Q/S262N/R283E; R219Q/S262N/K309A; R219Q/S262N/K309D; R219Q/S262N/K309E; R219Q/S262N/K317A; R219Q/S262N/K317D; R219Q/S262N/K317E; R219Q/S262N/K321A; R219Q/S262N/K321D; R219Q/S262N/K321E; R219Q/S262N/R352A; R219Q/S262N/R352D; R219Q/S262N/R352E; R219Q/S262N/R441A; R219Q/S262N/R441D; R219Q/S262N/R441E; R219Q/S262N/K444A; R219Q/S262N/K444D; R219Q/S262N/K444E; R219Q/S262N/K461A; R219Q/S262N/K461D; R219Q/S262N/K461E; R219Q/S262N/K469A; R219Q/S262N/K469D; R219Q/S262N/K469E; R219Q/S262N/K470A; R219Q/S262N/K470D; R219Q/S262N/K470E; R219Q/S262N/D86A; R219Q/S262N/D86C; R219Q/S262N/D86E; R219Q/S262N/D86F;

R219Q/S262N/D86G; R219Q/S262N/D86H; R219Q/S262N/D86I; R219Q/S262N/D86K; R219Q/S262N/D86L; R219Q/S262N/D86M; R219Q/S262N/D86N; R219Q/S262N/D86P; R219Q/S262N/D86Q; R219Q/S262N/D86R; R219Q/S262N/D86S; R219Q/S262N/D86T; R219Q/S262N/D86V; R219Q/S262N/D86W; R219Q/S262N/D86Y; R219Q/S262N/E179C; R219Q/S262N/E179F; R219Q/S262N/E179H; R219Q/S262N/E179I; R219Q/S262N/E179K; R219Q/S262N/E179L; R219Q/S262N/E179M; R219Q/S262N/E179N; R219Q/S262N/E179P; R219Q/S262N/E179Q; R219Q/S262N/E179R; R219Q/S262N/E179W; R219Q/S262N/E179Y; R219C/S262N; R219D/S262N; R219E/S262N; R219F/S262N; R219G/S262N; R219H/S262N; R219I/S262N; R219L/S262N; R219M/S262N; R219P/S262N; R219S/S262N; R219T/S262N; R219V/S262N; R219W/S262N; R219Y/S262N; R219Q/S262N/L221C; R219Q/S262N/L221D; R219Q/S262N/L221E; R219Q/S262N/L221F; R219Q/S262N/L221H; R219Q/S262N/L221I; R219Q/S262N/L221K; R219Q/S262N/L221M; R219Q/S262N/L221N; R219Q/S262N/L221P; R219Q/S262N/L221Q; R219Q/S262N/L221R; R219Q/S262N/L221S; R219Q/S262N/L221T; R219Q/S262N/L221W; R219Q/S262N/L221Y; R219Q/S262C; R219Q/S262D; R219Q/S262E; R219Q/S262F; R219Q/S262G; R219Q/S262H; R219Q/S262I; R219Q/S262K; R219Q/S262L; R219Q/S262P; R219Q/S262Q; R219Q/S262R; R219Q/S262T; R219Q/S262W; R219Q/S262Y; R219Q/S262N/H264C; R219Q/S262N/H264D; R219Q/S262N/H264E; R219Q/S262N/H264F; R219Q/S262N/H264I; R219Q/S262N/H264K; R219Q/S262N/H264L; R219Q/S262N/H264M; R219Q/S262N/H264P; R219Q/S262N/H264R; R219Q/S262N/H264S; R219Q/S262N/H264T; R219Q/S262N/H264V; R219Q/S262N/H264W; R219Q/S262N/H264Y; R219Q/S262N/S266A; R219Q/S262N/S266C; R219Q/S262N/S266D; R219Q/S262N/S266E; R219Q/S262N/S266F; R219Q/S262N/S266G; R219Q/S262N/S266H; R219Q/S262N/S266I; R219Q/S262N/S266K; R219Q/S262N/S266L; R219Q/S262N/S266M; R219Q/S262N/S266N; R219Q/S262N/S266P; R219Q/S262N/S266Q; R219Q/S262N/S266R; R219Q/S262N/S266T; R219Q/S262N/S266V; R219Q/S262N/S266W; R219Q/S262N/S266Y;

R219Q/S262N/K267A; R219Q/S262N/K267C; R219Q/S262N/K267D; R219Q/S262N/K267E; R219Q/S262N/K267F; R219Q/S262N/K267G; R219Q/S262N/K267H; R219Q/S262N/K267I; R219Q/S262N/K267L; R219Q/S262N/K267M; R219Q/S262N/K267N; R219Q/S262N/K267P; R219Q/S262N/K267Q; R219Q/S262N/K267R; R219Q/S262N/K267S; R219Q/S262N/K267T; R219Q/S262N/K267V; R219Q/S262N/K267W; R219Q/S262N/K267Y; R219Q/S262N/V296A; R219Q/S262N/V296C; R219Q/S262N/V296D; R219Q/S262N/V296E; R219Q/S262N/V296F; R219Q/S262N/V296G; R219Q/S262N/V296H; R219Q/S262N/V296I; R219Q/S262N/V296K; R219Q/S262N/V296L; R219Q/S262N/V296M; R219Q/S262N/V296N; R219Q/S262N/V296P; R219Q/S262N/V296Q; R219Q/S262N/V296R; R219Q/S262N/V296S; R219Q/S262N/V296T; R219Q/S262N/V296W; R219Q/S262N/V296Y; R219Q/K11A/R20A; R219Q/K11A/R20A/K371A; R219Q/R20A/K371A; R219Q/K11A/K371A; S262N/K11A/R20A; S262N/K11A/R20A/K371A; S262N/R20A/K371A; and S262N/K11A/K371A.

Examples of these combination mutants containing these replacements: K11A/R20A (SEQ ID NO:55); K11A/R20A/K371A (SEQ ID NO:56); R20A/K371A (SEQ ID NO:57); K11A/K371A (SEQ ID NO:58); S262N/K371D (SEQ ID NO:59); S262N/K371E (SEQ ID NO:60); S262N/R20E (SEQ ID NO:61); S262N/R20E/K371D (SEQ ID NO:62); S262N/R20E/K371E (SEQ ID NO:63); R219Q/K371E (SEQ ID NO:263); R219Q/K371D (SEQ ID NO:264); R219Q/R20E (SEQ ID NO:265); R219Q/K371E/R20E (SEQ ID NO:266); R219Q/K371D/R20E (SEQ ID NO:267); R219Q/S262N/K371E (SEQ ID NO:268); R219Q/S262N/K371D (SEQ ID NO:269); R219Q/S262N/R20E (SEQ ID NO:270); R219Q/S262N/K371E/R20E (SEQ ID NO:271); R219Q/S262N/K371D/R20E (SEQ ID NO:272); R219Q/S262N (SEQ ID NO:273); R219Q/S262N/K11A (SEQ ID NO:659); R219Q/S262N/K11D (SEQ ID NO:660); R219Q/S262N/K11E (SEQ ID NO:661); R219Q/S262N/K13A (SEQ ID NO:662); R219Q/S262N/K13D (SEQ ID NO:663); R219Q/S262N/K13E (SEQ ID NO:682); R219Q/S262N/K371A (SEQ ID NO:683); R219Q/S262N/K372A (SEQ ID NO:684); R219Q/S262N/K372D (SEQ ID NO:685); R219Q/S262N/K372E (SEQ ID NO:686); R219Q/S262N/K452A (SEQ ID

NO:687); R219Q/S262N/K452D (SEQ ID NO:688); R219Q/S262N/K452E (SEQ ID NO:689); R219Q/S262N/R20A (SEQ ID NO:690); R219Q/S262N/R20D (SEQ ID NO:691); R219Q/S262N/R366A (SEQ ID NO:692); R219Q/S262N/R366D (SEQ ID NO:693); R219Q/S262N/R366E (SEQ ID NO:694); R219Q/S262N/H264A (SEQ ID NO:695); R219Q/S262N/H264Q (SEQ ID NO:696); R219Q/S262N/H264N (SEQ ID NO:697); R219Q/S262N/H264G (SEQ ID NO:698); R219K/S262N (SEQ ID NO:699); R219N/S262N (SEQ ID NO:700); R219A/S262N (SEQ ID NO:701); R219Q/S262N/L221A (SEQ ID NO:702); R219Q/S262N/L221V (SEQ ID NO:703); R219Q/S262N/L221G (SEQ ID NO:704); R219Q/S262N/E179D (SEQ ID NO:705); R219Q/S262N/E179A (SEQ ID NO:706); R219Q/S262N/E179S (SEQ ID NO:707); R219Q/S262N/E179T (SEQ ID NO:708); R219Q/S262N/E179V (SEQ ID NO:709); R219Q/S262N/E179G (SEQ ID NO:710); R219Q/S262A (SEQ ID NO:711); R219Q/S262V (SEQ ID NO:712); R219Q/S262M (SEQ ID NO:713); R219Q/S262N/K11A/R20A (SEQ ID NO:714); R219Q/S262N/K11A/R20A/K371A (SEQ ID NO:715); R219Q/S262N/R20A/K371A (SEQ ID NO:716); R219Q/S262N/K11A/K371A (SEQ ID NO:717); R219Q/S262N/K26A (SEQ ID NO:718); R219Q/S262N/K26D (SEQ ID NO:719); R219Q/S262N/K26E (SEQ ID NO:720); R219Q/S262N/R217A (SEQ ID NO:721); R219Q/S262N/R217D (SEQ ID NO:722); R219Q/S262N/R217E (SEQ ID NO:723); R219Q/S262N/K258A (SEQ ID NO:724); R219Q/S262N/K258D (SEQ ID NO:725); R219Q/S262N/K258E (SEQ ID NO:726); R219Q/S262N/R277A (SEQ ID NO:727); R219Q/S262N/R277D (SEQ ID NO:728); R219Q/S262N/R277E (SEQ ID NO:729); R219Q/S262N/R283A (SEQ ID NO:730); R219Q/S262N/R283D (SEQ ID NO:731); R219Q/S262N/R283E (SEQ ID NO:732); R219Q/S262N/K309A (SEQ ID NO:733); R219Q/S262N/K309D (SEQ ID NO:734); R219Q/S262N/K309E (SEQ ID NO:735); R219Q/S262N/K317A (SEQ ID NO:736); R219Q/S262N/K317D (SEQ ID NO:737); R219Q/S262N/K317E (SEQ ID NO:738); R219Q/S262N/K321A (SEQ ID NO:739); R219Q/S262N/K321D (SEQ ID NO:740); R219Q/S262N/K321E (SEQ ID NO:741); R219Q/S262N/R352A (SEQ ID NO:742); R219Q/S262N/R352D (SEQ ID NO:743); R219Q/S262N/R352E (SEQ ID NO:744); R219Q/S262N/R441A (SEQ ID NO:745); R219Q/S262N/R441D (SEQ ID NO:746); R219Q/S262N/R441E (SEQ ID NO:747); R219Q/S262N/K444A (SEQ ID NO:748); R219Q/S262N/K444D (SEQ ID NO:749); R219Q/S262N/K444E (SEQ ID

NO:750); R219Q/S262N/K461A (SEQ ID NO:751); R219Q/S262N/K461D (SEQ ID NO:752); R219Q/S262N/K461E (SEQ ID NO:753); R219Q/S262N/K469A (SEQ ID NO:754); R219Q/S262N/K469D (SEQ ID NO:755); R219Q/S262N/K469E (SEQ ID NO:756); R219Q/S262N/K470A (SEQ ID NO:757); R219Q/S262N/K470D (SEQ ID NO:758); R219Q/S262N/K470E (SEQ ID NO:759); R219Q/S262N/D86A (SEQ ID NO:760); R219Q/S262N/D86C (SEQ ID NO:761); R219Q/S262N/D86E (SEQ ID NO:762); R219Q/S262N/D86F (SEQ ID NO:763); R219Q/S262N/D86G (SEQ ID NO:764); R219Q/S262N/D86H (SEQ ID NO:765); R219Q/S262N/D86I (SEQ ID NO:766); R219Q/S262N/D86K (SEQ ID NO:767); R219Q/S262N/D86L (SEQ ID NO:768); R219Q/S262N/D86M (SEQ ID NO:769); R219Q/S262N/D86N (SEQ ID NO:770); R219Q/S262N/D86P (SEQ ID NO:771); R219Q/S262N/D86Q (SEQ ID NO:772); R219Q/S262N/D86R (SEQ ID NO:773); R219Q/S262N/D86S (SEQ ID NO:774); R219Q/S262N/D86T (SEQ ID NO:775); R219Q/S262N/D86V (SEQ ID NO:776); R219Q/S262N/D86W (SEQ ID NO:777); R219Q/S262N/D86Y (SEQ ID NO:778); R219Q/S262N/E179C (SEQ ID NO:779); R219Q/S262N/E179F (SEQ ID NO:780); R219Q/S262N/E179H (SEQ ID NO:781); R219Q/S262N/E179I (SEQ ID NO:782); R219Q/S262N/E179K (SEQ ID NO:783); R219Q/S262N/E179L (SEQ ID NO:784); R219Q/S262N/E179M (SEQ ID NO:785); R219Q/S262N/E179N (SEQ ID NO:786); R219Q/S262N/E179P (SEQ ID NO:787); R219Q/S262N/E179Q (SEQ ID NO:788); R219Q/S262N/E179R (SEQ ID NO:789); R219Q/S262N/E179W (SEQ ID NO:790); R219Q/S262N/E179Y (SEQ ID NO:791); R219C/S262N (SEQ ID NO:792); R219D/S262N (SEQ ID NO:793); R219E/S262N (SEQ ID NO:794); R219F/S262N (SEQ ID NO:795); R219G/S262N (SEQ ID NO:796); R219H/S262N (SEQ ID NO:797); R219I/S262N (SEQ ID NO:798); R219L/S262N (SEQ ID NO:799); R219M/S262N (SEQ ID NO:800); R219P/S262N (SEQ ID NO:801); R219S/S262N (SEQ ID NO:802); R219T/S262N (SEQ ID NO:803); R219V/S262N (SEQ ID NO:804); R219W/S262N (SEQ ID NO:805); R219Y/S262N (SEQ ID NO:806); R219Q/S262N/L221C (SEQ ID NO:807); R219Q/S262N/L221D (SEQ ID NO:808); R219Q/S262N/L221E (SEQ ID NO:809); R219Q/S262N/L221F (SEQ ID NO:810); R219Q/S262N/L221H (SEQ ID NO:811); R219Q/S262N/L221I (SEQ ID NO:812); R219Q/S262N/L221K (SEQ ID NO:813); R219Q/S262N/L221M (SEQ ID NO:814); R219Q/S262N/L221N (SEQ ID NO:815); R219Q/S262N/L221P (SEQ ID

NO:816); R219Q/S262N/L221Q (SEQ ID NO:817); R219Q/S262N/L221R (SEQ ID NO:818); R219Q/S262N/L221S (SEQ ID NO:819); R219Q/S262N/L221T (SEQ ID NO:820); R219Q/S262N/L221W (SEQ ID NO:821); R219Q/S262N/L221Y (SEQ ID NO:822); R219Q/S262C (SEQ ID NO:823); R219Q/S262D (SEQ ID NO:824); R219Q/S262E (SEQ ID NO:825); R219Q/S262F (SEQ ID NO:826); R219Q/S262G (SEQ ID NO:827); R219Q/S262H (SEQ ID NO:828); R219Q/S262I (SEQ ID NO:829); R219Q/S262K (SEQ ID NO:830); R219Q/S262L (SEQ ID NO:831); R219Q/S262P (SEQ ID NO:832); R219Q/S262Q (SEQ ID NO:833); R219Q/S262R (SEQ ID NO:834); R219Q/S262T (SEQ ID NO:835); R219Q/S262W (SEQ ID NO:836); R219Q/S262Y (SEQ ID NO:837); R219Q/S262N/H264C (SEQ ID NO:838); R219Q/S262N/H264D (SEQ ID NO:839); R219Q/S262N/H264E (SEQ ID NO:840); R219Q/S262N/H264F (SEQ ID NO:841); R219Q/S262N/H264I (SEQ ID NO:842); R219Q/S262N/H264K (SEQ ID NO:843); R219Q/S262N/H264L (SEQ ID NO:844); R219Q/S262N/H264M (SEQ ID NO:845); R219Q/S262N/H264P (SEQ ID NO:846); R219Q/S262N/H264R (SEQ ID NO:847); R219Q/S262N/H264S (SEQ ID NO:848); R219Q/S262N/H264T (SEQ ID NO:849); R219Q/S262N/H264V (SEQ ID NO:850); R219Q/S262N/H264W (SEQ ID NO:851); R219Q/S262N/H264Y (SEQ ID NO:852); R219Q/S262N/S266A (SEQ ID NO:853); R219Q/S262N/S266C (SEQ ID NO:854); R219Q/S262N/S266D (SEQ ID NO:855); R219Q/S262N/S266E (SEQ ID NO:856); R219Q/S262N/S266F (SEQ ID NO:857); R219Q/S262N/S266G (SEQ ID NO:858); R219Q/S262N/S266H (SEQ ID NO:859); R219Q/S262N/S266I (SEQ ID NO:860); R219Q/S262N/S266K (SEQ ID NO:861); R219Q/S262N/S266L (SEQ ID NO:862); R219Q/S262N/S266M (SEQ ID NO:863); R219Q/S262N/S266N (SEQ ID NO:864); R219Q/S262N/S266P (SEQ ID NO:865); R219Q/S262N/S266Q (SEQ ID NO:866); R219Q/S262N/S266R (SEQ ID NO:867); R219Q/S262N/S266T (SEQ ID NO:868); R219Q/S262N/S266V (SEQ ID NO:869); R219Q/S262N/S266W (SEQ ID NO:870); R219Q/S262N/S266Y (SEQ ID NO:871); R219Q/S262N/K267A (SEQ ID NO:872); R219Q/S262N/K267C (SEQ ID NO:873); R219Q/S262N/K267D (SEQ ID NO:874); R219Q/S262N/K267E (SEQ ID NO:875); R219Q/S262N/K267F (SEQ ID NO:876); R219Q/S262N/K267G (SEQ ID NO:877); R219Q/S262N/K267H (SEQ ID NO:878); R219Q/S262N/K267I (SEQ ID NO:879); R219Q/S262N/K267L (SEQ ID NO:880); R219Q/S262N/K267M (SEQ ID NO:881); R219Q/S262N/K267N (SEQ ID

NO:882); R219Q/S262N/K267P (SEQ ID NO:883); R219Q/S262N/K267Q (SEQ ID NO:884); R219Q/S262N/K267R (SEQ ID NO:885); R219Q/S262N/K267S (SEQ ID NO:886); R219Q/S262N/K267T (SEQ ID NO:887); R219Q/S262N/K267V (SEQ ID NO:888); R219Q/S262N/K267W (SEQ ID NO:889); R219Q/S262N/K267Y (SEQ ID NO:890); R219Q/S262N/V296A (SEQ ID NO:891); R219Q/S262N/V296C (SEQ ID NO:892); R219Q/S262N/V296D (SEQ ID NO:893); R219Q/S262N/V296E (SEQ ID NO:894); R219Q/S262N/V296F (SEQ ID NO:895); R219Q/S262N/V296G (SEQ ID NO:896); R219Q/S262N/V296H (SEQ ID NO:897); R219Q/S262N/V296I (SEQ ID NO:898); R219Q/S262N/V296K (SEQ ID NO:899); R219Q/S262N/V296L (SEQ ID NO:900); R219Q/S262N/V296M (SEQ ID NO:901); R219Q/S262N/V296N (SEQ ID NO:902); R219Q/S262N/V296P (SEQ ID NO:903); R219Q/S262N/V296Q (SEQ ID NO:904); R219Q/S262N/V296R (SEQ ID NO:905); R219Q/S262N/V296S (SEQ ID NO:906); R219Q/S262N/V296T (SEQ ID NO:907); R219Q/S262N/V296W (SEQ ID NO:908); R219Q/S262N/V296Y (SEQ ID NO:909); R219Q/K11A/R20A (SEQ ID NO:910); R219Q/K11A/R20A/K371A (SEQ ID NO:911); R219Q/R20A/K371A (SEQ ID NO:912); R219Q/K11A/K371A (SEQ ID NO:913); S262N/K11A/R20A (SEQ ID NO:914); S262N/K11A/R20A/K371A (SEQ ID NO:915); S262N/R20A/K371A (SEQ ID NO:916); and S262N/K11A/K371A (SEQ ID NO:917).

In an embodiment, there is provided a variant Adenosine Deaminase 2 (ADA2) protein or a catalytically active portion thereof, comprising one or more modifications in the sequence of amino acids of an unmodified ADA2 protein or a catalytically active portion thereof, wherein: i) the unmodified ADA2 protein or catalytically active portion thereof comprises a sequence of amino acids selected from the group consisting of: a) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383; b) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383; c) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383 with all or a part of the ADA2 putative receptor binding domain (PRB domain) deleted, wherein the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; d) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383 with all or a part of the PRB domain deleted and a linker inserted in place of deleted residues, wherein: the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; and the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20; e) the ADA2 catalytic domain, and, optionally, additional contiguous amino acids from the

ADA2 protein contiguous to the catalytic domain, wherein the ADA2 catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; f) the sequence of amino acids selected from the group consisting of the catalytic domain of the polypeptides of any one of SEQ ID NOS: 5 and 380-383, wherein the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; g) the sequence of amino acids comprising the catalytic domain with all or a part of the PRB domain deleted, wherein: the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; h) the sequence of amino acids selected from the group consisting of the catalytic domain of any one of SEQ ID NOS: 5 and 380-383 with all or a part of the PRB domain deleted, wherein: the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; i) the ADA2 catalytic domain, and, optionally, additional contiguous amino acids from the ADA2 protein contiguous to the catalytic domain, with all or a part of the PRB domain deleted, and a linker in place of deleted residues, wherein: the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; and the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20; and j) the sequence of amino acids selected from the group consisting of the catalytic domain of any one of SEQ ID NOS: 5 and 380-383 with all or a part of the PRB domain deleted, and a linker in place of deleted residues, wherein: the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; and the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20; ii) the variant ADA2 protein or catalytically active portion thereof has 1 to 10 amino acid modifications, in addition to any deletion of all or a portion of the PRB domain, as compared to the unmodified ADA2 protein or catalytically active portion thereof as set forth in i) parts a) to j), wherein modifications comprise insertions, deletions, and replacements of amino acid residues; iii) the variant ADA2 protein or the catalytically active portion thereof, when in dimer form, exhibits reduced heparin binding compared to the corresponding dimer form of the unmodified ADA2 protein or catalytically active portion thereof as

set forth in i) parts a) to j); iv) the variant ADA2 protein or catalytically active portion thereof comprises amino acid replacements that confer reduced heparin binding selected from the group consisting of R20A, R20D, R20E, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E, K452E, K11A/R20A, K11A/R20A/K371A, R20A/K371A, and K11A/K371A, with reference to amino acid positions set forth in SEQ ID NO:5; v) the variant ADA2 protein or the catalytically active portion thereof, when in dimer form, exhibits adenosine deaminase activity to convert adenosine to inosine; and vi) the variant ADA2 protein or the catalytically active portion thereof does not comprise any of the amino acid replacements R219K, R219Q, R219N, S262M or S262N.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1F depict exemplary alignments of precursor human adenosine deaminase 2 (ADA2) set forth in SEQ ID NO:2 (residues 30-511 corresponding to mature ADA2 set forth in SEQ ID NO:5) with other ADA2 proteins. A "*" means that the aligned residues are identical, a ":" means that aligned residues are not identical, but are similar and contain conservative amino acids residues at the aligned position, and a "." means that the aligned residues are similar and contain semi- conservative amino acid residues at the aligned position. Residues corresponding to the putative receptor binding domain (PRB) are underlined. Exemplary, non-limiting, corresponding positions for amino acid replacements are indicated by highlighting. For example, **Figure 1A** depicts the alignment of ADA2 set forth in SEQ ID NO:2 with chimpanzee ADA2 set forth in SEQ ID NO:286. **Figure 1B** depicts the alignment of a ADA2 set forth in SEQ ID NO:2 with gorilla ADA2 set forth in SEQ

ID NO:287. **Figure 1C** depicts the alignment of a ADA2 set forth in SEQ ID NO:2 with pygmy chimpanzee set forth in SEQ ID NO:288. **Figure 1D** depicts the alignment of ADA2 set forth in SEQ ID NO:2 with Sumatran orangutan ADA2 set forth in SEQ ID NO:289. **Figure 1E** depicts the alignment of ADA2 set forth in SEQ ID NO:2 with northern white-cheeked gibbon ADA2 set forth in SEQ ID NO:290. **Figure 1F** depicts the alignment of ADA2 set forth in SEQ ID NO:2 with crab-eating macaque ADA2 set forth in SEQ ID NO:291.

Figure 2 depicts the biosynthesis and catabolism of extracellular adenosine, and adenosine receptor signaling (adapted from Antonioli *et al.* (2013) Nat Rev Can 13:842-857). Physiological conditions such as hypoxia, ischemia, inflammation, tumor environment or trauma can promote the extracellular accumulation of ATP, which is metabolized to AMP by the cell surface enzyme CD39. AMP is in turn metabolized to adenosine by CD73. Extracellular adenosine can bind to four different G-protein-coupled adenosine receptors (ADRs; i.e. A1, A2A, A2B and A3), expressed on the cell surface of nearby immune, tumor or other cells, to mediate various downstream adenosine-mediated signaling and activities, such as immunosuppression, cancer cell proliferation, cancer cell migration and/or metastasis, angiogenesis, and other effects. Nucleoside transporters (NTs) facilitate uptake of extracellular adenosine into cells. Adenosine deaminase 2 (ADA2), including exogenous ADA2 or variants as provided herein, can breakdown extracellular adenosine by catalyzing the conversion of adenosine to inosine.

DETAILED DESCRIPTION

Outline

A. Definitions
B. Adenosine Deaminase 2 (ADA2) and Modulation of Adenosine-Mediated Tumor Immunosuppression
1. Tumor Immunity and Immune Evasion
2. Adenosine Immunomodulation in Cancer and Tumor Microenvironment (TME)
3. Adenosine Deaminase and Targeting Adenosine in Treatment of Cancer
C. Adenosine Deaminase 2 (ADA2) and Variants Thereof
1. Structure and Activity of ADA2
a. Structure of ADA2
b. Activities of ADA2

2. ADA2 Variants
a. Exemplary Modifications
i. Amino Acid Replacements
ii. Modification(s) of PRB Domain
iv. Hyperglycosylation
b. Nucleic Acid Molecules
c. Production of Variant ADA2 Proteins
D. ADA2 Conjugates and Fusion Proteins
1. Half-Life Extending Moieties
a. Low Complexity Polypeptides
b. C-terminal peptide (CTP) of the β Subunit of Human Chorionic Gonadotropin
c. Immunoglobulin Constant Region (Fc) or Portions Thereof
d. Albumin or Fragment, or Variant Thereof
e. Albumin Binding Moiety
f. PAS Sequences
g. HAP Sequences
h. XTEN Sequences
i. Transferrin or Fragment thereof
j. Polymer conjugation
i. Polyethylene Glycol (PEG)
ii. Hydroxyethyl Starch (HES)
iii. Polysialic Acids (PSA)
iv. Other polymers
2. Methods of Producing Conjugates or Fusion Proteins
Linkers
i. Peptide Linkers
ii. Heterobifunctional linking agents
E. Methods of Producing Nucleic Acids Encoding ADA2 and Polypeptides Thereof
1. Isolation or Preparation of Nucleic Acids Encoding ADA2 Polypeptides
2. Generation of Mutant or Modified Nucleic Acid and Encoding Polypeptides
3. Vectors and Cells
Immune cells that encode and express the ADA2 variants provided herein
4. Expression
a. Prokaryotic Cells
b. Yeast Cells
c. Insect Cells
d. Mammalian Cells

Date Reçue/Date Received 2021-07-28

e. Plants
5. Purification Techniques
F. Methods of Assessing Activity and Physical Properties of ADA2
1. Adenosine Deaminase Assay
2. Methods of Assessing Heparin Binding
a. Affinity Assay
b. ELISA Assay
c. Dot Blot and other Radiolabeled Heparin Binding Assays
3. Methods for Assessing Stability
a. Conditions
i. Stability in Plasma
ii. Thermal Stability
iii. Stability in pH or pH Optima
iv. Other Conditions
b. Determination of Physical Properties
i. Enzymatic Activity
ii. Chromatographic Analysis of Protein Purity
iii. Differential Scanning Calorimetry
iv. Differential Scanning Fluorimetry
v. Intrinsic Fluorescence Spectroscopy
vi. Circular Dichroism
vii. Dynamic Light Scattering
viii. Static Light Scattering
ix. Turbidity Measurements
x. Other Methods to Determine Stability
4. Assay for Therapeutic Activity
a. *In vitro* Tests
b. *In vivo* Animal Models
i. Tumor Metabolic Activity
ii. Tumor Size and Volume
c. Clinical Monitoring
5. Pharmacodynamics/Pharmacokinetics and Tolerability
G. Pharmaceutical Compositions and Formulations
1. Formulations – liquids, injectables, emulsions
Lyophilized Powders
2. Compositions for Other Routes of Administration
3. Dosages and Administration
4. Packaging and Articles of Manufacture
H. Methods of Treatment with an Adenosine Deaminase 2 (ADA2)
1. Exemplary Diseases and Conditions
a. Cancers and Tumors
b. Non-Cancer Hyperproliferative Diseases

c. Fibrotic Diseases
d. Infectious Diseases
e. Other Diseases and Conditions
2. Methods of Patient Selection
a. Adenosine-associated Biomarkers
i. Plasma Adenosine Levels
ii. Adenosine Receptors (ADRs)
iii. Ectonucleotidases CD39 and CD73
b. Patient Selection
3. Dosages and Administration
4. Combination Therapies
a. Anticancer Agents
i. Anticancer Antibodies
ii. Chemotherapeutic Agents
iii. Radiation Therapy
iv. Anti-angiogenic Agents
v. Immune Checkpoint Inhibitors
(a) Anti-CTLA4 Therapies
(b) Anti-PD-1 and Anti-PD-L1 Therapies
b. Other Immunomodulatory Agents
c. Hyaluronan-Degrading Enzyme
Soluble hyaluronan degrading enzymes (*e.g.* soluble PH20)
d. Antibodies to Treat Infectious Diseases
e. Antibiotics and Antifungals
I. Examples

A. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which the invention(s) belong. In the event that there are a plurality of definitions for terms herein, those in this section prevail. Where reference is made to a URL or other such identifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

As used herein, "adenosine" refers to a purine nucleoside that is composed of a molecule of adenine attached to a ribose sugar molecule (ribofuranose) moiety via a β-N_9-glycosidic bond. Adenosine can modulate a variety of physiological processes through its interaction with adenosine receptors.

As used herein, "Michaelis constant" or K_m is a measure of the substrate concentration required for effective catalysis to occur. For example, an enzyme with a high K_m can require a higher substrate concentration to achieve a given reaction velocity than an enzyme with a lower K_m for the substrate. K_m can represent the affinity of the enzyme for a substrate.

As used herein, "catalytic efficiency" is the efficiency with which an enzyme reacts with a substrate to form a product. It is represented by the k_{cat}/K_m ($M^{-1}s^{-1}$ or 1/Ms). Methods to assess kinetic parameters of catalytic activity, including k_{cat}/K_m, are well known to a skilled artisan. Generally, k_{cat}/K_m is measured under steady state conditions.

As used herein, "adenosine deaminase" or "ADA" refers to an enzyme that catalyzes the hydrolytic deamination of adenosine to form inosine. An ADA also can deaminate 2'deoxyadenosine to 2'deoxyinosine, and hence includes enzymes that have 2'deoxyadenosine deaminase activity. In humans there are two ADA isozymes, designated ADA1 and ADA2, that differ in their molecular weight, catalytic parameters and other properties.

As used herein, "adenosine deaminase 1" or ADA1 refers to an ADA that lacks a signal peptide and is ubiquitously expressed inside cells. It is produced as a monomer. Exemplary of ADA1 is human ADA1 having the sequence of nucleotides set forth in SEQ ID NO:11 and encoding the sequence of amino acids set forth in SEQ ID NO:12. In humans, wild-type ADA1 is characterized by a K*m* of or of about 5.2 x 10^{-5} M, has a pH optimum of from or from about 7 to 7.5, and exhibits a similar affinity for both adenosine and 2'deoxyadenosine. For example, ADA1 has a 2'deoxyadenosine/adenosine deaminase ratio of at least or at least about 0.70, such at least or at least about 0.75. Reference to ADA1 includes wild-type or native ADA1 present in mammalian, including human and non-human, subjects. For example, reference to ADA1 includes human ADA1 that contains a polypeptide having the sequence of amino acids set forth in SEQ ID NO:12. Reference to ADA1 also

includes variants thereof, such as allelic variants, species variants, splice variants and other variants that include a polypeptide having a sequence of amino acids that has at least 65%, 70%, 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:12, and that exhibit adenosine deaminase activity.

As used herein, "adenosine deaminase 2," or "ADA2" refers to an ADA that is present in extracellular environments, including in the plasma. ADA2 is produced from a precursor polypeptide that contains a signal peptide (e.g. ADA2 set forth in SEQ ID NO:2), which is removed to yield a mature protein lacking the signal peptide (e.g. ADA2 set forth in SEQ ID NO:5). The secreted ADA2 is a homodimer containing two identical polypeptide chains that interact via non-polar interactions between residues of each subunit. In humans, wild-type ADA2 is characterized with a Km that is or is about 200 x 10^{-5} M, has a pH optimum of or of about 6.5 ± 0.2, and exhibits a weak affinity for 2'deoxyadenosine. For example, ADA2 has a 2'deoxyadenosine/adenosine deaminase ratio of less than 0.40, such less than or about 0.30 or less than or about 0.25. Reference to ADA2 includes wild-type or native ADA2 present in mammalian, including human and non-human, subjects. For example, reference to ADA2 includes human ADA2 that contains a polypeptide having the sequence of amino acids set forth in SEQ ID NO:2, the mature form set forth in SEQ ID NO:5, catalytically active portions of SEQ ID NO:5, and dimer forms thereof. Reference to ADA2 also includes precursor, mature, catalytically active forms, and dimer forms that are variants thereof, such as allelic variants, species variants, splice variants and other variants that include a polypeptide having a sequence of amino acids that has at least 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 85%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the precursor polypeptide set forth in SEQ ID NO:2 or the mature form thereof set forth in SEQ ID NO:5, and that, when in active form, exhibit adenosine deaminase activity. Such variants, when in active form, exhibit at least 40%, 50%, 70%, 90%, 90%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200% 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more activity than the activity of a native or wildtype ADA2 polypeptide. As used herein, wild-type" or "native" with reference to ADA2 refers to a ADA2 protein

containing a polypeptide encoded by a native or naturally occurring ADA2 gene, including allelic variants, that is present in an organism, including a human and other animals, in nature. Reference to wild-type ADA2 without reference to a species is intended to encompass any species of a wild-type ADA2. Included among wild-type ADA2 polypeptides are the encoded precursor polypeptide, fragments thereof, and processed forms thereof, such as a mature form lacking the signal peptide, as well as any pre- or post- translationally processed or modified forms thereof. Also included among native ADA2 proteins are those that are post-translationally modified, including, but not limited to, modification by glycosylation, carboxylation and hydroxylation. Native ADA2 proteins also include the polypeptide monomer as well as dimer forms. For example, humans express native ADA2. Wild-type human ADA2 is set forth in SEQ ID NO:2 (precursor) and SEQ ID NO:5 (mature), and includes catalytically active forms thereof as described herein, and allelic variants (precursor or mature) set forth in any of SEQ ID NOS:376-383, or isoforms of an ADA2 such as ADA2 set forth in SEQ ID NO:68. Wildtype or native ADA2 from non-human species include, but are not limited to, ADA2 from *Pan troglodytes* (chimpanzee; precursor form SEQ ID NO:286, mature form SEQ ID NO:326; NCBI Acc. No. XP_003317127.1); *Gorilla gorilla* (gorilla; precursor form SEQ ID NO:287, mature form SEQ ID NO:327; NCBI Acc. No. XP_004063024.1); *Pan paniscus* (pygmy chimpanzee; precursor form SEQ ID NO:288, mature form SEQ ID NO:328; NCBI Acc. No. XP_003828345.1); *Pongo abelii* (Sumatran orangutan; precursor form SEQ ID NO:289, mature form SEQ ID NO:329; NCBI Acc. No. NP_001125360.1); *Nomascus leucogenys* (Northern white-cheeked gibbon; precursor form SEQ ID NO:290, mature form SEQ ID NO:330; NCBI Acc. No. XP_004088517.1); *Macaca fascicularis* (crab-eating macaque; precursor form SEQ ID NO:291, mature form SEQ ID NO:331; NCBI Acc. No. XP_005568111.1); *Chlorocebus sabaeus* (green monkey; precursor form SEQ ID NO:292, mature form SEQ ID NO:332; NCBI Acc. No. XP_007972990.1); *Macaca mulatta* (Rhesus macaque; precursor form SEQ ID NOS:293, 337, mature form SEQ ID NOS:333, 340; GenBank Acc. Nos. AFH32795.1, EHH20002.1); *Callithrix jacchus* (marmoset; precursor form SEQ ID NOS:294, 374, mature form SEQ ID NO:334, 375; NCBI Acc. No. XP_009004591.1, XP_009004586.1); *Xenopus laevis* (African clawed frog; precursor form SEQ ID

NO:295, mature form SEQ ID NO:335; NCBI Acc. No. NP_001090531.1); *Drosophila melanogaster* (fruit fly; precursor form SEQ ID NOS:296-300, mature form SEQ ID NOS:336, 338, 339; AAL40913.1, AAL40920.1, AAL40911.1, AAL40912.1, and AAL40910.1); *Bombyx mori* (silk moth; precursor form SEQ ID NO:301, mature form SEQ ID NO:341; NCBI Acc. No. NP_001098698.1); and *Sarcophaga perigrina* (flesh fly; precursor form SEQ ID NO:302, mature form SEQ ID NO:342; GenBank Acc. No. BAA11812.1).

As used herein, a precursor ADA2 refers to a non-secreted form of an ADA2 that contains an N-terminal signal peptide that targets the protein for secretion. The signal peptide is cleaved off in the endoplasmic reticulum. Exemplary of an ADA2 precursor polypeptide is the polypeptide set forth in SEQ ID NO:2, or an allelic or species variant or other variant thereof such as those set forth in any of SEQ ID NOS:286-302, 337 or 376-379.

As used herein, a "mature ADA2" refers to an ADA2 that lacks a signal sequence. An exemplary mature ADA2 is set forth in SEQ ID NO:5, and also includes variants thereof such as species and allelic variants and other variants, such as those set forth in any of SEQ ID NOS:326-336, 338-342, 375, and 380-383. Reference to mature ADA2 includes dimer forms thereof.

As used herein, species variants refer to variants in polypeptides among different species, including different mammalian species, such as mouse and human.

As used herein, allelic variants refer to variations in proteins among members of the same species.

As used herein, domain (typically a sequence of three or more, generally 5 or 7 or more amino acids) refers to a portion of a molecule, such as proteins or the encoding nucleic acids, that is structurally and/or functionally distinct from other portions of the molecule and is identifiable. For example, domains include those portions of a polypeptide chain that can form an independently folded structure within a protein made up of one or more structural motifs and/or that is recognized by virtue of a functional activity, such as proteolytic activity. A protein can have one, or more than one, distinct domains. For example, a domain can be identified, defined or distinguished by homology of the sequence therein to related family members, such as homology to motifs that define a protease domain. In another example, a domain can

be distinguished by its function, such as by proteolytic activity, or an ability to interact with a biomolecule, such as DNA binding, ligand binding, and dimerization. A domain independently can exhibit a biological function or activity such that the domain independently or fused to another molecule can perform an activity, such as, for example proteolytic activity or ligand binding. A domain can be a linear sequence of amino acids or a non-linear sequence of amino acids. Many polypeptides contain a plurality of domains. Such domains are known, and can be identified by those of skill in the art. For exemplification herein, definitions are provided, but it is understood that it is well within the skill in the art to recognize particular domains by name. If needed appropriate software can be employed to identify domains.

As used herein "catalytic domain" or "ADA domain" refers to the domain that confers adenosine deaminase activity. The catalytic domain of an enzyme contains all of the requisite properties of that protein required for its enzymatic, such as adenosine deaminase activity. The ADA domain is structurally composed of an eight-stranded, parallel β-sheet that closes into a barrel and is surrounded by classical α/β-TIM barrel motif helices and five additional, located between β1 and α1 (H1, H2, and H3) and at the C terminus (H4 and H5) (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377). Loops between β-strands and α-helices contain many of the active site residues required for activity. Active site residues include residues that coordinate zinc binding, active site proton donor and acceptor residues, and substrate binding residues. Exemplary of such residues in human ADA2 are set forth in Table 4. In reference to human ADA2, the ADA domain is contained in the region corresponding to residues 106-502 of the precursor sequence of amino acids set forth in SEQ ID NO:2 (corresponding to residues 77-473 of the mature protein set forth in SEQ ID NO:5), except that residues corresponding to the putative receptor binding (PRB) domain contained therein are not required for catalytic activity.

As used herein, "a catalytically active portion thereof" or "a catalytically active fragment thereof" refers to an ADA2 polypeptide that contains less than the full-length sequence of a mature ADA2 polypeptide, but contains a contiguous portion of amino acids of an ADA2, including all or part of the catalytic domain, sufficient for adenosine deaminase activity. For example, a catalytically active portion of ADA2 is one that includes a polypeptide containing a contiguous sequence of amino acids of

the mature sequence of an ADA2 polypeptide that includes amino acid residues corresponding to residues 83, 85, 327, 330, 355, and 412 with reference to amino acid residues set forth in SEQ ID NO:5, but does not include the full amino acid sequence of the mature ADA2 polypeptide. For example, a catalytically active portion is one that includes a polypeptide containing a contiguous sequence of amino acids of the mature sequence of ADA2 set forth in SEQ ID NO:5 that includes amino acid residues 83, 85, 327, 330, 355, and 412, but that does not include the full length sequence of amino acids set forth in SEQ ID NO:5. An ADA2 that contains a catalytically active portion of an ADA2 polypeptide, when in active form, exhibits at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more of the activity, such as at least 120%, 130%, 140%, 150%, 200%, 300%, 400%, 500% or more of the activity, compared to the ADA2 containing a full-length mature ADA2 polypeptide. In one example, a catalytically active portion of an ADA2 polypeptide includes a polypeptide that lacks all or a portion of the putative receptor binding (PRB) domain. In another example, a catalytically active portion of an ADA2 polypeptide includes a polypeptide that lacks one or more C-terminal amino acids of the mature polypeptide, i.e. is truncated at the C-terminus, by up to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more contiguous C-terminal amino acid residues compared to the mature ADA2 polypeptide. It is understood that reference herein to a variant ADA2 or catalytically active portion thereof means that the catalytically active portion contains the modification(s) (*e.g.* amino acid replacement(s)).

As used herein, "putative receptor binding domain" or "PRB domain" refers to a portion of ADA2 that forms an independently folded structure composed of an α- and β-fold containing a three-stranded antiparallel β-sheets designated SR1-SR2-SR3, surrounded by the HR and partially H2 α-helices on one side and the proline-rich SR2-SR3 loop on the other side (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377). The PRB domain contains conserved cysteine residues that form a disulfide bond between C137 and C159 of precursor ADA2 set forth in SEQ ID NO:2

(positions C108 and C130 of mature ADA2 set forth in SEQ ID NO:5). The PRB domain is reported to be involved in binding of ADA2 to its receptors. It is understood that the particular residues that make up the domain can vary (*e.g.* longer or shorter), for example, depending on methods used to identify the domain. In reference to human ADA2, the PRB domain is reported to correspond to residues 127-185 or 134-177 of precursor ADA2 set forth in SEQ ID NO:2 (residues 98-156 or 105-148, respectively, of mature ADA2 set forth in SEQ ID NO:5).

As used herein, a protein lacking all or a portion of a domain, such as all or a portion of the PRB domain, refers a polypeptide that has a deletion of one or more amino acids or all of the amino acids of the domain, such as the PRB domain, compared to a reference or unmodified protein. Amino acids deleted in a polypeptide lacking all or part of a domain can be contiguous, but need not be contiguous amino acids within the domain of the cognate polypeptide. Polypeptides that lack all or a part of a domain can include the loss or reduction of an activity of the polypeptide compared to the activity of a reference or unmodified protein.

As used herein, "active form" refers to any ADA2 enzyme that exhibits adenosine deaminase activity. An active form of an enzyme can contain a full-length sequence of amino acids or can be a catalytically active portion thereof. An active form of an enzyme can be a monomer or a dimer. Typically, an active enzyme is a dimer. An active enzyme is any form that exhibits a catalytic efficiency (k_{cat}/K_M) that is at least or at least about 5 x 10^3 $M^{-1}s^{-1}$, 6 x 10^3 $M^{-1}s^{-1}$, 7 x 10^3 $M^{-1}s^{-1}$, 8 x 10^3 $M^{-1}s^{-1}$, 9 x 10^3 $M^{-1}s^{-1}$, 1 x 10^4 $M^{-1}s^{-1}$, 2 x 10^4 $M^{-1}s^{-1}$, 3 x 10^4 $M^{-1}s^{-1}$, 4 x 10^4 $M^{-1}s^{-1}$, 5 x 10^4 $M^{-1}s^{-1}$, 6 x 10^4 $M^{-1}s^{-1}$, 7 x 10^4 $M^{-1}s^{-1}$, 8 x 10^4 $M^{-1}s^{-1}$, 9 x 10^4 $M^{-1}s^{-1}$, 1 x 10^5 $M^{-1}s^{-1}$, 2 x 10^5 $M^{-1}s^{-1}$, 3 x 10^5 $M^{-1}s^{-1}$, 4 x 10^5 $M^{-1}s^{-1}$, 5 x 10^5 $M^{-1}s^{-1}$, 6 x 10^5 $M^{-1}s^{-1}$, 7 x 10^5 $M^{-1}s^{-1}$, 8 x 10^5 $M^{-1}s^{-1}$, 9 x 10^5 $M^{-1}s^{-1}$, 1 x 10^6 $M^{-1}s^{-1}$, 2 x 10^6 $M^{-1}s^{-1}$, 3 x 10^6 $M^{-1}s^{-1}$, 4 x 10^6 $M^{-1}s^{-1}$, 5 x 10^6 $M^{-1}s^{-1}$ or greater.

As used herein, a "multimer" refers to a molecule composed of several identical or different subunits held together or associated, for example, by non-covalent interactions.

As used herein, a "dimer" refers to a molecule that contains two polypeptides linked together. Typically, the polypeptides are non-covalently linked. For example,

an ADA2 dimer is formed by nonpolar intersubunit interactions, including hydrophobic interaction, between residues of two polypeptides.

As used herein, a "homodimer" refers to a dimer that is formed by two identical polypeptides.

As used herein, a "heterodimer" refers to a dimer that is formed by two different polypeptides.

As used herein, a "monomer" refers to a single protein or polypeptide unit. A monomer has a relatively low molecular weight compared to dimers or other multimers. A monomer can exist independently, or it can associate with other molecules to form a dimer or other multimer.

As used herein a "corresponding form" with reference to an ADA2 protein means that when comparing a property or activity of two ADA2 proteins, the property is compared using the same structural form of the protein. For example, if its stated that an ADA2 protein has less activity compared to the activity of the corresponding form of a first ADA2 protein, that means that a particular form, such as a dimer, has less activity compared to the dimer of the first ADA2 protein.

As used herein, a "polypeptide" refers to a linear organic polymer containing a large number of amino acid residues bonded together in a chain, forming part of, or the whole of, a protein molecule.

As used herein, a "protein" or "protein molecule" or variations thereof refers to a large molecule composed of one or more polypeptide chains made up of a linear sequence of amino acids. Hence, a protein can be a monomer, or can be a dimer or other multimer. A protein can exhibit structural, mechanical, biochemical or signaling activities.

As used herein, a "polypeptide subunit" or "protein subunit" refers to a single polypeptide or monomer that is capable of assembling with other polypeptides or monomers to form a protein molecule that is a multimeric complex. One subunit is made up of one polypeptide chain.

As used herein, "variant ADA2 protein" refers to an ADA2 protein, including any form thereof such as a full-length, catalytically active portion, monomer, or dimer, that has one or more amino acid differences compared to an unmodified ADA2 protein. The one or more amino acid differences can be amino acid mutations, such

as one or more amino acid replacements (substitutions), insertions or deletions, or can be insertions or deletions of entire domains, and any combination thereof. Typically, a variant ADA2 protein has one or more modifications in primary sequence compared to an unmodified ADA2 protein. For example, a variant ADA2 provided herein can have at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 or more amino acid differences compared to an unmodified ADA2 protein. Any modification is contemplated as long as the resulting protein exhibits adenosine deaminase activity.

As used herein, modification refers to modification of a sequence of amino acid residues of a polypeptide or a sequence of nucleotides in a nucleic acid molecule and includes deletions, insertions, and replacements of amino acids and nucleotides, respectively. Modifications also can include post-translational modifications or other changes to the molecule that can occur due to conjugation or linkage, directly or indirectly, to another moiety. Methods of modifying a polypeptide are routine to those of skill in the art, such as by using recombinant DNA methodologies.

As used herein, "deletion," when referring to modification of a nucleic acid or polypeptide sequence, refers to the removal of one or more nucleotides or amino acids compared to a sequence, such as a target polynucleotide or polypeptide or a native or wild-type sequence. Thus, an amino acid sequence or nucleic acid molecule that contains one or more deletions compared to a wild-type sequence, contains one or more fewer amino acids or nucleotides within the linear length of the sequence.

As used herein, "insertion" when referring to modification of a nucleic acid or amino acid sequence, describes the inclusion of one or more additional nucleotides or amino acids, within a target, native, wild-type or other related sequence. Thus, an amino acid or nucleic acid molecule that contains one or more insertions compared to a wild-type sequence, contains one or more additional amino acids or nucleotides within the linear length of the sequence.

As used herein, "additions" to nucleic acid and amino acid sequences describe addition of nucleotides or amino acids onto either termini compared to another sequence.

As used herein, "substitution" or "replacement" with respect to a modification refers to the replacing of one or more nucleotides or amino acids in a native, target,

wild-type or other nucleic acid or polypeptide sequence with an alternative nucleotide or amino acid, without changing the length (as described in numbers of residues) of the molecule. Thus, one or more substitutions in a molecule does not change the number of amino acid residues or nucleotides of the molecule. Amino acid replacements compared to a particular polypeptide can be expressed in terms of the number of the amino acid residue along the length of the polypeptide sequence or a reference polypeptide sequence. For example, a modified polypeptide having a modification in the amino acid at the 19th position of the amino acid sequence that is a substitution of Isoleucine (Ile; I) for cysteine (Cys; C) can be expressed as "replacement with Cys or C at a position corresponding to position 19," I19C, Ile19Cys, or simply C19, to indicate that the amino acid at the modified 19th position is a cysteine. In this example, the molecule having the substitution has a modification at Ile 19 of the unmodified polypeptide.

As used herein, "unmodified polypeptide" or "unmodified ADA2" and grammatical variations thereof refer to a starting polypeptide that is selected for modification as provided herein. The starting polypeptide can be a naturally-occurring, wild-type form of a polypeptide. Exemplary of an unmodified ADA2 polypeptide is human ADA2 set forth in SEQ ID NO:5, or a catalytically active portion thereof. In addition, the starting polypeptide can be altered or mutated, such that it differs from a native wild type isoform but is nonetheless referred to herein as a starting unmodified polypeptide relative to the subsequently modified polypeptides produced herein. Thus, existing proteins known in the art that have been modified to have a desired increase or decrease in a particular activity or property compared to an unmodified reference protein can be selected and used as the starting unmodified polypeptide. For example, a protein that has been modified from its native form by one or more single amino acid changes and possesses either an increase or decrease in a desired property, such as a change in an amino acid residue or residues to alter glycosylation, can be a target protein, referred to herein as unmodified, for further modification of either the same or a different property.

As used herein, "corresponding residues" refers to residues that occur at aligned loci. For purposes herein, the amino acid sequence of a protein is aligned to precursor ADA2 set forth in SEQ ID NO:2 or its mature form set forth in SEQ ID

NO:5 (see Figure 1) or to the ADA2 sequence used for Zavialov numbering, the numbering of residues used in Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377 and in PDB accession Nos. 3LGG and 3LGD, set forth in SEQ ID NO:4. Related or variant polypeptides are aligned by any method known to those of skill in the art. Such methods typically maximize matches, and include methods such as using manual alignments and by using the numerous alignment programs available (for example, BLASTP) and others known to those of skill in the art. By aligning the sequences of ADA2 polypeptides, one of skill in the art can identify corresponding residues, using conserved and identical amino acid residues as guides. Generally, recitation that amino acids of a polypeptide correspond to amino acids in a disclosed sequence refers to amino acids identified upon alignment of the polypeptide with the disclosed sequence to maximize identity or homology (where conserved amino acids are aligned) using a standard alignment algorithm, such as the GAP algorithm.

As used herein, a "property" of an ADA2 refers to a physical or structural property, such as three-dimensional structure, pI, half-life, conformation and other such physical characteristics.

As used herein, an "activity" of an ADA2 or "ADA2 activity" refers to any activity exhibited by the active form of an ADA2 protein, typically the dimer form. Such activities can be tested *in vitro* and/or *in vivo* and include, but are not limited to, adenosine deaminase activity, growth factor activity, ability to bind heparin and/or ability to bind to an adenosine receptor (ADR). Activity can be assessed *in vitro* or *in vivo* using recognized assays, for example, by measuring adenosine deaminase activity *in vitro* or *in vivo*. The results of such assays indicate that a polypeptide exhibits an activity that can be correlated to activity of the polypeptide *in vivo*, in which *in vivo* activity can be referred to as biological activity. Assays to determine functionality or activity of modified forms of ADA2 are known to those of skill in the art, and exemplary assays are described herein.

As used herein, "adenosine deaminase activity" refers to the ability of an enzyme to catalyze the hydrolytic deamination of adenosine to form inosine. ADA2 activity can be assessed, directly or indirectly, by measuring the rate of production of a product of the enzymatic reaction. For example, the production of inosine or ammonia can be directly or indirectly measured. In other examples, the decrease of

the substrate of the enzyme, *e.g.*, adenosine or 2-deoxyadenosine, is measured. Assays to assess adenosine deaminase activity are known to those of skill in the art, and include but are not limited to, assays in which the decrease of the substrate, or the increase of the product, is measured directly by spectrophotometry, or indirectly, by subsequent enzymatic or oxidation-reduction reactions that use chromogenic substrates or change the absorbance spectra of the reaction.

As used herein, "increased adenosine deaminase activity" refers to an enhanced ability of an ADA2 protein, for example a variant ADA2 protein, to exhibit adenosine deaminase activity as compared with a reference protein. For example, the ability of a variant ADA2 protein to exhibit adenosine deaminase activity can be greater than the adenosine deaminase activity of the unmodified ADA2 protein. The adenosine deaminase activity can be increased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, or more compared to the adenosine deaminase activity of reference or an unmodified protein.

As used herein, a glycosylation site refers to an amino position in a polypeptide to which a carbohydrate moiety can be attached. Typically, a glycosylated protein contains one or more amino acid residues, such as asparagine or serine, for the attachment of the carbohydrate moieties.

As used herein, a native glycosylation site refers to an amino position to which a carbohydrate moiety is attached in a wild-type polypeptide. There are four N-linked native glycosylation sites in ADA2 corresponding to residues N98, N145, N156 and N349 with reference to SEQ ID NO:5.

As used herein, a non-native glycosylation site refers to an amino position to which a carbohydrate moiety is attached in a modified polypeptide that is not present in a wild-type polypeptide. Non-native glycosylation sites can be introduced into a ADA2 polypeptide by amino acid replacement. O-glycosylation sites can be created, for example, by amino acid replacement of a native residue with a serine or threonine. N-glycosylation sites can be created, for example, by establishing the motif Asn-Xaa-Ser/Thr/Cys, where Xaa is not proline. Creation of this consensus sequence by amino acid modification can involve, for example, a single amino acid replacement of a native amino acid residue with an asparagine, a single amino acid replacement of a

Date Reçue/Date Received 2021-07-28

native amino acid residue with a serine, threonine or cysteine, or a double amino acid replacement involving a first amino acid replacement of a native residue with an asparagine and a second amino acid replacement of native residue with a serine, threonine or cysteine, or an insertion of a non-native N-glycosylation motif, such as the motif Asn-Xaa-Ser/Thr/Cys, where Xaa is not proline.

As used herein, "level of glycosylation" refers to the number of glycosylation sites capable of being occupied by a glycan, for example, upon expression in a host cell capable of glycosylation.

As used herein, increases with reference to the level of glycosylation means that there is a greater number of glycosylation sites capable of being occupied by a glycan with reference to an unmodified or wildtype ADA2. A variant ADA2 that exhibits an increased level of glycosylation can be hyperglycosylated if there is a greater number of glycosylation sites occupied by a glycan compared to the unmodified or wildtype ADA2.

As used herein, "protein stability" refers to a measure of the maintenance of one or more physical properties of a protein in response to an environmental condition (*e.g.* an elevated temperature). In one embodiment, the physical property is the maintenance of the covalent structure of the protein (*e.g.* the absence of proteolytic cleavage, unwanted oxidation or deamidation). In another embodiment, the physical property is the presence of the protein in a properly folded state (*e.g.* the absence of soluble or insoluble aggregates or precipitates). In one embodiment, stability of a protein is measured by assaying a biophysical property of the protein, for example thermal stability, pH unfolding profile, stable removal of glycosylation, solubility, biochemical function (*e.g.*, ability to bind to a protein such as a receptor or enzymatic activity) and/or combinations thereof. In another embodiment, biochemical function is demonstrated by the binding affinity of an interaction. Stability can be measured using methods known in the art and/or described herein.

As used herein, "half-life" refers to the time that a living body requires to eliminate one half of the quantity of an administered substance through its normal channels of elimination. The normal channels of elimination generally include the kidneys and liver, but can include other excretion pathways. A half-life can be described as the time it takes the concentration of a substance to halve its

concentration from steady state or from a certain point on the elimination curve. A half-life typically is measured in the plasma and can be determined by giving a single dose of drug, and then measuring the concentration of the drug in the plasma at times to determine the relationship between time and decline in concentration as the substance is eliminated.

As used herein, "increased half-life" refers to a longer half-life of a protein molecule compared to a reference protein. Hence, it means that the time that it takes the concentration of a substance to halve its concentration is longer than for the time it takes the concentration of a reference protein to halve. The half-life can be increased by at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, 1100%, 1200%, 1300%, 1400%, 1500%, 1600%, 1700%, 1800%, 1900%, 2000%, 3000%, 4000%, 5000%, 6000%, 7000%, 8000%, 9000%, 10000% or more compared to the half-life of an unmodified polypeptide. Assays to assess half-life are known and standard in the art.

As used herein, "thermal stability" refers to the measure of the resistance to denaturation of a protein that occurs upon exposure to high or elevated temperatures, and hence is the ability of a protein to function at a particular temperature. A polypeptide is thermally stable at a temperature if it retains at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of an activity or a property of the polypeptide at the temperature. Thermal stability can be measured either by known procedures or by the methods described herein. In certain embodiments, thermal stability is evaluated by measuring the melting temperature (Tm) of a protein or by a thermal challenge assay (Tc).

As used herein, "increased thermal stability" refers to a higher degree of resistance to denaturation of a protein. For example, it can mean that a protein is thermally stable at a higher temperature than a reference proteins. It also can mean that a protein exhibits greater retained activity at a particular temperature compared to the activity of a reference protein at the same temperature. In some cases, an increased thermal stability means that a protein has a greater melting temperature Tm compared to a reference protein. For example, the thermal stability is increased if the Tm of a protein is at least 0.1°C, 0.2°C, 0.3°C, 0.4°C, 0.5°C, 0.6°C, 0.7°C, 0.8°C,

0.9°C, 1.0°C, 1.5°C, 2.0°C. 2.5°C. 3.0°C, 4.0°C, 5.0°C or more greater than a reference or unmodified protein.

As used herein, the melting temperature (Tm; also called transition temperature) is the temperature at the midpoint of a thermal transition curve where 50% of molecules of a composition are in a folded state. Hence, it is the temperature at which 50% of a macromolecule becomes denatured, and is a standard parameter for describing the thermal stability of a protein. Methods to determine Tm are well-known to a skilled artisan and include, for example, analytical spectroscopy methods such as, but are not limited to, differential scanning calorimetry (DSC), circular dichroism (CD) spectroscopy), fluorescence emission spectroscopy or nuclear magnetic resonance (NMR) spectroscopy.

As used herein, "pH optima" or "pH optimum" refers to the pH at which any enzymatic reaction, such as adenosine deaminase activity, is most effective under a given set of conditions. With respect to its adenosine deaminase activity, ADA2 exhibits a pH optima that is or is about 6.5.

As used herein, "altered pH optima" or "altered pH optimum" refers to a change (increased or decreased) in the pH that is the optimal pH for adenosine deaminase activity. An increased pH optimum occurs if the pH optimum is greater than 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.0, 2.5 or more compared to the pH optimum of a reference or unmodified protein. A decreased pH optimum occurs if the pH optimum is lower than or less than 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.0, 2.5 or more less to the pH optimum of a reference or unmodified protein.

As used herein, "bind," "bound" or grammatical variations thereof refers to the participation of a molecule in any attractive interaction with another molecule, resulting in a stable association in which the two molecules are in close proximity to one another. Binding includes, but is not limited to, non-covalent bonds, covalent bonds (such as reversible and irreversible covalent bonds), and includes interactions between molecules such as, but not limited to, proteins, nucleic acids, carbohydrates, lipids, and small molecules, such as chemical compounds including drugs. Typically, bind involves the association of two or more molecules by one or more noncovalent bonds. Binding can be assessed by standard methods known in the art, including but

not limited to, equilibrium dialysis, radioimmunoassay radiolabeled target antigen, immunoassays (*e.g.* enzyme linked immunosorbent assay (ELISA)), surface plasmon resonance (SPR), isothermal titration calorimetry (ITC) and other methods well known to a skilled artisan.

As used herein, binding activity refer to characteristics of a molecule, e.g. a polypeptide, relating to whether or not, and how, it binds one or more binding partners. Binding activities include the ability to bind the binding partner(s), the affinity with which it binds to the binding partner (e.g. high affinity), the avidity with which it binds to the binding partner, the strength of the bond with the binding partner and/or specificity for binding with the binding partner.

As used herein, "heparin binding" refers to the ability of ADA2 to bind heparin, which is a highly sulfated glycosaminoglycan made up of variably sulfated repeating disaccharide units. Commonly, a heparin disaccharide unit is made up of a 2-O-sulfated iduronic acid and 6-O-sulfated, N-sulfated glucosamine, IdoA(2S)-GlcNS(6S).

As used herein, "reduced heparin binding" or "attenuated heparin binding" refers to a lessening or reduced binding activity for heparin. For example, it can mean that the level or degree of binding of an ADA2 protein, such as a variant ADA2, is less than a reference protein. For example, the heparin binding is reduced if the level or degree of binding of an ADA2 protein to heparin is no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the binding of a reference or unmodified ADA2 protein to heparin. In some cases, heparin binding is reduced at least or at least about 0.5-fold, 1-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or more compared to the heparin binding of a reference or unmodified ADA2 protein.

As used herein, "adenosine receptor" or ADR refers to a class of G-protein coupled receptors that bind adenosine. Adenosine receptors also can bind to ADA2. There are four types of adenosine receptors. For example, in humans, the ADRs are designated A_1 (SEQ ID NO:533), A_{2A} (SEQ ID NO:534), A_{2B} (SEQ ID NO:535) and A_3 (SEQ ID NOS:536-538).

Date Reçue/Date Received 2021-07-28

As used herein, "receptor binding" refers to the ability of ADA2 to bind an adenosine receptor.

As used herein, "reduced receptor binding" refers to a lessening or reduced binding activity for an adenosine receptor. For example, it can mean that the level or degree of binding of an ADA2 protein, such as a variant ADA2, is less than the binding of a reference protein for the same adenosine receptor. For example, receptor binding is reduced if the level or degree of an ADA2 protein for an adenosine receptor is no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the binding of a reference or unmodified ADA2 protein for the same adenosine receptor. In some cases, receptor binding is reduced at least or at least about 0.5-fold, 1-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or more compared to the receptor binding of a reference or unmodified ADA2 protein for the same adenosine receptor.

As used herein, recitation that proteins are "compared under the same conditions" means that different proteins are treated identically or substantially identically such that any one or more conditions that can influence the activity or properties of a protein or agent are not varied or not substantially varied between the test agents. For example, when the adenosine deaminase activity of an ADA2 is compared to an unmodified ADA2 protein any one or more conditions such as the amount or concentration of the protein; presence, including amount, of excipients, carriers or other components in a formulation other than the active agent; temperature; pH and/or other conditions are identical or substantially identical between and among the compared polypeptides.

As used herein, "immune checkpoints" refer to inhibitory pathways of the immune system that are responsible for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in peripheral tissues in order to minimize collateral tissue damage. Immune checkpoints are regulated by immune checkpoint proteins.

An "immune checkpoint protein" is a protein, typically a receptor (*e.g.*, CTLA4 or PD-1) or a ligand (*e.g.*, PD-L1) that regulates or modulates the extent of an immune response. The immune checkpoint proteins can be inhibitory or stimulatory.

Date Reçue/Date Received 2021-07-28

In particular, the immune checkpoint proteins are inhibitory to the activation of the immune response. Thus, inhibition of an inhibitory immune checkpoint protein acts to stimulate or activate an immune response, such as T cell activation and proliferation.

As used herein, an "immune checkpoint inhibitor" or "immune checkpoint inhibiting agent," or "immune checkpoint blocking agent" refers to an agent that binds an inhibitory immune checkpoint protein and blocks its activity. The inhibition can be competitive or non-competitive inhibition that can be steric or allosteric. In cases where an immune checkpoint protein is an immune stimulating protein, an immune checkpoint inhibitor acts to promote the activity of the immune stimulating protein, such as by binding and activating the stimulatory immune checkpoint protein or by inhibiting by interfering with, such as by binding or deactivating, inhibitors of the stimulatory immune checkpoint protein. An example of an immune checkpoint inhibitor is an anti-immune checkpoint protein antibody.

A "target" of an immune checkpoint inhibitor is the immune checkpoint protein to which the immune checkpoint inhibitor or immune checkpoint inhibiting agent binds to block activity. Typically, the immune checkpoint inhibitor specifically binds to the target. For example, the target of the exemplary anti-CTLA4 antibody designated ipilimumab is CTLA4.

As used herein, an anti-immune checkpoint protein antibody, refers to any antibody that specifically binds to an immune checkpoint protein or a soluble fragment thereof and blocks. An anti-immune checkpoint protein antibody typically binds an immune checkpoint ligand protein or an immune checkpoint receptor protein and blocks the binding of a receptor to the target immune checkpoint ligand protein or a ligand to the target immune checkpoint receptor protein, thereby preventing the inhibitory signal transduction that suppresses an immune response. Hence, anti-immune checkpoint protein antibodies are immune checkpoint inhibitors. Reference to anti-immune checkpoint protein antibodies herein include full-length antibodies and antigen-binding fragments thereof that specifically bind to an immune checkpoint ligand or receptor protein. Exemplary anti-immune checkpoint protein antibodies include, but are not limited to, anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) antibodies and anti-programmed cell death protein 1 (PD-1) antibodies.

Date Reçue/Date Received 2021-07-28

As used herein, an antigen-binding fragment of an anti-immune checkpoint protein antibody refers to an antibody derived from an anti-immune checkpoint protein antibody but that is less than the full length sequence of the anti-immune checkpoint protein antibody but contains at least a portion of the variable regions (heavy and light) of the antibody sufficient to form an antigen binding site (*e.g.*, one or more CDRs, and generally all CDRs) and thus retains the binding specificity and/or activity of the anti-immune checkpoint protein antibody.

As used herein, anti-CTLA4 antibody refers to any antibody that specifically binds to cytotoxic T-lymphocyte-associated protein 4 (CTLA4) or a soluble fragment thereof and blocks the binding of ligands to CTLA4, thereby resulting in competitive inhibition of CTLA4 and inhibition of CTLA4-mediated inhibition of T cell activation. Hence, anti-CTLA4 antibodies are CTLA4 inhibitors. Reference to anti-CTLA4 antibodies herein include a full-length antibody and derivatives thereof, such as antigen-binding fragments thereof that specifically bind to CTLA4. Exemplary anti-CTLA4 antibodies include, but are not limited to, Ipilimumab or Tremelimumab, or a derivative or antigen-binding fragment thereof.

As used herein, anti-PD-1 antibody refers to any antibody that specifically binds to programmed cell death protein 1 (PD-1) or a soluble fragment thereof and blocks the binding of ligands to PD-1, thereby resulting in competitive inhibition of PD-1 and inhibition of PD-1-mediated inhibition of T cell activation. Hence, anti-PD-1 antibodies are PD-1 inhibitors. Reference to anti-PD-1 antibodies herein include a full-length antibody and derivatives thereof, such as antigen-binding fragments thereof that specifically bind to PD-1. Exemplary anti-PD-1 antibodies include, but are not limited to, Nivolumab, MK-3475, Pidilizumab, or a derivative or antigen-binding fragment thereof.

As used herein, anti-PD-L1 antibody refers to an antibody that specifically binds to programed death-ligand 1 (PD-L1) or a soluble fragment thereof and blocking the binding of the ligand to PD-1, thereby resulting in competitive inhibition of PD-1 and inhibition of PD-1-mediated inhibition of T cell activity. Hence, anti-PD-L1 antibodies are PD-1 inhibitors. Reference to anti-PD-L1 antibodies herein include a full-length antibody and derivatives thereof, such as antigen-binding fragments thereof that specifically bind to PD-L1. Exemplary anti-PD-L1 antibodies include,

but are not limited to, BMS-936559, MPDL3280A, MEDI4736 or a derivative or antigen-binding fragment thereof.

As used herein, "antibody" refers to immunoglobulins and immunoglobulin fragments, whether natural or partially or wholly synthetically, such as recombinantly, produced, including any fragment thereof containing at least a portion of the variable heavy chain and light region of the immunoglobulin molecule that is sufficient to form an antigen binding site and, when assembled, to specifically bind antigen. Hence, an antibody includes any protein having a binding domain that is homologous or substantially homologous to an immunoglobulin antigen-binding domain (antibody combining site). For example, an antibody refers to an antibody that contains two heavy chains (which can be denoted H and H') and two light chains (which can be denoted L and L'), where each heavy chain can be a full-length immunoglobulin heavy chain or a portion thereof sufficient to form an antigen binding site (*e.g.*, heavy chains include, but are not limited to, VH chains, VH-CH1 chains and VH-CH1-CH2-CH3 chains), and each light chain can be a full-length light chain or a portion thereof sufficient to form an antigen binding site (*e.g.*, light chains include, but are not limited to, VL chains and VL-CL chains). Each heavy chain (H and H') pairs with one light chain (L and L', respectively). Typically, antibodies minimally include all or at least a portion of the variable heavy (VH) chain and/or the variable light (VL) chain. The antibody also can include all or a portion of the constant region.

For purposes herein, the term antibody includes full-length antibodies and portions thereof including antibody fragments. Antibody fragments, include, but are not limited to, Fab fragments, Fab' fragments, $F(ab')_2$ fragments, Fv fragments, disulfide-linked Fvs (dsFv), Fd fragments, Fd' fragments, single-chain Fvs (scFv), single-chain Fabs (scFab), diabodies, anti-idiotypic (anti-Id) antibodies, or antigen-binding fragments of any of the above. Antibody also includes synthetic antibodies, recombinantly produced antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), human antibodies, non-human antibodies, humanized antibodies, chimeric antibodies, and intrabodies. Antibodies provided herein include members of any immunoglobulin type (*e.g.*, IgG, IgM, IgD, IgE, IgA and IgY), any class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass (*e.g.*, IgG2a and IgG2b).

As used herein, the phrase "derived from" or "derivative" when referring to antibody fragments derived from another antibody, such as a monoclonal antibody, refers to the engineering of antibody fragments (*e.g.*, Fab, F(ab'), $F(ab')_2$, single-chain Fv (scFv), Fv, dsFv, diabody, Fd and Fd' fragments) that retain the binding specificity of the original or parent antibody. Such fragments can be derived by a variety of methods known in the art, including, but not limited to, enzymatic cleavage, chemical crosslinking, recombinant means or combinations thereof. Generally, the derived antibody fragment shares the identical or substantially identical heavy chain variable region (V_H) and light chain variable region (V_L) of the parent antibody, such that the antibody fragment and the parent antibody bind the same epitope.

As used herein, an anti-hyaluronan agent refers to any agent that modulates hyaluronan (HA) synthesis or degradation, thereby altering hyaluronan levels in a tissue or cell. For purposes herein, anti-hyaluronan agents reduce hyaluronan levels in a tissue or cell compared to the absence of the agent. Such agents include compounds that modulate the expression of genetic material encoding HA synthase (HAS) and other enzymes or receptors involved in hyaluronan metabolism, or that modulate the proteins that synthesize or degrade hyaluronan including HAS function or activity. The agents include small-molecules, nucleic acids, peptides, proteins or other compounds. For example, anti-hyaluronan agents include, but are not limited to, antisense or sense molecules, antibodies, enzymes, small molecule inhibitors and HAS substrate analogs.

As used herein, a hyaluronan degrading enzyme refers to an enzyme that catalyzes the cleavage of a hyaluronan polymer (also referred to as hyaluronic acid or HA) into smaller molecular weight fragments. Exemplary hyaluronan degrading enzymes are hyaluronidases, and particular chondroitinases and lyases that have the ability to depolymerize hyaluronan. Exemplary chondroitinases that are hyaluronan degrading enzymes include, but are not limited to, chondroitin ABC lyase (also known as chondroitinase ABC), chondroitin AC lyase (also known as chondroitin sulfate lyase or chondroitin sulfate eliminase) and chondroitin C lyase.

As used herein, hyaluronidase refers to a class of hyaluronan degrading enzymes. Hyaluronidases include bacterial hyaluronidases (EC 4.2.2.1 or EC 4.2.99.1), hyaluronidases from leeches, other parasites, and crustaceans (EC 3.2.1.36),

and mammalian-type hyaluronidases (EC 3.2.1.35). Hyaluronidases include any of non-human origin including, but not limited to, murine, canine, feline, leporine, avian, bovine, ovine, porcine, equine, piscine, ranine, bacterial, and any from leeches, other parasites, and crustaceans. For example, hyaluronidases include those of human origin. Exemplary human hyaluronidases include HYAL1, HYAL2, HYAL3, HYAL4, and PH20 (SEQ ID NO:480 and 551). Also included amongst hyaluronidases are soluble hyaluronidases, including, ovine and bovine PH20, soluble human PH20 and soluble rHuPH20. Examples of commercially available bovine or ovine soluble hyaluronidases include Vitrase® (ovine hyaluronidase), Amphadase® (bovine hyaluronidase) and Hydase™ (bovine hyaluronidase).

Reference to hyaluronan degrading enzymes or hyaluronidase includes precursor hyaluronan degrading enzyme polypeptides and mature hyaluronan degrading enzyme polypeptides (such as those in which a signal sequence has been removed), truncated forms thereof that have activity, and includes allelic variants and species variants, variants encoded by splice variants, and other variants, including polypeptides that have at least 40%, 45%, 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the precursor polypeptides, or the mature forms thereof. Hyaluronan degrading enzymes and hyaluronidase also include those that contain chemical or posttranslational modifications and those that do not contain chemical or posttranslational modifications. Such modifications include, but are not limited to, PEGylation, albumination, glycosylation, farnesylation, carboxylation, hydroxylation, phosphorylation, and other polypeptide modifications known in the art. A truncated PH20 hyaluronidase is any C-terminal shortened form thereof, particularly forms that are truncated and neutral active when N-glycosylated.

As used herein, "bovine PH20" refers to a bovine hyaluronidase purified from bovine testicular extracts (see U.S. Patent Nos. 2,488,564, 2,488,565, 2,806,815, 2,808,362, 2,676,139, 2,795,529, 5,747,027 and 5,827,721). Examples of commercially available purified bovine testicular hyaluronidases include Amphadase® and Hydase™, and bovine hyaluronidases, including, but not limited to, those available from Sigma Aldrich, Abnova, EMD Chemicals, GenWay Biotech,

Inc., Raybiotech, Inc., and Calzyme. Also included are recombinantly produced bovine hyaluronidases.

As used herein, "ovine PH20" refers to an ovine hyaluronidase purified from ovine testicular extracts (see U.S. Patent Nos. 2,488,564, 2,488,565 and 2,806,815 and International PCT Publication No. WO2005/118799). Examples of commercially available purified ovine testicular extract include Vitrase®, and ovine hyaluronidases, including, but not limited to, those available from Sigma Aldrich, Cell Sciences, EMD Chemicals, GenWay Biotech, Inc., Mybiosource.com and Raybiotech, Inc. Also included are recombinantly produced ovine hyaluronidases.

As used herein, "PH20" refers to a type of hyaluronidase that occurs in sperm and is neutral-active. PH-20 occurs on the sperm surface, and in the lysosome-derived acrosome, where it is bound to the inner acrosomal membrane. PH20 includes those of any origin including, but not limited to, human, chimpanzee, Cynomolgus monkey, Rhesus monkey, murine, bovine, ovine, guinea pig, rabbit and rat origin. Exemplary PH20 polypeptides include those from human (precursor set forth in SEQ ID NO:551 and mature set forth in SEQ ID NO:480).

As used herein, a "soluble PH20" refers to any form of PH20 that is soluble under physiologic conditions. A soluble PH20 can be identified, for example, by its partitioning into the aqueous phase of a Triton® X-114 solution at 37 °C (Bordier *et al.*, (1981) *J. Biol. Chem.*, 256:1604-7). Membrane-anchored PH20, such as lipid-anchored PH20, including GPI-anchored PH20, will partition into the detergent-rich phase, but will partition into the detergent-poor or aqueous phase following treatment with Phospholipase-C. Included among soluble PH20 are membrane-anchored PH20 in which one or more regions associated with anchoring of the PH20 to the membrane has been removed or modified, where the soluble form retains hyaluronidase activity. Soluble PH20 also includes recombinant soluble PH20 and those contained in or purified from natural sources, such as, for example, testes extracts from sheep or cows. An example of such soluble PH20 is soluble human PH20. Exemplary soluble human PH20 polypeptides are set forth in any of SEQ ID NOS:481-488, 493-514, or 526-532, or has a sequence of amino acids that is at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to a

sequence of amino acids set forth in any of SEQ ID NOS:481-488, 493-514, or 526-532 and is soluble and retains hyaluronidase activity.

As used herein, "soluble recombinant human PH20 (rHuPH20)" refers to a composition containing soluble form of human PH20 as recombinantly expressed and secreted in Chinese Hamster Ovary (CHO) cells. Soluble rHuPH20 is encoded by nucleic acid molecule that includes the signal sequence and encodes the polypeptide set forth in SEQ ID NO:481. The nucleic acid encoding soluble rHuPH20 is expressed in CHO cells which secrete the mature polypeptide. As produced in the culture medium, there is heterogeneity at the C-terminus so that the product includes a mixture of species that can include any one or more of SEQ ID NO:481-486 in various abundance.

As used herein, "hyaluronidase activity" refers to the ability to enzymatically catalyze the cleavage of hyaluronic acid. The United States Pharmacopeia (USP) XXII assay for hyaluronidase determines hyaluronidase activity indirectly by measuring the amount of higher molecular weight hyaluronic acid, or hyaluronan, (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37°C (USP XXII-NF XVII (1990) 644-645 United States Pharmacopeia Convention, Inc., Rockville, MD). A Reference Standard solution can be used in an assay to ascertain the relative activity, in units, of any hyaluronidase. *In vitro* assays to determine the hyaluronidase activity of hyaluronidases, such as PH20, including soluble PH20 and esPH20, are known in the art and described herein. Exemplary assays include the microturbidity assay that measures cleavage of hyaluronic acid by hyaluronidase indirectly by detecting the insoluble precipitate formed when the uncleaved hyaluronic acid binds with serum albumin and the biotinylated-hyaluronic acid assay that measures the cleavage of hyaluronic acid indirectly by detecting the remaining biotinylated-hyaluronic acid non-covalently bound to microtiter plate wells with a streptavidin-horseradish peroxidase conjugate and a chromogenic substrate. Reference Standards can be used, for example, to generate a standard curve to determine the activity in Units of the hyaluronidase being tested.

As used herein, "neutral active" refers to the ability of a PH20 polypeptide to enzymatically catalyze the cleavage of hyaluronic acid at neutral pH (*e.g.,* at or about pH 7.0).

As used herein, an anti-cancer agent or chemotherapeutic agent refers to an agent that is capable of killing cells that divide rapidly, such as cancer cells. One of skill in the art is familiar with anti-cancer agents, including chemotherapeutic agents. Exemplary agents are described herein.

As used herein, "biological activity" refers to the *in vivo* activities of a compound or physiological responses that result upon *in vivo* administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions and mixtures. Biological activities can be observed in *in vitro* systems designed to test or use such activities. Thus, for purposes herein a biological activity of a ADA2 encompasses adenosine deaminase activity.

As used herein the term "assess", and grammatical variations thereof, is intended to include quantitative and qualitative determination in the sense of obtaining an absolute value for the activity of a polypeptide, and also of obtaining an index, ratio, percentage, visual or other value indicative of the level of the activity. Assessment can be direct or indirect. For example, detection of cleavage of a substrate by a polypeptide can be by direct measurement of the product, or can be indirectly measured by determining the resulting activity of the cleaved substrate.

As used herein, "mature numbering" or "standard numbering" refers to the numbering of residues in order based on a mature ADA2 polypeptide. For purposes herein, mature numbering is based on the numbering of residues of mature ADA2 set forth in SEQ ID NO:5.

As used herein, "Zavialov numbering" refers to the numbering of residues used in Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377 and in PDB accession Nos. 3LGG and 3LGD. Zavialov numbering is based on the numbering of residues of ADA2 as set forth in SEQ ID NO:4. Hence Zavialov numbering can be determined by alignment with SEQ ID NO:4. Table 1 below sets forth the corresponding position numbers between mature numbering and Zavialov numbering. Table 1 provides the sequence of amino acids set forth in SEQ ID NO:4 (reference sequence for Zavialov numbering), its position numbers and the corresponding position numbers for SEQ ID NO:5 (reference sequence for mature numbering as used herein).

Table 1. Corresponding Position Numbers for Mature Numbering (SEQ ID NO:5) and Zavialov Numbering (SEQ ID NO:4)

SEQ ID NO:5 (mature)				1	2	3	4	5	6	7
SEQ ID NO:4 (Zavialov)	1	2	3	4	5	6	7	8	9	10
Sequence (SEQ ID NO:4)	G	G	S	I	D	E	T	R	A	H
SEQ ID NO:5 (mature)	8	9	10	11	12	13	14	15	16	17
SEQ ID NO:4 (Zavialov)	11	12	13	14	15	16	17	18	19	20
Sequence (SEQ ID NO:4)	L	L	L	K	E	K	M	M	R	L
SEQ ID NO:5 (mature)	18	19	20	21	22	23	24	25	26	27
SEQ ID NO:4 (Zavialov)	21	22	23	24	25	26	27	28	29	30
Sequence (SEQ ID NO:4)	G	G	R	L	V	L	N	T	K	E
SEQ ID NO:5 (mature)	28	29	30	31	32	33	34	35	36	37
SEQ ID NO:4 (Zavialov)	31	32	33	34	35	36	37	38	39	40
Sequence (SEQ ID NO:4)	E	L	A	N	E	R	L	M	T	L
SEQ ID NO:5 (mature)	38	39	40	41	42	43	44	45	46	47
SEQ ID NO:4 (Zavialov)	41	42	43	44	45	46	47	48	49	50
Sequence (SEQ ID NO:4)	K	I	A	E	M	K	E	A	M	R
SEQ ID NO:5 (mature)	48	49	50	51	52	53	54	55	56	57
SEQ ID NO:4 (Zavialov)	51	52	53	54	55	56	57	58	59	60
Sequence (SEQ ID NO:4)	T	L	I	F	P	P	S	M	H	F
SEQ ID NO:5 (mature)	58	59	60	61	62	63	64	65	66	67
SEQ ID NO:4 (Zavialov)	61	62	63	64	65	66	67	68	69	70
Sequence (SEQ ID NO:4)	F	Q	A	K	H	L	I	E	R	S
SEQ ID NO:5 (mature)	68	69	70	71	72	73	74	75	76	77
SEQ ID NO:4 (Zavialov)	71	72	73	74	75	76	77	78	79	80
Sequence (SEQ ID NO:4)	Q	V	F	N	I	L	R	M	M	P
SEQ ID NO:5 (mature)	78	79	80	81	82	83	84	85	86	87
SEQ ID NO:4 (Zavialov)	81	82	83	84	85	86	87	88	89	90
Sequence (SEQ ID NO:4)	K	G	A	A	L	H	L	H	D	I
SEQ ID NO:5 (mature)	88	89	90	91	92	93	94	95	96	97
SEQ ID NO:4 (Zavialov)	91	92	93	94	95	96	97	98	99	100
Sequence (SEQ ID NO:4)	G	I	V	T	M	D	W	L	V	R
SEQ ID NO:5 (mature)	98	99	100	101	102	103	104	105	106	107
SEQ ID NO:4 (Zavialov)	101	102	103	104	105	106	107	108	109	110
Sequence (SEQ ID NO:4)	N	V	T	Y	R	P	H	C	H	I
SEQ ID NO:5 (mature)	108	109	110	111	112	113	114	115	116	117
SEQ ID NO:4 (Zavialov)	111	112	113	114	115	116	117	118	119	120
Sequence (SEQ ID NO:4)	C	F	T	P	R	G	I	M	Q	F
SEQ ID NO:5 (mature)	118	119	120	121	122	123	124	125	126	127
SEQ ID NO:4 (Zavialov)	121	122	123	124	125	126	127	128	129	130
Sequence (SEQ ID NO:4)	R	F	A	H	P	T	P	R	P	S
SEQ ID NO:5 (mature)	128	129	130	131	132	133	134	135	136	137
SEQ ID NO:4 (Zavialov)	131	132	133	134	135	136	137	138	139	140
Sequence (SEQ ID NO:4)	E	K	C	S	K	W	I	L	L	E
SEQ ID NO:5 (mature)	138	139	140	141	142	143	144	145	146	147
SEQ ID NO:4 (Zavialov)	141	142	143	144	145	146	147	148	149	150

Table 1. Corresponding Position Numbers for Mature Numbering (SEQ ID NO:5) and Zavialov Numbering (SEQ ID NO:4)

Sequence (SEQ ID NO:4)	D	Y	R	K	R	V	Q	N	V	T
SEQ ID NO:5 (mature)	148	149	150	151	152	153	154	155	156	157
SEQ ID NO:4 (Zavialov)	151	152	153	154	155	156	157	158	159	160
Sequence (SEQ ID NO:4)	E	F	D	D	S	L	L	R	N	F
SEQ ID NO:5 (mature)	158	159	160	161	162	163	164	165	166	167
SEQ ID NO:4 (Zavialov)	161	162	163	164	165	166	167	168	169	170
Sequence (SEQ ID NO:4)	T	L	V	T	Q	H	P	E	V	I
SEQ ID NO:5 (mature)	168	169	170	171	172	173	174	175	176	177
SEQ ID NO:4 (Zavialov)	171	172	173	174	175	176	177	178	179	180
Sequence (SEQ ID NO:4)	Y	T	N	Q	N	V	V	W	S	K
SEQ ID NO:5 (mature)	178	179	180	181	182	183	184	185	186	187
SEQ ID NO:4 (Zavialov)	181	182	183	184	185	186	187	188	189	190
Sequence (SEQ ID NO:4)	F	E	T	I	F	F	T	I	S	G
SEQ ID NO:5 (mature)	188	189	190	191	192	193	194	195	196	197
SEQ ID NO:4 (Zavialov)	191	192	193	194	195	196	197	198	199	200
Sequence (SEQ ID NO:4)	L	I	H	Y	A	P	V	F	R	D
SEQ ID NO:5 (mature)	198	199	200	201	202	203	204	205	206	207
SEQ ID NO:4 (Zavialov)	201	202	203	204	205	206	207	208	209	210
Sequence (SEQ ID NO:4)	Y	V	F	R	S	M	Q	E	F	Y
SEQ ID NO:5 (mature)	208	209	210	211	212	213	214	215	216	217
SEQ ID NO:4 (Zavialov)	211	212	213	214	215	216	217	218	219	220
Sequence (SEQ ID NO:4)	E	D	N	V	L	Y	M	E	I	R
SEQ ID NO:5 (mature)	218	219	220	221	222	223	224	225	226	227
SEQ ID NO:4 (Zavialov)	221	222	223	224	225	226	227	228	229	230
Sequence (SEQ ID NO:4)	A	R	L	L	P	V	Y	E	L	S
SEQ ID NO:5 (mature)	228	229	230	231	232	233	234	235	236	237
SEQ ID NO:4 (Zavialov)	231	232	233	234	235	236	237	238	239	240
Sequence (SEQ ID NO:4)	G	E	H	H	D	E	E	W	S	V
SEQ ID NO:5 (mature)	238	239	240	241	242	243	244	245	246	247
SEQ ID NO:4 (Zavialov)	241	242	243	244	245	246	247	248	249	250
Sequence (SEQ ID NO:4)	K	T	Y	Q	E	V	A	Q	K	F
SEQ ID NO:5 (mature)	248	249	250	251	252	253	254	255	256	257
SEQ ID NO:4 (Zavialov)	251	252	253	254	255	256	257	258	259	260
Sequence (SEQ ID NO:4)	V	E	T	H	P	E	F	I	G	I
SEQ ID NO:5 (mature)	258	259	260	261	262	263	264	265	266	267
SEQ ID NO:4 (Zavialov)	261	262	263	264	265	266	267	268	269	270
Sequence (SEQ ID NO:4)	K	I	I	Y	S	D	H	R	S	K
SEQ ID NO:5 (mature)	268	269	270	271	272	273	274	275	276	277
SEQ ID NO:4 (Zavialov)	271	272	273	274	275	276	277	278	279	280
Sequence (SEQ ID NO:4)	D	V	A	V	I	A	E	S	I	R
SEQ ID NO:5 (mature)	278	279	280	281	282	283	284	285	286	287
SEQ ID NO:4 (Zavialov)	281	282	283	284	285	286	287	288	289	290
Sequence (SEQ ID NO:4)	M	A	M	G	L	R	I	K	F	P
SEQ ID NO:5 (mature)	288	289	290	291	292	293	294	295	296	297

Table 1. Corresponding Position Numbers for Mature Numbering (SEQ ID NO:5) and Zavialov Numbering (SEQ ID NO:4)										
SEQ ID NO:4 (Zavialov)	291	292	293	294	295	296	297	298	299	300
Sequence (SEQ ID NO:4)	T	V	V	A	G	F	D	L	V	G
SEQ ID NO:5 (mature)	298	299	300	301	302	303	304	305	306	307
SEQ ID NO:4 (Zavialov)	301	302	303	304	305	306	307	308	309	310
Sequence (SEQ ID NO:4)	H	E	D	T	G	H	S	L	H	D
SEQ ID NO:5 (mature)	308	309	310	311	312	313	314	315	316	317
SEQ ID NO:4 (Zavialov)	311	312	313	314	315	316	317	318	319	320
Sequence (SEQ ID NO:4)	Y	K	E	A	L	M	I	P	A	K
SEQ ID NO:5 (mature)	318	319	320	321	322	323	324	325	326	327
SEQ ID NO:4 (Zavialov)	321	322	323	324	325	326	327	328	329	330
Sequence (SEQ ID NO:4)	D	G	V	K	L	P	Y	F	F	H
SEQ ID NO:5 (mature)	328	329	330	331	332	333	334	335	336	337
SEQ ID NO:4 (Zavialov)	331	332	333	334	335	336	337	338	339	340
Sequence (SEQ ID NO:4)	A	G	E	T	D	W	Q	G	T	S
SEQ ID NO:5 (mature)	338	339	340	341	342	343	344	345	346	347
SEQ ID NO:4 (Zavialov)	341	342	343	344	345	346	347	348	349	350
Sequence (SEQ ID NO:4)	I	D	R	N	I	L	D	A	L	M
SEQ ID NO:5 (mature)	348	349	350	351	352	353	354	355	356	357
SEQ ID NO:4 (Zavialov)	351	352	353	354	355	356	357	358	359	360
Sequence (SEQ ID NO:4)	L	N	T	T	R	I	G	H	G	F
SEQ ID NO:5 (mature)	358	359	360	361	362	363	364	365	366	367
SEQ ID NO:4 (Zavialov)	361	362	363	364	365	366	367	368	369	370
Sequence (SEQ ID NO:4)	A	L	S	K	H	P	A	V	R	T
SEQ ID NO:5 (mature)	368	369	370	371	372	373	374	375	376	377
SEQ ID NO:4 (Zavialov)	371	372	373	374	375	376	377	378	379	380
Sequence (SEQ ID NO:4)	Y	S	W	K	K	D	I	P	I	E
SEQ ID NO:5 (mature)	378	379	380	381	382	383	384	385	386	387
SEQ ID NO:4 (Zavialov)	381	382	383	384	385	386	387	388	389	390
Sequence (SEQ ID NO:4)	V	C	P	I	S	N	Q	V	L	K
SEQ ID NO:5 (mature)	388	389	390	391	392	393	394	395	396	397
SEQ ID NO:4 (Zavialov)	391	392	393	394	395	396	397	398	399	400
Sequence (SEQ ID NO:4)	L	V	S	D	L	R	N	H	P	V
SEQ ID NO:5 (mature)	398	399	400	401	402	403	404	405	406	407
SEQ ID NO:4 (Zavialov)	401	402	403	404	405	406	407	408	409	410
Sequence (SEQ ID NO:4)	A	T	L	M	A	T	G	H	P	M
SEQ ID NO:5 (mature)	408	409	410	411	412	413	414	415	416	417
SEQ ID NO:4 (Zavialov)	411	412	413	414	415	416	417	418	419	420
Sequence (SEQ ID NO:4)	V	I	S	S	D	D	P	A	M	F
SEQ ID NO:5 (mature)	418	419	420	421	422	423	424	425	426	427
SEQ ID NO:4 (Zavialov)	421	422	423	424	425	426	427	428	429	430
Sequence (SEQ ID NO:4)	G	A	K	G	L	S	Y	D	F	Y
SEQ ID NO:5 (mature)	428	429	430	431	432	433	434	435	436	437
SEQ ID NO:4 (Zavialov)	431	432	433	434	435	436	437	438	439	440

Table 1. Corresponding Position Numbers for Mature Numbering (SEQ ID NO:5) and Zavialov Numbering (SEQ ID NO:4)										
Sequence (SEQ ID NO:4)	E	V	F	M	G	I	G	G	M	K
SEQ ID NO:5 (mature)	438	439	440	441	442	443	444	445	446	447
SEQ ID NO:4 (Zavialov)	441	442	443	444	445	446	447	448	449	450
Sequence (SEQ ID NO:4)	A	D	L	R	T	L	K	Q	L	A
SEQ ID NO:5 (mature)	448	449	450	451	452	453	454	455	456	457
SEQ ID NO:4 (Zavialov)	451	452	453	454	455	456	457	458	459	460
Sequence (SEQ ID NO:4)	M	N	S	I	K	Y	S	T	L	L
SEQ ID NO:5 (mature)	458	459	460	461	462	463	464	465	466	467
SEQ ID NO:4 (Zavialov)	461	462	463	464	465	466	467	468	469	470
Sequence (SEQ ID NO:4)	E	S	E	K	N	T	F	M	E	I
SEQ ID NO:5 (mature)	468	469	470	471	472	473	474	475	476	477
SEQ ID NO:4 (Zavialov)	471	472	473	474	475	476	477	478	479	480
Sequence (SEQ ID NO:4)	W	K	K	R	W	D	K	F	I	A
SEQ ID NO:5 (mature)	478	479	480	481	482					
SEQ ID NO:4 (Zavialov)	481	482	483	484	485					
Sequence (SEQ ID NO:4)	D	V	A	T	K					

As used herein, a "conjugate" refers to a polypeptide linked directly or indirectly to one or more other polypeptides or chemical moieties. Such conjugates include fusion proteins, those produced by chemical conjugates and those produced by any other methods. For example, a conjugate refers to an ADA2 protein linked directly or indirectly to one or more other polypeptides or chemical moieties, whereby at least one ADA2 polypeptide subunit is linked, directly or indirectly to another polypeptide or chemical moiety so long as the conjugate retains adenosine deaminase activity.

As used herein, "coupled" or "conjugated" means attached via a covalent or noncovalent interaction.

As used herein, a chimeric polypeptide refers to a polypeptide that contains portions from at least two different polypeptides or from two non-contiguous portions of a single polypeptide. Thus, a chimeric polypeptide generally includes a sequence of amino acid residues from all or part of one polypeptide and a sequence of amino acids from all or part of another different polypeptide. The two portions can be linked directly or indirectly and can be linked via peptide bonds, other covalent bonds covalent interactions of sufficient strength to maintain the integrity of a substantial portion of the chimeric polypeptide under equilibrium conditions and physiologic conditions, such as in isotonic pH 7 buffered saline.

As used herein, a fusion protein is a polypeptide engineered to contain sequences of amino acids corresponding to two distinct polypeptides, which are joined together, such as by expressing the fusion protein from a vector containing two nucleic acids, encoding the two polypeptides, in close proximity, *e.g.*, adjacent, to one another along the length of the vector. Accordingly, a fusion protein refers to a chimeric protein containing two, or portions from two, or more proteins or peptides that are linked directly or indirectly via peptide bonds. The two molecules can be adjacent in the construct or separated by a linker, or spacer polypeptide.

As used herein, "linker" or "spacer" peptide refers to short sequences of amino acids that join two polypeptide sequences (or nucleic acid encoding such an amino acid sequence). "Peptide linker" refers to the short sequence of amino acids joining the two polypeptide sequences. Exemplary of polypeptide linkers are linkers joining a peptide transduction domain to an antibody or linkers joining two antibody chains in a synthetic antibody fragment such as an scFv fragment. Linkers are well-known and any known linkers can be used in the provided methods. Exemplary of polypeptide linkers are $(Gly\text{-}Ser)_n$ amino acid sequences, with some Glu or Lys residues dispersed throughout to increase solubility. Other exemplary linkers are described herein; any of these and other known linkers can be used with the provided compositions and methods.

As used herein, a multimerization domain refers to a sequence of amino acids that promotes stable interaction of a polypeptide molecule with one or more additional polypeptide molecules, each containing a complementary multimerization domain, which can be the same or a different multimerization domain to form a stable multimer with the first domain. Generally, a polypeptide is joined directly or indirectly to the multimerization domain. Exemplary multimerization domains include the immunoglobulin sequences or portions thereof, leucine zippers, hydrophobic regions, hydrophilic regions, and compatible protein-protein interaction domains. The multimerization domain, for example, can be an immunoglobulin constant region or domain, such as, for example, the Fc domain or portions thereof from IgG, including IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD and IgM and modified forms thereof.

As used herein, a "moiety" or "heterologous moiety" refers to a molecule that is capable of associating with another molecule, either directly or indirectly by a covalent or non-covalent interaction. Typically, the molecule is derived from a distinct entity from that of the entity to which it is being associated. In one embodiment, a heterologous moiety can be a polypeptide fused to another polypeptide to produce a fusion polypeptide or protein. In another embodiment, a heterologous moiety can be a non-polypeptide such as a polymer, such as a PEG conjugated to a polypeptide or protein.

As used herein, a "half-life extending moiety" is heterologous moiety that facilitates the increased half-life of the molecule to which it is conjugated.

As used herein, "Fc" or "Fc region" or "Fc domain" refers to a polypeptide containing the constant region of an antibody heavy chain, excluding the first constant region immunoglobulin domain. Thus, Fc refers to the last two constant region immunoglobulin domains of IgA, IgD, and IgE, or the last three constant region immunoglobulin domains of IgE and IgM. Optionally, an Fc domain can include all or part of the flexible hinge N-terminal to these domains. For IgA and IgM, Fc can include the J chain. For an exemplary Fc domain of IgG, Fc contains immunoglobulin domains Cγ2 and Cγ3, and optionally, all or part of the hinge between Cγ1 and Cγ2. The boundaries of the Fc region can vary, but typically, include at least part of the hinge region. In addition, Fc also includes any allelic or species variant or any variant or modified form, such as any variant or modified form that alters the binding to an FcR or alters an Fc-mediated effector function.

As used herein, "Fc chimera" refers to a chimeric polypeptide in which one or more polypeptides is linked, directly or indirectly, to an Fc region or a derivative thereof. Typically, an Fc chimera combines the Fc region of an immunoglobulin with another polypeptide. Derivatives of or modified Fc polypeptides are known to those of skill in the art.

As used herein, a "polymer" refers to any high molecular weight natural or synthetic moiety that is conjugated to, *i.e.* stably linked directly or indirectly via a linker, to a polypeptide. Such polymers, typically increase serum half-life, and include, but are not limited to sialic moieties, PEGylation moieties, dextran, and sugar

and other moieties, such as for glycosylation. For example, ADA2 proteins, such as a variant ADA2, can be conjugated to a polymer.

As used herein, "PEGylated" refers to covalent or other stable attachment of polymeric molecules, such as polyethylene glycol (PEGylation moiety PEG) to proteins, including an ADA2, such as a variant ADA2. PEGylation can increase half-life of the ADA2.

As used herein, nucleic acids include DNA, RNA and analogs thereof, including peptide nucleic acids (PNA) and mixtures thereof. Nucleic acids can be single or double-stranded. When referring to probes or primers, which are optionally labeled, such as with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that their target is statistically unique or of low copy number (typically less than 5, generally less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous nucleotides of sequence complementary to or identical to a gene of interest. Probes and primers can be 10, 20, 30, 50, 100 or more nucleotides long.

As used herein, a peptide refers to a polypeptide that is from 2 to 40 amino acids in length.

As used herein, the amino acids that occur in the various sequences of amino acids provided herein are identified according to their known, three-letter or one-letter abbreviations (Table 2). The nucleotides which occur in the various nucleic acid fragments are designated with the standard single-letter designations used routinely in the art.

As used herein, an "amino acid" is an organic compound containing an amino group and a carboxylic acid group. A polypeptide contains two or more amino acids. For purposes herein, amino acids include the twenty naturally-occurring amino acids, non-natural amino acids and amino acid analogs (i.e., amino acids wherein the α-carbon has a side chain).

In keeping with standard polypeptide nomenclature described in *J. Biol. Chem.*, 243: 3557-3559 (1968), and adopted in 37 C.F.R. §§ 1.821-1.822, abbreviations for the amino acid residues are shown in Table 2:

Table 2. Table of Correspondence		
SYMBOL		
1-Letter	3-Letter	AMINO ACID
Y	Tyr	Tyrosine
G	Gly	Glycine
F	Phe	Phenylalanine
M	Met	Methionine
A	Ala	Alanine
S	Ser	Serine
I	Ile	Isoleucine
L	Leu	Leucine
T	Thr	Threonine
V	Val	Valine
P	Pro	proline
K	Lys	Lysine
H	His	Histidine
Q	Gln	Glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	Tryptophan
R	Arg	Arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	Cysteine
X	Xaa	Unknown or other

It should be noted that all amino acid residue sequences represented herein by formulae have a left to right orientation in the conventional direction of amino-terminus to carboxyl-terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence (Table 2) and modified and unusual amino acids, such as those referred to in 37 C.F.R. §§ 1.821-1.822. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues, to an amino-terminal group such as NH_2 or to a carboxyl-terminal group such as COOH.

As used herein, a "hydrophobic amino acid" includes any one of the amino acids determined to be hydrophobic using the Eisenberg hydrophobicity consensus scale. Exemplary are the naturally occurring hydrophobic amino acids, such as isoleucine, phenylalanine, valine, leucine, tryptophan, methionine, alanine, glycine, cysteine and tyrosine (Eisenberg *et al.*, (1982) Faraday Symp. Chem. Soc. 17:109-120). Non-naturally-occurring hydrophobic amino acids also are included.

As used herein, an "acidic amino acid" includes among the naturally-occurring amino acids aspartic acid and glutamic acid residues. Non-naturally-occurring acidic amino acids also are included.

As used herein, a "polar amino acid" refers to an amino acid that is a hydrophile, such that the side chains prefer to reside in an aqueous (*i.e.* water) environment. Such amino acids generally are located on the surface of a protein. Such amino acids generally are classified if they include those with polar side chains that have a functional group such as an acid, amide, alcohol or amine that contains oxygens or nitrogens that can participate in hydrogen bonding with water. Exemplary of such amino acids are Arg (R), Asn (N), Asp (D), Glu (E), Gln (Q), His (H), Lys (K), Ser (S), Thr (T), and Tyr (Y). Cys (C) and Trp (W), which are also considered to be weakly polar.

As used herein, a polar and neutral amino acid is a polar amino acid that contains a neutral side chain. Exemplary of such amino acid residues for replacement are Asn (N), Gln (Q), Ser (S), Thr (T), Cys (C) or Tyr (Y).

As used herein, "naturally occurring amino acids" refer to the 20 L-amino acids that occur in polypeptides.

As used herein, "non-natural amino acid" refers to an organic compound containing an amino group and a carboxylic acid group that is not one of the naturally-occurring amino acids listed in Table 2. Non-naturally occurring amino acids thus include, for example, amino acids or analogs of amino acids other than the 20 naturally-occurring amino acids and include, but are not limited to, the D-stereoisomer of amino acids. Exemplary non-natural amino acids are known to those of skill in the art and can be included in a modified ADA2 polypeptide.

As used herein, suitable conservative substitutions of amino acids are known to those of skill in the art and can be made generally without altering the biological activity of the resulting molecule. Those of skill in the art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, *e.g.*, Watson *et al.* Molecular Biology of the Gene, 4th Edition, 1987, The Benjamin/Cummings Pub. co., p.224). Such substitutions can be made in accordance with those set forth in Table 3 as follows:

Table 3	
Original residue	Exemplary conservative substitution
Ala (A)	Gly; Ser
Arg (R)	Lys
Asn (N)	Gln; His
Cys (C)	Ser
Gln (Q)	Asn
Glu (E)	Asp
Gly (G)	Ala; Pro
His (H)	Asn; Gln
Ile (I)	Leu; Val
Leu (L)	Ile; Val
Lys (K)	Arg; Gln; Glu
Met (M)	Leu; Tyr; Ile
Phe (F)	Met; Leu; Tyr
Ser (S)	Thr
Thr (T)	Ser
Trp (W)	Tyr
Tyr (Y)	Trp; Phe
Val (V)	Ile; Leu

Other substitutions also are permissible and can be determined empirically or in accord with known conservative substitutions.

As used herein, a DNA construct is a single or double stranded, linear or circular DNA molecule that contains segments of DNA combined and juxtaposed in a manner not found in nature. DNA constructs exist as a result of human manipulation, and include clones and other copies of manipulated molecules.

As used herein, a DNA segment is a portion of a larger DNA molecule having specified attributes. For example, a DNA segment encoding a specified polypeptide is a portion of a longer DNA molecule, such as a plasmid or plasmid fragment, which, when read from the 5' to 3' direction, encodes the sequence of amino acids of the specified polypeptide.

As used herein, the term polynucleotide means a single- or double-stranded polymer of deoxyribonucleotides or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and can be isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules. The length of a polynucleotide molecule is given herein in terms of nucleotides (abbreviated "nt") or base pairs (abbreviated "bp"). The term nucleotides is used for single- and double-stranded molecules where the context permits. When the term is applied to double-stranded molecules it is used to denote overall length

and will be understood to be equivalent to the term base pairs. It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide can differ slightly in length and that the ends thereof can be staggered; thus all nucleotides within a double-stranded polynucleotide molecule cannot be paired. Such unpaired ends will, in general, not exceed 20 nucleotides in length.

As used herein, "primary sequence" refers to the sequence of amino acid residues in a polypeptide.

As used herein, "similarity" between two proteins or nucleic acids refers to the relatedness between the sequence of amino acids of the proteins or the nucleotide sequences of the nucleic acids. Similarity can be based on the degree of identity and/or homology of sequences of residues and the residues contained therein. Methods for assessing the degree of similarity between proteins or nucleic acids are known to those of skill in the art. For example, in one method of assessing sequence similarity, two amino acid or nucleotide sequences are aligned in a manner that yields a maximal level of identity between the sequences. "Identity" refers to the extent to which the amino acid or nucleotide sequences are invariant. Alignment of amino acid sequences, and to some extent nucleotide sequences, also can take into account conservative differences and/or frequent substitutions in amino acids (or nucleotides). Conservative differences are those that preserve the physico-chemical properties of the residues involved. Alignments can be global (alignment of the compared sequences over the entire length of the sequences and including all residues) or local (the alignment of a portion of the sequences that includes only the most similar region or regions).

As used herein, the terms "homology" and "identity"" are used interchangeably, but homology for proteins can include conservative amino acid changes. In general to identify corresponding positions the sequences of amino acids are aligned so that the highest order match is obtained (see, *e.g.*: Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and

Devereux, J., eds., M Stockton Press, New York, 1991; Carrillo *et al.* (1988) *SIAM J Applied Math* 48:1073).

As use herein, "sequence identity" refers to the number of identical amino acids (or nucleotide bases) in a comparison between a test and a reference polypeptide or polynucleotide. Homologous polypeptides refer to a pre-determined number of identical or homologous amino acid residues. Homology includes conservative amino acid substitutions as well as identical residues. Sequence identity can be determined by standard alignment algorithm programs used with default gap penalties established by each supplier. Homologous nucleic acid molecules refer to a pre-determined number of identical or homologous nucleotides. Homology includes substitutions that do not change the encoded amino acid (*i.e.*, "silent substitutions") as well identical residues. Substantially homologous nucleic acid molecules hybridize typically at moderate stringency or at high stringency all along the length of the nucleic acid or along at least about 70%, 80% or 90% of the full-length nucleic acid molecule of interest. Also contemplated are nucleic acid molecules that contain degenerate codons in place of codons in the hybridizing nucleic acid molecule. (For determination of homology of proteins, conservative amino acids can be aligned as well as identical amino acids; in this case, percentage of identity and percentage homology varies). Whether any two nucleic acid molecules have nucleotide sequences (or any two polypeptides have amino acid sequences) that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson *et al. Proc. Natl. Acad. Sci. USA* 85: 2444 (1988) (other programs include the GCG program package (Devereux, J., *et al.*, *Nucleic Acids Research* 12(I): 387 (1984)), BLASTP, BLASTN, FASTA (Altschul, S.F., *et al.*, *J. Molec. Biol.* 215:403 (1990); Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego (1994), and Carrillo *et al. SIAM J Applied Math* 48: 1073 (1988)). For example, the BLAST function of the National Center for Biotechnology Information database can be used to determine identity. Other commercially or publicly available programs include DNAStar "MegAlign" program (Madison, WI) and the University of Wisconsin Genetics Computer Group (UWG) "Gap" program (Madison WI)). Percent homology or identity of proteins and/or nucleic acid molecules can be determined, for example, by

comparing sequence information using a GAP computer program (*e.g.*, Needleman *et al. J. Mol. Biol.* 48: 443 (1970), as revised by Smith and Waterman (*Adv. Appl. Math.* 2: 482 (1981)). Briefly, a GAP program defines similarity as the number of aligned symbols (*i.e.*, nucleotides or amino acids) that are similar, divided by the total number of symbols in the shorter of the two sequences. Default parameters for the GAP program can include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non identities) and the weighted comparison matrix of Gribskov *et al. Nucl. Acids Res.* 14: 6745 (1986), as described by Schwartz and Dayhoff, eds., Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. In one non-limiting example, "at least 90% identical to" refers to percent identities from 90 to 100% relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids are compared, no more than 10% (*i.e.*, 10 out of 100) of amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons can be made between a test and reference polynucleotides. Such differences can be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they can be clustered in one or more locations of varying length up to the maximum allowable, *e.g.*, 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, insertions or deletions. At the level of homologies or identities above about 85-90%, the result should be independent of the program and gap parameters set; such high levels of identity can be assessed readily, often without relying on software.

As used herein, it also is understood that the terms "substantially identical" or "similar" varies with the context as understood by those skilled in the relevant art, but that those of skill can assess such.

As used herein, an aligned sequence refers to the use of homology (similarity and/or identity) to align corresponding positions in a sequence of nucleotides or

amino acids. Typically, two or more sequences that are related by 50% or more identity are aligned. An aligned set of sequences refers to 2 or more sequences that are aligned at corresponding positions and can include aligning sequences derived from RNAs, such as ESTs and other cDNAs, aligned with genomic DNA sequence.

As used herein, "specifically hybridizes" refers to annealing, by complementary base-pairing, of a nucleic acid molecule (e.g. an oligonucleotide) to a target nucleic acid molecule. Those of skill in the art are familiar with *in vitro* and *in vivo* parameters that affect specific hybridization, such as length and composition of the particular molecule. Parameters particularly relevant to *in vitro* hybridization further include annealing and washing temperature, buffer composition and salt concentration. Exemplary washing conditions for removing non-specifically bound nucleic acid molecules at high stringency are 0.1 x SSPE, 0.1% SDS, 65°C, and at medium stringency are 0.2 x SSPE, 0.1% SDS, 50°C. Equivalent stringency conditions are known in the art. The skilled person can readily adjust these parameters to achieve specific hybridization of a nucleic acid molecule to a target nucleic acid molecule appropriate for a particular application.

As used herein, isolated or purified polypeptide or protein or biologically-active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. Preparations can be determined to be substantially free if they appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound, however, can be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

Hence, reference to an isolated or purified protein or catalytically active protein thereof means that it is substantially free of cellular material or other

contaminating proteins from the cell of tissue from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. Preparations can be determined to be substantially free if they appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as proteolytic and biological activities, of the substance. Methods for purification of the proteins to produce substantially pure polypeptides are known to those of skill in the art.

The term substantially free of cellular material includes preparations of proteins in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly-produced. In one embodiment, the term substantially free of cellular material includes preparations of protease proteins having less than about 30% (by dry weight) of non-protease proteins (also referred to herein as a contaminating protein), generally less than about 20% of non-protease proteins or 10% of non-protease proteins or less that about 5% of non-protease proteins. When the protease protein or active portion thereof is recombinantly produced, it also is substantially free of culture medium, *i.e.*, culture medium represents less than, about, or equal to 20%, 10% or 5% of the volume of the protease protein preparation.

As used herein, the term substantially free of chemical precursors or other chemicals includes preparations of protease proteins in which the protein is separated from chemical precursors or other chemicals that are involved in the synthesis of the protein. The term includes preparations of protease proteins having less than about 30% (by dry weight), 20%, 10%, 5% or less of chemical precursors or non-protease chemicals or components.

As used herein, production by recombinant methods by using recombinant DNA methods refers to the use of the well-known methods of molecular biology for expressing proteins encoded by cloned DNA.

As used herein, vector (or plasmid) refers to discrete elements that are used to introduce heterologous nucleic acid into cells for either expression or replication thereof. The vectors typically remain episomal, but can be designed to effect

integration of a gene or portion thereof into a chromosome of the genome. Also contemplated are vectors that are artificial chromosomes, such as bacterial artificial chromosomes, yeast artificial chromosomes and mammalian artificial chromosomes. Selection and use of such vehicles are well known to those of skill in the art.

As used herein, expression refers to the process by which nucleic acid is transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the nucleic acid is derived from genomic DNA, expression can, if an appropriate eukaryotic host cell or organism is selected, include processing, such as splicing of the mRNA.

As used herein, an expression vector includes vectors capable of expressing DNA that is operatively linked with regulatory sequences, such as promoter regions, that are capable of effecting expression of such DNA fragments. Such additional segments can include promoter and terminator sequences, and optionally can include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, and the like. Expression vectors are generally derived from plasmid or viral DNA, or can contain elements of both. Thus, an expression vector refers to a recombinant DNA or RNA construct, such as a plasmid, a phage, recombinant virus or other vector that, upon introduction into an appropriate host cell, results in expression of the cloned DNA. Appropriate expression vectors are well known to those of skill in the art and include those that are replicable in eukaryotic cells and/or prokaryotic cells and those that remain episomal or those which integrate into the host cell genome.

As used herein, vector also includes "virus vectors" or "viral vectors." Viral vectors are engineered viruses that are operatively linked to exogenous genes to transfer (as vehicles or shuttles) the exogenous genes into cells.

As used herein, "operably" or "operatively linked" when referring to DNA segments means that the segments are arranged so that they function in concert for their intended purposes, *e.g.*, transcription initiates downstream of the promoter and upstream of any transcribed sequences. The promoter is usually the domain to which the transcriptional machinery binds to initiate transcription and proceeds through the coding segment to the terminator.

As used herein, a human protein is one encoded by a nucleic acid molecule, such as DNA, present in the genome of a human, including all allelic variants and conservative variations thereof. A variant or modification of a protein is a human protein if the modification is based on the wildtype or prominent sequence of a human protein.

As used herein, a "composition" refers to any mixture of two or more products or compounds. It can be a solution, a suspension, liquid, powder, a paste, aqueous, non-aqueous, or any combination thereof.

As used herein, "a combination" refers to any association between two or among more items or elements, for example, two or more items that can be used together. For example, a combination can include an ADA2 protein and another therapeutic agent. Such combinations can be packaged as kits.

As used herein, a kit is a packaged combination, optionally, including instructions for use of the combination and/or other reactions and components for such use.

As used herein, an "article of manufacture" is a product that is made and sold. As used throughout this application, the term is intended to encompass ADA2 proteins, for example variant ADA2 proteins contained in articles of packaging.

As used herein, direct administration refers to a composition that is administered without dilution.

As used herein, a single dosage formulation refers to a formulation for use only once. Typically, a single dosage formulation is for direct administration.

As used herein, a multiple dosage formulation refers to a formulation for use in repeat administrations.

As used herein, when referencing dosage based on mg/kg of the subject, an average human subject is considered to have a mass of about 70 kg-75 kg, such as 70 kg and a body surface area (BSA) of 1.73 m^2.

As used herein, "disease or disorder" refers to a pathological condition in an organism resulting from cause or condition including, but not limited to, infections, acquired conditions, genetic conditions, and characterized by identifiable symptoms. Diseases and disorders of interest herein are any associated with aberrant or high adenosine levels.

As used herein, a tumor, also known as a neoplasm, is an abnormal mass of tissue that results when cells proliferate at an abnormally high rate. Tumors can show partial or total lack of structural organization and functional coordination with normal tissue. Tumors can be benign (not cancerous), or malignant (cancerous). As used herein, a tumor is intended to encompass hematopoietic tumors as well as solid tumors.

Malignant tumors can be broadly classified into three major types. Carcinomas are malignant tumors arising from epithelial structures (*e.g.* breast, prostate, lung, colon, pancreas). Sarcomas are malignant tumors that originate from connective tissues, or mesenchymal cells, such as muscle, cartilage, fat or bone. Leukemias and lymphomas are malignant tumors affecting hematopoietic structures (structures pertaining to the formation of blood cells) including components of the immune system. Other malignant tumors include, but are not limited to, tumors of the nervous system (*e.g.* neurofibromatomas), germ cell tumors, and blastic tumors.

As used herein, neoplastic disease refers to any disorder involving cancer, including tumor development, growth, metastasis and progression.

As used herein, cancer is a term for diseases caused by or characterized by any type of malignant tumor, including metastatic cancers, lymphatic tumors, and blood cancers. Exemplary cancers include, but are not limited to, cancers of the bladder, brain, breast, bone marrow, cervix, colon/rectum, kidney, liver, lung/bronchus, ovary, pancreas, prostate, skin, stomach, thyroid, or uterus.

As used herein, "intravenous administration" refers to delivery of a therapeutic directly into a vein.

As used herein, a control refers to a sample that is substantially identical to the test sample, except that it is not treated with a test parameter, or, if it is a plasma sample, it can be from a normal volunteer not affected with the condition of interest. A control also can be an internal control.

As used herein, normal levels or values can be defined in a variety of ways known to one of skill in the art. Typically, normal levels refer to the expression levels of a marker (e.g. adenosine, ADR or nucleosidase) across a healthy population. The normal levels (or reference levels) are based on measurements of healthy subjects, such as from a specified source (*i.e.,* blood, serum, tissue, or other source). Often, a normal level will be specified as a "normal range", which typically refers to the range of values of the median 95% of the healthy population. Reference value is used

interchangeably herein with normal level but can be different from normal levels depending on the subjects or the source. Reference levels are typically dependent on the normal levels of a particular segment of the population. Thus, for purposes herein, a normal or reference level is a predetermined standard or control by which a test patient can be compared.

As used herein, elevated level refers to the any level of amount or expression of a marker above a recited or normal threshold.

As used herein, biological sample refers to any sample obtained from a living or viral source or other source of macromolecules and biomolecules, and includes any cell type or tissue of a subject from which nucleic acid or protein or other macromolecule can be obtained. The biological sample can be a sample obtained directly from a biological source or to sample that is processed. For example, isolated nucleic acids that are amplified constitute a biological sample. Biological samples include, but are not limited to, body fluids, such as blood, plasma, serum, cerebrospinal fluid, synovial fluid, urine and sweat, and tissue and organ samples from animals, including biopsied tumor samples.

As used herein, detection includes methods that permit visualization (by eye or equipment) of a protein or marker. A protein can be visualized using an antibody specific to the protein. Detection of a protein can also be facilitated by fusion of a protein with a tag including an epitope tag or label.

As used herein, "treating" a subject with a disease or condition means that the subject's symptoms are partially or totally alleviated, or remain static following treatment. Hence treatment encompasses prophylaxis, therapy and/or cure. Prophylaxis refers to prevention of a potential disease and/or a prevention of worsening of symptoms or progression of a disease.

As used herein, a pharmaceutically effective agent includes any therapeutic agent or bioactive agents, including, but not limited to, for example, chemotherapeutics, anesthetics, vasoconstrictors, dispersing agents, conventional therapeutic drugs, including small molecule drugs and therapeutic proteins.

As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease or other indication, are ameliorated or otherwise beneficially altered.

As used herein, therapeutic effect means an effect resulting from treatment of a subject that alters, typically improves or ameliorates the symptoms of a disease or condition or that cures a disease or condition. A therapeutically effective amount refers to the amount of a composition, molecule or compound which results in a therapeutic effect following administration to a subject.

As used herein, amelioration of the symptoms of a particular disease or disorder by a treatment, such as by administration of a pharmaceutical composition or other therapeutic, refers to any lessening, whether permanent or temporary, lasting or transient, of the symptoms or, adverse effects of a condition, such as, for example, reduction of adverse effects associated with or that occur upon administration of an ADA2, such as a variant ADA2.

As used herein, prevention or prophylaxis refers to reduction in the risk of developing a disease or condition.

As used herein, a "therapeutically effective amount" or a "therapeutically effective dose" refers to the quantity of an agent, compound, material, or composition containing a compound that is at least sufficient to produce a therapeutic effect. Hence, it is the quantity necessary for preventing, curing, ameliorating, arresting or partially arresting a symptom of a disease or disorder.

As used herein, unit dose form refers to physically discrete units suitable for human and animal subjects and packaged individually as is known in the art.

As used herein, the term "subject" refers to an animal, including a mammal, such as a human being. The subject can include any animal, such as, but are not limited to primates including humans, gorillas and monkeys; rodents, such as mice and rats; fowl, such as chickens; ruminants, such as goats, cows, deer, sheep; pigs and other animals. Non-human animals exclude humans as the contemplated animal.

As used herein, a patient refers to a human subject exhibiting symptoms of a disease or disorder.

As used herein, about the same means within an amount that one of skill in the art would consider to be the same or to be within an acceptable range of error. For example, typically, for pharmaceutical compositions, within at least 1%, 2%, 3%, 4%, 5% or 10% is considered about the same. Such amount can vary depending upon the tolerance for variation in the particular composition by subjects.

As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to a compound comprising or containing "an extracellular domain" includes compounds with one or a plurality of extracellular domains.

As used herein, ranges and amounts can be expressed as "about" a particular value or range. About also includes the exact amount. Hence "about 5 bases" means "about 5 bases" and also "5 bases."

As used herein, "optional" or "optionally" means that the subsequently described event or circumstance does or does not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally substituted group means that the group is unsubstituted or is substituted.

As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature (see, (1972) *Biochem.* 11:1726).

B. ADENOSINE DEAMINASE 2 (ADA2) AND MODULATION OF ADENOSINE-MEDIATED TUMOR IMMUNOSUPPRESSION

Provided herein are methods of treating diseases or conditions such as a cancer or a tumor by administering any Adenosine Deaminase 2 (ADA2) protein, including variants or conjugates thereof, to a subject. Extracellular adenosine is responsible for the regulation of critical biological processes, such as immunomodulation (Blay, J. (2012) Encyclopedia of Cancer pp.49-52). In pathophysiological conditions such as the tumor microenvironment (TME), extracellular adenosine concentration rapidly increases in certain parts of the TME, generating an immunosuppressive niche that promotes tumor growth. ADA2 modulates adenosine levels in the extracellular environment, thereby affecting adenosine signaling and adenosine-dependent immunosuppression. ADA2 can decrease the extracellular adenosine levels by converting adenosine to inosine, to overcome the immunosuppressive effects in the TME. For example, as shown herein, administration of ADA2 can reverse the adenosine-dependent immunosuppression and can reduce tumor growth *in vivo*.

1. Tumor Immunity and Immune Evasion

Cancerous cells contain tumor-specific antigens that are recognized by the immune system. In tumor immunity, the goal of the immune system is to attack and eradicate these cancerous cells through the actions of immune cells, including cytotoxic T cells, Natural Killer (NK) cells and macrophages. For example, CD4+ and CD8+ T cells can become activated upon recognition of antigenic peptides presented on antigen-presenting cells on major histocompatibility complex (MHC) class I or class II molecules, respectively. Activated CD8+ cells, or cytotoxic T cells, can kill tumor cells expressing the antigen, which can be helped by the presence of CD4+ T cells. In addition to the direct killing effects of cytotoxic T cells, T cells also produce various cytokines and chemokines that can recruit other effector immune cells, such as neutrophils, macrophages or NK cells to the tumor microenvironment. NK cells also can directly kill cancer cells.

Studies have demonstrated that the immune system can prevent tumor growth. For example, immunodeficient mice develop more cancers than wild-type mice (Dunn *et al.* (2004) *Immunity*, 21:137-48). Lymphocytes and IFN-gamma have been shown to collaborate to prevent the formation of carcinogen-induced sarcoma and spontaneous epithelial carcinomas (Shankaran *et al.* (2001) *Nature*, 410:1107-1111). Further, gene-targeted and lymphocyte subset-depleted mice have demonstrated a role for NK cells in tumor rejection. For example, mice depleted for both NK and NK1.1+ T cells were found to have increased susceptibility to tumor formation compared to control mice, and a similar result was observed upon treatment of mice with antisialo-GM1, which selectively eliminates NK cells (Smyth *et al.* (2001) *Int Immunol.*, 13;459-63). In addition, the number, type and location of tumor immune infiltrates in primary tumors are prognostic factors for survival of cancer in human patients (Pages *et al.* (2005) *N Engl J Med*, 353:2654-2666).

Most tumors, however, can evade the immune system. The tumor microenvironment is complex, and includes a variety of immunosuppressive mechanisms that can be intrinsic to tumor cells or mediated by other cells or molecules. Through these mechanisms, alone or in combination, the immune system can promote tumor progression. These mechanisms include, but are not limited to, eliminating tumor cell antigens that elicit immune response; preventing or

downregulating the expression of ligands required for immune activation, such as major histocompatibility complex class I (MHC I); production of immunosuppressive mediators, such as interleukin-10 (IL-10), transforming growth factor-β or adenosine; recruitment of immune cell subsets that suppress effector immune cell function, such as T regulatory cells (Tregs) or myeloid-derived suppressor cell (MDSC); or the upregulation of checkpoint inhibitors, such as cytotoxic T-lymphocyte antigen 4 (CTLA4), that can attenuate effector T-cell function. For example, adenosine is a prominent immunosuppressive agent in the tumor microenvironment.

2. Adenosine Immunomodulation in Cancer and Tumor Microenvironment (TME)

Adenosine (adenine-9-β-D-ribofuranoside; Ado) is a nucleoside that exists as a part of adenine nucleotides (AMP, ADP, and ATP) which participate widely in cellular energy metabolism and act as precursor molecules in many processes. Adenosine can exist in the free form both inside and outside of cells.

Adenosine is an important *in vivo* signaling molecule, especially for the immune system. In particular, adenosine is a well-known effector of immune function. In T-cells, adenosine decreases T-cell receptor induced activation of NF-κB, and inhibits IL-2, IL-4, and IFN-γ. Adenosine decreases T-cell cytotoxicity, increases T-cell anergy, and increases T-cell differentiation to $Foxp3^+$ or $Lag\text{-}3^+$ regulatory T cells. Adenosine decreases IFN-γ production by NK cells and suppresses NK cell cytotoxicity. Adenosine blocks neutrophil adhesion and extravasation, decreases phagocytosis, and attenuates levels of superoxide and nitric oxide. Adenosine also decreases the expression of TNF-α, IL-12, and MIP-1α on macrophages, attenuates MHC Class II expression, and increases levels of IL-10 and IL-6. In addition, adenosine decreases phagocytosis and superoxide and nitric oxide levels on macrophages (Stagg and Smyth (2010) Oncogene 29:5346-5358).

Figure 2 sets forth the biosynthesis and catabolism of adenosine. Extracellular adenosine is produced by the sequential activities of membrane associated ectoenzymes, CD39 and CD73, which together produce adenosine by phosphohydrolysis of ATP or ADP produced from dead or dying cells. CD39 (also called ectonucleoside triphosphate diphosphohydrolase; SEQ ID NO:542) converts extracellular ATP (or ADP) to 5'AMP. Then, CD73 (also called 5'nucleotidase; SEQ

Date Reçue/Date Received 2021-07-28

ID NO:543) converts 5'AMP to adenosine. The activity of CD39 is reversible by the actions of NDP kinase and adenylate kinase, whereas the activity of CD73 is irreversible. CD39 and CD73 are expressed on tumor stromal cells, including endothelial cells and Tregs, and also on many cancer cells. For example, the expression of CD39 and CD73 on endothelial cells is increased under the hypoxic conditions of the tumor microenvironment. Tumor hypoxia can result from inadequate blood supply and disorganized tumor vasculature, impairing delivery of oxygen (Carroll and Ashcroft (2005), *Expert. Rev. Mol. Med.* 7(6):1-16). Hypoxia also inhibits adenylate kinase (AK), which converts adenosine to AMP, leading to very high extracellular adenosine concentration. Thus, adenosine is released at high concentrations in response to hypoxia, which is a condition that frequently occurs the tumor microenvironment (TME), in or around solid tumors.

Thus, while the concentration of adenosine is typically low in the tissues and blood, the local adenosine concentration can increase significantly as a result of damage or stress, e.g., inflammation, ischemia, and hypoxia. For example, the extracellular concentration of adenosine in the hypoxic tumor microenvironment can be up to 10 μM adenosine, which is up to about 100-fold higher than the typical extracellular adenosine concentration of approximately 0.1 μM (Antonioli *et al.* (2013) Nat Rev Can 13:842-857). Since the hypoxic regions in tumors are centered around the microvessels, the local concentration of adenosine in regions of the tumor can actually be higher.

Adenosine immunomodulation activity occurs after its release into the extracellular space of the tumor and activation of adenosine receptors (ADRs) on the surface of target immune cells, cancer cells or endothelial cells. There are four types of ADRs, A_1 (SEQ ID NO:533), A_{2A} (SEQ ID NO:534), A_{2B} (SEQ ID NO:535) and A_3 (SEQ ID NOS:536-538), which are each G-protein coupled receptors with different affinity for adenosine and different downstream signaling pathways. Activation of the A_1 and A_3 receptors decrease intracellular cyclic AMP (cAMP) levels, and the activation of A_{2A} and A_{2B} receptors increase cAMP levels through the activation of adenylyl cyclase. Each of the A_1, A_{2A}, and A_3 can be activated at physiological concentrations of adenosine (e.g. 30-300 nM), but A_{2B} has a lower affinity for adenosine and requires higher levels of adenosine for activation (Stagg *et al.* (2010)

Date Reçue/Date Received 2021-07-28

Oncogene, 29:5346-5358). The result of activation of the ADRs differs depending on the cell type and the receptor type: it can lead to activation or suppression of cell function and cell death (Antonioli *et al.* (2013) Nat Rev Can 13:842-857). All four types of receptors can exist on cells in the tumor microenvironment, including on cancer cells, stromal cells, endothelial cells, and inflammatory and immune cells, and all can be activated at adenosine concentrations present in the tumor microenvironment.

The high adenosine levels in the tumor microenvironment results in local immunosuppression, which limits the capacity of the immune system to eliminate cancer cells. For example, adenosine can suppress various functions of T lymphocytes, natural killer (NK) cells, polymorphonuclear granulocytes, and phagocytic cells such as tissue macrophages. In particular, the A_{2A} receptor is known to be expressed on monocytes, macrophages, mast cells, granulocytes, lymphocytes, dendritic cells (DCs), NK cells and endothelial cells, and its expression on many cell types is induced by hypoxia (Stagg and Smyth (2010) Oncogene, 29:5346-5358). Activation of A_{2A} has been shown to suppress NK cell functions, inhibit T-cell proliferation, inhibit T cell cytotoxicity and cytokine production, and inhibit macrophage activation (Stagg and Smyth (2010); Antonioli *et al.* (2013)). Activation of A_{2B} has been shown to suppress DC differentiation to limit T cell activation and to promote expansion and accumulation of MSDC (Stagg and Smyth (2010); Antonioli *et al.* (2013)).

In addition to direct effects to inhibit the immune system, adenosine also can control cancer cell growth and dissemination by effects on cancer cell proliferation, apoptosis and angiogenesis. For example, adenosine can promote angiogenesis, primarily through the stimulation of A_{2A} and A_{2B} receptors. Stimulation of the receptors on endothelial cells can regulate the expression of intercellular adhesion molecule 1 (ICAM-1) and E-selectin on endothelial cells, maintain vascular integrity, and promote vessel growth (Antonioli *et al.* (2013)). In addition, activation of one or more of A_{2A}, A_{2B} or A_3 on various cells by adenosine can stimulate the production of the pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8) or angiopoietin 2 (Antonioli *et al.* (2013)).

Adenosine also can directly regulate tumor cell proliferation, apoptosis and metastasis through interaction with receptors on cancer cells. For example, studies have shown that the activation of A_1 and A_{2A} receptors promote tumor cell proliferation in some breast cancer cell lines, and activation of A_{2B} receptors have cancer growth-promoting properties in colonic carcinoma cells (Antonioli *et al.* (2013)). Adenosine also can trigger apoptosis of cancer cells, and various studies have correlated this activity to activation of the extrinsic apoptotic pathway through A_3 or the intrinsic apoptotic pathway through A_{2A} and A_{2B} (Antonioli *et al.* (2013)). In addition, adenosine can promote tumor cell migration and metastasis, by increasing cell motility, adhesion to the extracellular matrix, and expression of cell attachment proteins and receptors to promote cell movement motility.

3. Adenosine Deaminase and Targeting Adenosine in Treatment of Cancer

The levels of adenosine can be regulated by the actions of adenosine deaminase (ADA), which is an enzyme that converts adenosine to inosine or 2'-deoxyadenosine to 2'-deoxyinosine. In particular, ADA converts either adenosine or deoxyadenosine, in the presence or water, into inosine or dexoyinosine and ammonia as follows: adenosine + H_2O = inosine + NH_3 or 2'-deoxyadenosine + H_2O = 2'-deoxyinosine + NH_3. The increase in ammonia in the local tumor microenvironment can increase the pH.

There are two types of ADA in humans, ADA1 and ADA2. ADA1 is ubiquitously present inside cells, and exhibits a similar binding affinity for adenosine and 2'deoxyadenosine with a Km of about 5.2 x 10^{-5} M. ADA1 principally functions intracellularly, to reduce the levels of adenosine that can be toxic to cells, such as lymphocytes. For example, deficiency of adenosine deaminase 1 (ADA1) is associated with mild immunodeficiency to severe combined immunodeficiency (SCID), due to the toxic accumulation of adenosine in immature lymphoid cells, thereby resulting in apoptotic death of lymphocytes and a profound depletion of T, B, and NK cells (Hershfield, M.S. (2005) Eur. J. Immunol. 35:25-30). In contrast, ADA2 contains a secretion signal sequence, and is the predominant extracellular ADA. The majority of ADA activity in normal human serum or plasma are from ADA2 (Neidzwicki and Aberneth (1991) *Biochemical Pharmacology* 41:1615-1624).

ADA2 has a much lower binding affinity for adenosine with a Km of about 200 x 10^{-5} M, and exhibits even weaker affinity for 2'deoxyadenosine. Also, unlike ADA1, ADA2 has an acidic pH optimum.

Decreasing the tumor-specific accumulation of adenosine in the TME is an attractive therapeutic option for treating tumors and cancers. It is found herein that recombinant forms of ADA2 can be administered to a subject to selectively target the TME, where it can decrease the extracellular adenosine levels by deaminating adenosine to inosine, thereby reversing the immunosuppressive effect of adenosine. In particular, ADA2 is an extracellular adenosine deaminase adapted for high adenosine concentrations. As discussed above, adenosine is actively produced in the TME, and regions of the TME can have up to about a 100-fold higher adenosine concentration than other tissue environments. Because of the hydrophobic subpocket for substrate binding, discussed further below, the K_m of ADA2 for adenosine is approximately 100 times higher than that of ADA1. The turnover rate (k_{cat}), however, is similar to that of ADA1. Because ADA2 has a similar turnover rate but a lower affinity to adenosine, it can be specifically active in environments with high adenosine concentrations, such as the TME or site of inflammation, without affecting adenosine metabolism in normal microenvironments that have lower adenosine concentrations.

The results herein demonstrate that recombinant ADA2 is selectively targeted to the tumor environment. In addition, results provided herein confirm that adenosine-mediates immunosuppression in T cells and NK cells, and that this suppression can be reversed by administered adenosine deaminase 2 (ADA2). The selective activity of ADA2 for decreasing adenosine levels in the TME can limit undesired or unwanted side effects, which can occur if the activity of ADA2 were more ubiquitous. For example, many existing tumor therapeutics are limited because they can result in adverse side effects in the subject due to lack of specificity or selectivity. The use of ADA2, or variants or conjugates thereof, in methods provided herein can result in fewer or lesser undesirable side-effects and/or exhibit improved efficacy by virtue of the ability to dose higher.

Thus, ADA2 offers advantages compared to ADA1. In addition to differences in binding affinity for adenosine that permits the use of ADA2 as a selective tumor-targeting molecule, ADA1 also is not adapted for use in an extracellular environment.

For example, ADA1 is primarily intracellular *in vivo* and is substantially less stable in the extracellular environment, such as in the plasma, as shown in the results provided herein. In contrast, ADA2 shows increased stability in the extracellular environment due to the extensive glycosylation that protect the molecule from proteolysis in the extracellular environment and conserved disulfide bonds. ADA2 also is substantially more stable at higher temperature compared to ADA1 (Daddona and Kelley (1981) Biochim. Biophys. Acta 658:280-290). It is found herein that ADA2 has a higher thermal stability, and that ADA2 is also more stable than ADA1 in extracellular environments, such as the plasma.

ADA2 also shows optimal activity in environments commonly found in the TME, such as environments having an acidic pH. For example, the optimal pH of wildtype ADA2 is approximately pH 6.5, whereas it is pH 7.5 for ADA1. The TME is a complex microenvironment in and surrounding the tumor that is made of diverse cell types and extracellular conditions. The TME commonly has regions where the extracellular environment is acidic, caused by lactic acid and other acidic metabolites produced by anaerobic glycolysis in hypoxic conditions of the tumor (Kato *et al.* (2013) Cancer Cell International 13:89).

In addition, ADA2 also overcomes other problems encountered with existing therapeutics, including those that target adenosine. For example, since adenosine has multiple receptors, it is difficult to target adenosine using an anti-ADR antibody, since all four ADR receptors are present in the TME and can be activated by adenosine. Hence, targeting of a single receptor would not achieve complete attenuation of adenosine immunomodulation activity.

Thus, the methods provided include methods of treatment using any ADA2, for example recombinant human ADA2 (rHuADA2) or variants and/or conjugates thereof, for treatment of diseases or conditions, such as a cancer or a tumor and other diseases or conditions involving aberrant or accumulated production of adenosine. Also provided herein are ADA2 variants and modified forms that possess altered properties, such as decreased heparin binding, increased catalytic efficiency, increased stability, altered glycosylation state and/or altered pH optimum. Any of the ADA2 proteins can be used in the methods of treatment provided herein. Also provided herein are methods of combination therapy using any ADA2 and other

immunomodulatory agents, chemotherapeutic agents, immune checkpoint inhibitors or hyaluronan-degrading enzyme, such as a soluble hyaluronidase or polymer-conjugated soluble hyaluronidase (*e.g.* PEGPH20).

C. ADENOSINE DEAMINASE 2 (ADA2) AND VARIANTS THEREOF

Provided herein are methods of treatment using an adenosine deaminase 2 (ADA2), including wildtype human ADA2, ADA2 variants and/or conjugates or other modified forms thereof. Also provided herein are variants of ADA2 with altered properties. ADA2 can be used to regulate adenosine levels in environments where regulation of adenosine-dependent immunomodulation or other adenosine-dependent activity is needed, such as in a tumor microenvironment or for inflammation.

1. Structure and Activity of ADA2

Adenosine deaminases are enzymes that convert adenosine to inosine. There are three known ADAs: ADA1, ADA2 and ADA3, although the activity of ADA3 is not known. With respect to proteins with known adenosine deaminase activity, humans have both ADA2 and ADA1, whereas in flies, homologues of ADA2 (known as ADGF homologues) are the only active adenosine deaminase enzymes, and rodents only have ADA1, indicating that the two proteins have overlapping yet also distinct functions. Distinct functions relate to the difference in expression, cellular location and kinetic properties of the enzymatic activity, difference in other structural features, as well as the additional growth factor and heparin binding properties (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377).

ADA1 and ADA2 are structurally similar, and exhibit a shared catalytic mechanism to convert adenosine to inosine, but exhibit little sequence similarity. ADA2 has a nucleotide sequence set forth in SEQ ID NO:1, which encodes a 511 amino acid protein, set forth in SEQ ID NO:2, that contains a signal sequence (corresponding to amino acid residues 1-29 of SEQ ID NO:2). Mature ADA2 is a secreted protein that lacks the signal sequence and has the sequence of amino acids set forth in SEQ ID NO:5. ADA1 has a nucleotide sequence set forth in SEQ ID NO:11, which encodes a 363 amino acid protein that does not contain a signal sequence, and has the sequence of amino acids set forth in SEQ ID NO:12. The N-terminal methionine residue is cleaved, resulting in the mature 362 amino acid protein, set forth in SEQ ID NO:66.

As discussed in more detail below, compared to ADA1, ADA2 is considerably longer and includes an 80–100-amino acid extension at the N terminus that is involved in dimerization and glycosaminoglycan (*e.g.* heparin) binding (Maier *et al.* (2005) Mol Evol 61:776–794). ADA2 also has an additional putative receptor binding (PRB) domain that is reported to mediate binding to cell surface receptors and/or contribute to its growth factor or other signaling functions. Also, unlike ADA1, ADA2 is dimeric and secreted, whereas ADA1 is monomeric and largely intracellular. ADA2 also is extensively glycosylated and has a conserved disulfide bond. The structural and functional features of ADA2 offer advantages as a therapeutic molecule, including, but not limited to, greater stability and increased tumor selectivity.

a. Structure of ADA2

ADA2, also known as dendritic cell derived growth factor (DCDGF) or adenosine deaminase growth factor (ADGF), is a member of the adenosine ADGF family of proteins. ADA2 is found only in eukaryotes, and primarily in multicellular organisms. In contrast, ADA1 is found in both prokaryotes and eukaryotes. In particular ADA2/ADGF homologs, have been characterized in insects and other vertebrates such as *Xenopus laevis,* as well as in humans. ADGF family proteins in insects were initially identified as proteins having growth factor activity, and later found to also possess adenosine deaminase activity.

In humans, ADA2 is encoded by the cat eye syndrome critical region gene 1 (CECR1) gene (Riazi *et al.* (2000) Genomics 64:277-285). The human CECR1 gene (nucleotide sequence of the coding region set forth in SEQ ID NO:1) encodes a 511 amino acid precursor protein (sequence set forth in SEQ ID NO:2; Uniprot Accession No. Q9NZK5). ADA2 has an N-terminal 29 residue signal sequence (amino acid residues positions 1-29 of SEQ ID NO:2) that is cleaved following transport to the ER to form the 482 amino acid mature protein (sequence set forth in SEQ ID NO:5). The mature ADA2 protein exists as a homodimer due to nonpolar interactions between two polypeptide chains. Other sequences of human ADA2 also have been reported, *see e.g.* U.S. Patent No. 5,968,780 (precursor form SEQ ID NO:376 and mature form SEQ ID NO:380), NCBI Acc. No. BAG369969.1 (precursor form SEQ ID NO:377 and mature form SEQ ID NO:381); NCBI Acc. No. AAF65941 (precursor form SEQ

ID NO:378 and mature form SEQ ID NO:382); and NCBI Acc. No. AAH51755 (precursor form SEQ ID NO:379 and mature form SEQ ID NO:383). A non-canonical second isoform, formed by alternative splicing of the mRNA, encodes a shorter protein of 270 amino acids (sequence set forth in SEQ ID NO:68; Uniprot Accession No. Q9NZK5-2), missing the N-terminal 241 amino acids and containing a 10-amino acid sequence in the N-terminus that is different from the canonical isoform.

Exemplary ADA2 homologs in other species include, but are not limited to, ADA2 from *Pan troglodytes* (chimpanzee; precursor form SEQ ID NO:286, mature form SEQ ID NO:326; NCBI Acc. No. XP_003317127.1); *Gorilla gorilla* (gorilla; precursor form SEQ ID NO:287, mature form SEQ ID NO:327; NCBI Acc. No. XP_004063024.1); *Pan paniscus* (pygmy chimpanzee; precursor form SEQ ID NO:288, mature form SEQ ID NO:328; NCBI Acc. No. XP_003828345.1); *Pongo abelii* (Sumatran orangutan; precursor form SEQ ID NO:289, mature form SEQ ID NO:329; NCBI Acc. No. NP_001125360.1); *Nomascus leucogenys* (Northern white-cheeked gibbon; precursor form SEQ ID NO:290, mature form SEQ ID NO:330; NCBI Acc. No. XP_004088517.1); *Macaca fascicularis* (crab-eating macaque; precursor form SEQ ID NO:291, mature form SEQ ID NO:331; NCBI Acc. No. XP_005568111.1); *Chlorocebus sabaeus* (green monkey; precursor form SEQ ID NO:292, mature form SEQ ID NO:332; NCBI Acc. No. XP_007972990.1); *Macaca mulatta* (Rhesus macaque; precursor form SEQ ID NOS:293, 337, mature form SEQ ID NOS:333, 340; GenBank Acc. Nos. AFH32795.1, EHH20002.1); *Callithrix jacchus* (marmoset; precursor form SEQ ID NOS:294, 374, mature form SEQ ID NO:334, 375; NCBI Acc. No. XP_009004591.1, XP_009004586.1); *Xenopus laevis* (African clawed frog; precursor form SEQ ID NO:295, mature form SEQ ID NO:335; NCBI Acc. No. NP_001090531.1); *Drosophila melanogaster* (fruit fly; precursor form SEQ ID NOS:296-300, mature form SEQ ID NOS:336, 338, 339; AAL40913.1, AAL40920.1, AAL40911.1, AAL40912.1, and AAL40910.1); *Bombyx mori* (silk moth; precursor form SEQ ID NO:301, mature form SEQ ID NO:341; NCBI Acc. No. NP_001098698.1); and *Sarcophaga perigrina* (flesh fly; precursor form SEQ ID NO:302, mature form SEQ ID NO:342; GenBank Acc. No. BAA11812.1).

The domain organization of ADA2 is described in Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377. ADA2 contains a core ADA domain or catalytic domain that makes up more than 70% of the amino acid sequence, and is structurally similar to the ADA domain in ADA1. In the monomer, the ADA domain is folded into eight strands of parallel α/β barrels, which surround a central deep pocket that is the active site. In addition, the ADA domain also contains three additional helices located between the β1 strand and the α1 helix (designated H1, H2 and H3) and two additional helices at the C terminus (designated H4 and H5). The ADA domain is contained in the region corresponding to residues 106-502 of the precursor ADA2 set forth in SEQ ID NO:2 (corresponding to residues 77-473 of the mature ADA2 set forth in SEQ ID NO:5). In the ADA region, ADA2 contains insertions of amino acid residues compared to ADA1, including residues that make up the putative receptor-binding (PRB) domain (discussed below), and which are not involved in the catalytic function of ADA2. The ADA domain does not have high sequence homology with that of ADA1 (18-21% identical residues), but the two ADA domains have high structural similarity. Table 4 summarizes the residues in the active site involved in substrate binding and catalysis.

Table 4

Residue (numbering of precursor set forth in SEQ ID NO:2)	**Active Site**
112	Zn^{2+} coordination, catalytic
114	Zn^{2+} coordination, catalytic
115	substrate binding
116	substrate binding
204	substrate binding
207	substrate binding
208	substrate binding
211	substrate binding
293	substrate binding
325	substrate binding
326	substrate binding
356	Zn^{2+} coordination, catalytic
359	active site proton donor, substrate binding
384	active site proton acceptor, substrate binding
415	substrate binding
441	Zn^{2+} coordination, catalytic, substrate binding
442	substrate binding

Based on the crystal structure as reported in Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377 of ADA2 with coformycin (CF), a transition state inhibitor that mimics the tetrahedral intermediate at the C6 position of adenine, residues involved in substrate binding have been identified. These include residues D115, I116, W204, F207, E208, F211, H293, V325, G326, E359, H384, L415, D441, and D442 of precursor ADA2 set forth in SEQ ID NO:2 (corresponding to residues D86, I87, W175, F178, E179, F182, H254, V296, G297, E330, H355, L386, D412 and D413 of mature ADA2 set forth in SEQ ID NO:5). Although the structural features of the catalytic site are similar between ADA2 and ADA1, the hydrophobic substrate-binding subpocket in the ADA domain of ADA2 is more open and contains fewer hydrophobic residues. These differences could account for the lower affinity of ADA2 for adenosine.

ADA2 is a zinc-dependent hydrolase that requires coordination with a bound zinc for activity, which acts as a powerful electrophile activating the attacking water to a hydroxide ion. Amino acid residues H112, H114, H356 and D441 of precursor ADA2 set forth in SEQ ID NO:2 (corresponding to H83, H85, H327, D412 of mature ADA2 set forth in SEQ ID NO:5) are involved in coordinating the zinc active center. During catalysis, the Zn^{++} promotes nucleophilic attack on the carbonyl carbon by the oxygen atom of a water molecule at the active site. The combination of E359, H384 and D441 of precursor ADA2 set forth in SEQ ID NO:2 (corresponding to E330, H355 and D412 of mature ADA2 set forth in SEQ ID NO:5) participate as zinc ligands. H384 and D441 position the attacking water, E359 is the active site catalytic proton donor residue that faciliates the reaction by extracting a proton from the attacking water molecule, and H384 serves as the proton acceptor. The catalytic active site residues structurally mirror the corresponding active site residues of ADA1, indicating that the catalytic mechanism is similar between the two adenosine deaminases (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377).

Active ADA2 exists as a homodimer. Dimerization of ADA2 is mediated by residues in the N-terminal α-helices of ADA2 designated HN1, HN2, HN3 and HN4 (corresponding to residues 29-105 of precursor ADA2 set forth in SEQ ID NO:2, or residues 1-76 of mature ADA2 set forth in SEQ ID NO:5), as well as residues in the C-terminal α-helix designated H5 (corresponding to residues 503-511 of precursor

ADA2 set forth in SEQ ID NO:2 or residues 474-482 of mature ADA2 set forth in SEQ ID NO:5). Because these regions are responsible for more than 70% of the nonpolar intersubunit interaction, they are designated the dimerization domain. The first N-terminal helix, HN1, forms a helix anchor due to ionic interactions between residues R34 and E41 (residues R5 and E12 of mature ADA2 set forth in SEQ ID NO:5) with D373 and H391 (residues D344 and H362 of mature ADA2 set forth in SEQ ID NO:5) of the neighboring subunit, and hydrophobic interactions between residues I30, T33, L37, L38, K40 and M44 (residues I1, T4, L8, L9, K11 and M15 of mature ADA2 set forth in SEQ ID NO:5) with residues in the neighboring subunit. A hydrophobic binding pocket is formed with residues M71, A74, M75, L78 and F80, which accomodates the W336 (residues M42, A45, M46, L49 and F51 of mature ADA2 set forth in SEQ ID NO:5) residue from the neighboring subunit.

ADA1, which does not form a dimer, does not contain the residues that make up the "dimerization domain." Also, compared to ADA1, residue W336 in ADA2 is inserted into a region of the active site between β5 and α5, where it indirectly contributes to catalytic activity due to its involvement in dimerization. Substitution W336G results in an ADA2 molecule that partly dissociates into monomers, and which exhibits altered catalytic activity (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377). In addition to affecting full enzymatic activity, dimerization also is involved in the secretion of ADA2. Deletion of amino acids T33 and E41 (corresponding to T4 and E12 of mature ADA2 set forth in SEQ ID NO:5) abolishes secretion of ADA2 into the culture medium (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377).

ADA2 binds glycosaminoglycans (GAGs), including heparin and its analogs, such as heparan sulfate, and chondroitin sulfate. Protein dimerization results in a large, highly positively charged surface at the interface of dimer, which forms the GAG-binding site (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377). In particular, the GAG-binding site involves amino acid residues near positions I30-R45, S389-T396 and R422-T428 of precursor ADA2 (corresponding to I1-R16, S360-T367, and R393-T399 of mature ADA2 set forth in SEQ ID NO:5). The interaction with GAGs appears to play a role in stabilizing the ADA2 dimer.

ADA2 has an insertion sequence within the catalytic domain, designated the putative receptor-binding (PRB) domain, which is reported to correspond to residues 127-185 or 134-177 of precursor ADA2 set forth in SEQ ID NO:2 (positions 98-156 or 105-148, respectively, of mature ADA2 set forth in SEQ ID NO:5). The PRB domain folds into a chemokine-like domain made up of an α- and β-fold composed of a three-stranded antiparallel β-sheet surrounded by α-helices on one side and a proline-rich loop on the other side. A disulfide bond between positions 137 and 159 of precursor ADA2 (positions 108 and 130 of mature ADA2 set forth in SEQ ID NO:5) is present in the PRB domain, which is required for ADA2 secretion and structural stability. The crystal structure of ADA2 shows that the PRB domain, while not involved in the catalytic function of ADA2, sits on the top of the adenosine deaminase (ADA) domain at the edge of the active site. When ADA2 dimerizes, the two PRB domains in the dimer are present on the same side of the dimer, and could bind dimeric receptors or induce receptor dimerization (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377; Zavialov *et al.* (2010) J. Leukoc. Biol. 88:279-290). ADA2 binds adenosine receptors (ADRs) which are dimeric receptors. The PRB domain is implicated in its growth factor activity through the receptor binding activity (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377; Zavialov *et al.* (2010) J. Leukoc. Biol. 88:279-290). Thus, elimination or modification of this domain can reduce, attenuate or eliminate this activity.

ADA2 has four (4) native N-linked glycosylation sites, at N127, N174, N185 and N378 of precursor ADA2 (corresponding to N98, N145, N156 and N349 of mature ADA2 set forth in SEQ ID NO:5). Three N-glycosylation sites are present in the PRB domain, at N127, N174, and N185, and one is present on the opposite side of the molecule, at N378. The oligosaccharide chains located on three different faces of the ADA2 molecule protects the enzyme against proteolysis in the extracellular environment, providing increased stability (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377).

b. Activities of ADA2

ADA2 has several activities. ADA2 has adenosine deaminase (ADA) activity, which catalyzes adenosine to inosine (adenosine + H_2O = inosine + NH_3) and 2'-deoxyadenosine to 2'-deoxyinosine (2'-deoxyadenosine + H_2O = 2'-deoxyinosine +

NH_3) reactions. Coformycin and 2'-deoxycoformycin are potent inhibitors of ADA1 and ADA2. Due to differences in the substrate binding pocket, however, the inhibitor (+)-erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) selectively inhibits ADA1, but does not inhibit ADA2. Also, the differences in the substrate binding pocket account for differences in substrate binding affinity between ADA1 and ADA2. For example, while the k_{cat} values for adenosine are similar due to the high structural similarity of the catalytic residues in ADA2 and ADA1, the K_m for adenosine are different. The K_m of ADA2 for adenosine is approximately 2.25 mM. Because ADA2 has a hydrophobic subpocket for substrate binding, the affinity for ADA2 for substrates is different from that of ADA1. The K_m of ADA2 for adenosine is approximately 100 times higher than that of ADA1, which is approximately 0.1 mM.

The optimal pH for activity of ADA2 is around pH 6.5, and its activity decreases at a pH higher than 7.0. In contrast, the optimal pH for ADA1 is around pH 7.5. Different substrate affinity and pH optimum indicate that ADA2 is adapted for specific microenvironments, and serve overlapping yet different functions in regulation of adenosine concentration and signaling (Zavialov *et al.* (2005) Biochem. J. 391:51-57). The acidic optimum pH for ADA2 and requirement for high adenosine concentration indicates that ADA2 can be active specific environments, such as sites of inflammation or tumors, where adenosine concentration is elevated and pH is lower. In the tumor microenvironment, tumor cells can undergo extensive glycolysis due to the hypoxic environment, and the extracellular microenvironment becomes acidic (pH 6.5-6.9) in certain regions.

In humans, ADA2 is widely expressed, with most abundant expression in adult heart, lung, lymphoblasts, and placenta as well as fetal lung, liver, and kidney. ADA2 is also detected in blood plasma at the protein level. The majority of ADA activity in normal human serum or plasma are from ADA2 (Neidzwicki and Aberneth (1991) Biochemical Pharmacology 41:1615-1624). ADA2 is secreted by activated cells, including activated monocytes and other immune cells, and to a more limited extent, by unstimulated lymphocytes (Iwaki-Egawa *et al.* (2006) Biol. Chem. 387:319-321). Immune cells, such as monocytes, are activated in inflammatory sites and tumors, where extracellular adenosine deaminase is accumulated (Sitkovsky *et al.* (2004) Annu. Rev. Immunol. 22:657-682). ADA2 could be involved in the regulation of

adenosine levels in these specific environments (Zavialov *et al.* (2010) J. Leukoc. Biol. 88:279-290). For example, ADA2 could function to reduce the level of adenosine in environments with high adenosine concentrations, such as at inflammation sites or in the tumor microenvironment with hypoxic conditions.

ADA2 activity is elevated in plasma from patients suffering from liver diseases, such as chronic hepatitis and cirrhosis, AIDS, adult T-cell leukemia, acute lymphoblastic leukemia, tuberculosis and diabetes mellitus. (Zavialov *et al.* (2005) Biochem. J. 391:51-57). In addition, ADA2 levels are elevated in tuberculosis pleural effusion in recent *Mycobacterium tuberculosis* (MTB) infections (Valdez) or in visceral leishmaniasis (Tripathi *et al.*, Clinica Chimica Acta 388 (2008) 135-138). The pleural effusion of MTB infection contain a high number of macrophages and CD4+ cells, indicating that ADA2 secretion by macrophages could modulate the immune response during MTB infection (Zavialov *et al.* (2010) J. Leukoc. Biol. 88:279-290).

ADA2 binds to the cell surface via GAG proteoglycans (*e.g.* heparin) and ADRs. Heparin analogs such as heparan sulfate proteoglycan (HSPG), or chondroitin sulfate (CS)-containing proteoglycans are present on the cell surface and are involved in protein localization and cell signaling. ADA2 can bind various types of cells via these heparin analogs, and the binding is tighter to a more highly sulfated heparin sulfate than to less sulfated heparin, indicating that the binding involves extensive ionic interaction. In contrast to ADA2, ADA1 does not bind to heparin (Zavialov *et al.* (2005) Biochem. J. 391:51-57, Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377).

In addition to heparin analogs containing proteoglycans, ADA2 dimer binds to adenosine receptors (ADRs), which function as dimers (Zavialov *et al.* (2005) Biochem. J. 391:51-57, Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377). ADA2 is reported to interact with cells to mediate growth factor activity. ADA2 can also directly bind to some dimeric adenosine receptors, stimulate proliferation of monocyte-activated CD4 T cells independently of its catalytic activity, induce T cell-dependent differentiation of monocytes into macrophages and stimulate macrophage proliferation. For example, ADA2 increases the rate of proliferation of monocyte-activated CD4 T cells independently of its catalytic activity, and induces T cell-

Date Reçue/Date Received 2021-07-28

dependent differentiation of monocytes into macrophages and stimulates macrophage proliferation (Zavialov *et al.* (2010) J. Leukoc. Biol. 88:279-290).

Defects or deficiencies in ADA2 have been associated with increased vascular inflammation and vasculopathy, in particular associated with Polyarteritis nodosa or Sneddon syndrome (Zhou *et al.* (2014) N. Engl. J. Med 370:911-920; Navon Elkan *et al.* (2014) N. Engl. J. Med 370:921-931; Garg *et al.* (2014) Eur. J. Pediatr 173:827-830; Bras *et al.* (2014) New Eng. J. Med., 371:479-48; Belot *et al.*(2014) Pediatric Rheumatology 12:44). For example, vasculitis is associated with recessive mutations in the gene encoding for ADA2 characterized by mutations G47A, G47R, G47V, A109D, H112Q, V119A, G142S, R169Q, P193L, P251L, W264S, Y453C with reference to precursor ADA2 set forth in SEQ ID NO:2 (Navon Elkan *et al.* (2014) N. Engl. J. Med 370:921-931; Zhou *et al.* (2014) N. Engl. J. Med 370:911-920; Bras *et al.* (2014) New Eng. J. Med., 371:479-480).

2. ADA2 Variants

Provided herein are variants or mutants of ADA2 containing a polypeptide that contains one or more amino acid modifications (i.e. changes in amino acid sequence) compared to a reference or unmodified ADA2. The modifications can be in any reference or unmodified ADA2 polypeptide, so long as the reference ADA2 does not already contain the amino acid change at the modified position(s). For example, the modification(s) can be in an ADA2 polypeptide that contains the sequence of amino acids set forth in any of SEQ ID NOS:5 or 326-336, 338-342, 375 or 380-383, a catalytically active fragment thereof or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:5 or 326-336, 338-342, 375 or 380-383 or a catalytically active fragment thereof but does not contain the modification(s).

In particular examples, the modifications are in an ADA2 polypeptide set forth in SEQ ID NO:5, a catalytically active fragment thereof or in a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to SEQ ID NO:5 or a catalytically active fragment thereof but does not contain the modification(s). For example, modifications can be in an ADA2 having a sequence of amino acids set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383. Modifications also can be in

a catalytically active portion of SEQ ID NO:5. For example, a catalytically active ADA2 can be one that lacks all or a portion of the PRB domain, such as those set forth in any of SEQ ID NOS:548-550 or 579. In particular examples, modifications are in a human ADA2 containing the sequence of amino acids set forth in SEQ ID NO:5.

In examples of a variant ADA2 polypeptide provided herein, the variant ADA2 does not have the sequence of amino acids set forth in any of SEQ ID NOS:1, 5, 68, 286-302, 326-342 or 374-383. Also, in examples herein, the variant ADA2 polypeptide does not contain modifications that are deletion R8-K14del→-- or that are an amino acid replacement H7R, G18A, G18R, G18V, I64T, A80D, H83Q, V90A, C108G, H121R, W133G, R140Q, K141R, P164L, P222L, W235S, H306R, E330G, W333G, V365L, Y424C, F464S, with numbering with reference to amino acid residues set forth in SEQ ID NO:5.

The variant ADA2 can be a monomer or can be a dimer, such as a heterodimer or a homodimer. The variant ADA2 polypeptides provided herein exhibit adenosine deaminase activity to catalyze the conversion of adenosine to inosine. It is understood that such activity is exhibited when the variant ADA2 polypeptide is in active form, such as when it is present as a dimer. Typically, such activity is present when the ADA2 is in dimer form. Hence, any of the variants provided herein can be used to regulate adenosine levels in environments where regulation of adenosine-dependent immunomodulation or other adenosine-dependent activity is needed, such as in a tumor microenvironment or for inflammation. Hence, any of the variants provided herein can be used in methods of treating tumor or cancer as described herein.

When in active form, such as when in dimer form, the variant ADA2 containing the variant ADA2 polypeptide can exhibit about 50% to 500%, such as about 50% to 400%, 50% to 300%, 50% to 200%, 50% to 150%, 50% to 100%, 50% to 80%, 80% to 400%, 80% to 300%, 80% to 200%, 80% to 150%, 80% to 100%, 100% to 400%, 100% to 300%, 100% to 200% or 100% to 150% of the adenosine deaminase activity compared to the corresponding form of the ADA2 polypeptide not containing the modification(s) (i.e. the unmodified ADA2), such as an ADA2 homodimer containing the sequence of amino acids set forth in SEQ ID NO:5, 326-334, 340, 375 or 380-383 or a catalytically active fragment thereof. For example,

when in active form, such as when in dimer form, the variant ADA2 containing the variant ADA2 polypeptide can exhibit at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 210%, 220%, 230%, 240%, 250%, 260%, 270%, 280%, 290%, 300%, 350%, 400%, 450%, 500% or more of the adenosine deaminase activity compared to the corresponding form of the ADA2 polypeptide not containing the modification(s) (i.e. the unmodified ADA2), such as an ADA2 homodimer containing the sequence of amino acids set forth in SEQ ID NO:5, 326-334, 340, 375 or 380-383 or a catalytically active fragment thereof. Typically, a variant ADA2 containing a variant ADA2 polypeptide provided herein, when in dimer form, retains adenosine deaminase activity of the corresponding form of the ADA2 homodimer containing the sequence of amino acids set forth in SEQ ID NO:5 or a catalytically active fragment thereof, such that the variant ADA2, when in dimer form, exhibits at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 210%, 220%, 230%, 240%, 250%, 260%, 270%, 280%, 290%, 300%, 350%, 400%, 450%, 500% or more of the adenosine deaminase activity of the ADA2 homodimer containing the sequence of amino acids set forth in SEQ ID NO:5 or a catalytically active fragment thereof.

Typically, the catalytic efficiency or k_{cat}/K_M ($M^{-1}s^{-1}$) of variant ADA2 containing a variant ADA2 polypeptide provided herein is at least 5,000, such is generally from or from about 5×10^3 to 5×10^6, 5×10^3 to 2.5×10^6, 5×10^3 to 1×10^6, 5×10^3 to 5×10^5, 5×10^3 to 2.5×10^5, 5×10^3 to 1×10^5, 5×10^3 to 8×10^4, 5×10^3 to 5×10^4, 5×10^3 to 2.5×10^4, 5×10^3 to 1×10^4, 1×10^4 to 5×10^5, 1×10^4 to 2.5×10^5, 1×10^4 to 1×10^5, 1×10^4 to 8×10^4, 1×10^4 to 5×10^4, 1×10^4 to 2.5×10^4, 2.5×10^4 to 5×10^6, 2.5×10^4 to 2.5×10^6, 2.5×10^4 to 1×10^6, 2.5×10^4 to 5×10^5, 2.5×10^4 to 2.5×10^5, 2.5×10^4 to 1×10^5, 2.5×10^4 to 8×10^4, 2.5×10^4 to 5×10^4, 5×10^4 to 5×10^6, 5×10^4 to 2.5×10^6, 5×10^4 to 1×10^6, 5×10^4 to 5×10^5, 5×10^4 to 2.5×10^5, 5×10^4 to 1×10^5, 5×10^4 to 8×10^4, 8×10^4 to 5×10^6, 8×10^4 to 2.5×10^6, 8×10^4 to 1×10^6, 8×10^4 to 5×10^5, 8×10^4 to 2.5×10^5, 8×10^4 to 1×10^5, 1×10^5 to 5×10^6, 1×10^5 to 2.5×10^6, 1×10^5 to 1×10^6, 1×10^5 to 5×10^5, 1×10^5 to 2.5×10^5, 2.5×10^5 to 5×10^6, 2.5×10^5 to 2.5×10^6, 2.5×10^5 to 1×10^6, 2.5×10^5 to 5×10^5, 5×10^5 to 5×10^6, 5×10^5 to 2.5×10^6, or 5×10^5 to 1×10^6 $M^{-1}s^{-1}$. For example, variant ADA2 containing a

Date Reçue/Date Received 2021-07-28

variant ADA2 polypeptide provided herein has a catalytic efficiency of k_{cat}/K_M ($M^{-1}s^{-1}$) of at least 5 x 10^3, 6 x 10^3, 7 x 10^3, 8 x 10^3, 9 x 10^3, 1 x 10^4, 2 x 10^4, 3 x 10^4, 4 x 10^4, 5 x 10^4, 6 x 10^4, 7 x 10^4, 8 x 10^4, 9 x 10^4, 1 x 10^5, 2 x 10^5, 3 x 10^5, 4 x 10^5, 5 x 10^5, or greater, or 6 x 10^5, 7 x 10^5, 8 x 10^5, 9 x 10^5, 1 x 10^6, 2 x 10^6, 3 x 10^6, 4 x 10^6, 5 x 10^6 $M^{-1}s^{-1}$ or greater.

The variant ADA2 polypeptide provided herein can contain amino acid replacements (i.e. substitutions), additions (i.e. insertions), deletions, truncations or combinations thereof. The variant ADA2 can contain modification(s) in any region or domain of an ADA2 polypeptide provided the resulting variant ADA2, when in active form, for example as a dimer, at least retains adenosine deaminase activity. For purposes herein, reference to modification(s) in an ADA2 polypeptide is with respect to residues of the mature ADA2 polypeptide set forth in SEQ ID NO:5. Amino acid replacements can be made at corresponding residues of any ADA2 polypeptide or catalytically active fragment thereof, including in any ADA2 polypeptide or variant ADA2 polypeptide known in the art. Corresponding residues can be identified by alignment with the mature polypeptide set forth in SEQ ID NO:5 (see e.g. Figure 1, Table 1). Reference also is made throughout the application and Examples to numbering based on Zavialov (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377), which is based on the numbering of amino acids residues set forth in SEQ ID NO:4. See Table 1, which sets forth the corresponding position numbers of Zavialov numbering (SEQ ID NO:4) and mature ADA2 numbering (SEQ ID NO:5).

To retain adenosine deaminase activity, modifications typically are not at those positions that are less tolerant to change. Such positions can be within domains or regions that are required for catalytic activity, substrate binding and/or dimerization. For example, such positions include regions that are highly conserved, such as residues required for zinc coordination or active site residues. A skilled artisan knows or can readily identify amino acid residues that are required for activity and should not be changed. Also, in some instances if a modification is at these positions, it generally is a conservative amino acid substitution. One of skill in the art understands conservative amino acid substitutions, such as those provided in Table 3, can be used to reduce the likelihood of a modification resulting in a reduction in activity.

Variant ADA2 proteins provided herein can contain a polypeptide subunit that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the polypeptide sequence of the unmodified or reference ADA2 polypeptide, such as those set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383, or a catalytically active fragment thereof. In particular, variant ADA2 proteins provided herein contain a polypeptide subunit that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to the polypeptide sequence set forth in SEQ ID NO:5 or a catalytically active fragment thereof. The variant ADA2 proteins provided herein can contain a polypeptide subunit that can contain at least or about or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more amino acid modification(s) compared to the polypeptide sequence of the unmodified or reference ADA2 polypeptide. It is understood that when present as a dimer or multimer, the variant ADA2 can contain at least or about or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 12, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100 or more amino acid modification(s).

For purposes herein, amino acid replacements are denoted by the replaced amino acid, the amino acid position and the replacing amino acid (*e.g.* K11A by mature numbering, which represents that the amino acid at a position corresponding to amino acid residue 11 in SEQ ID NO:5, *e.g.* lysine, is replaced by alanine). For purposes herein, amino acid replacements can also be denoted by the replaced amino acid, the amino acid position and the replacing amino acid (*e.g.* K14A by Zavialov numbering, which represents that the amino acid at a position corresponding to amino acid residue 14 in SEQ ID NO:4, *e.g.* lysine, is replaced by alanine). See Table 1, which sets forth the corresponding position numbers of Zavialov numbering (SEQ ID NO:4) and mature ADA2 numbering (SEQ ID NO:5). Nomenclature also is employed herein to represent the insertion (--→ followed by position of insertion) or deletion (*e.g.* position of deletion (del) followed by →--) of an amino acid residue at a

corresponding position in SEQ ID NO:5, by mature numbering, and/or by SEQ ID NO:4, by Zavialov numbering. For example, ---→N1 by mature numbering means that the residue at position 1 is inserted compared to the corresponding sequence of mature ADA2 set forth in SEQ ID NO:5. For example, ---→N4 by Zavialov numbering means that the residue at position 4 is inserted compared to the corresponding sequence of ADA2 set forth in SEQ ID NO:4. It is understood that in some cases, due to deletions or insertions of amino acid residues, the numbering of residues in a variant ADA2 polypeptide is altered compared to the numbering of residues set forth in SEQ ID NO:5. In such instances, it is within the level of a skilled artisan to identify residues in the corresponding variant ADA2 polypeptide that correspond to residues in SEQ ID NO:5, for example by alignment as demonstrated in Figure 1. For example, the numbering of residues in a variant ADA2 polypeptide can be numbered based on Zavialov (Zavialov *et al.* (2010) J. Biol. Chem. 285:12367-12377), which is based on the numbering of amino acids residues set forth in SEQ ID NO:4. See Table 1, which sets forth the corresponding position numbers of Zavialov numbering (SEQ ID NO:4) and mature ADA2 numbering (SEQ ID NO:5).

Exemplary modifications in a variant ADA2 polypeptide provided herein are described in further detail below. The variant ADA2 provided herein include those that, when in active form, exhibit altered or improved activities or properties compared to the corresponding form of the reference or wildtype ADA2 not containing the modification(s) (i.e. the unmodified ADA2). For example, the variant ADA2 provided herein include those that, when in active form, exhibit altered or improved activities or properties compared to the corresponding form of an unmodified ADA2 containing an ADA2 polypeptide having a sequence of amino acids that exhibits at least 85% sequence identity to SEQ ID NO:5 or a catalytically active fragment thereof, such as those set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383, or a catalytically active fragment thereof. In particular, the modifications provided herein can affect any one or more activities from among increased adenosine deaminase activity, attenuated heparin binding, increased half-life, altered pH optimum, increased thermal stability, reduced receptor binding, or hyperglycosylation compared to the corresponding form of the ADA2 not containing the modifications (i.e. the unmodified ADA2).

In particular, the active form is the dimer form, such as the homodimer form, that contains the variant ADA2 polypeptide. Thus, in examples herein, variant ADA2 proteins containing a variant ADA2 polypeptide provided herein, when in dimer form, exhibit altered or improved activities or properties compared to the corresponding dimer form of the reference or wildtype ADA2 not containing the modifications. For example, the variant ADA2 provided herein include those that, when in dimer form, exhibit altered or improved activities or properties compared to the corresponding dimer form of an unmodified ADA2 containing an ADA1 polypeptide having a sequence of amino acids that exhibits at least 85% sequence identity to SEQ ID NO:5 or a catalytically active fragment thereof, such as those set forth in any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383, or a catalytically active portion thereof. For example, provided are variant ADA2 containing a variant ADA2 polypeptide provided herein that, when in dimer form, exhibit altered or improved activities or properties compared to the ADA2 homodimer containing the sequence of amino acids set forth in SEQ ID NO:5 or a catalytically active fragment thereof. In particular, the modifications provided herein can affect any one or more activities from among increased adenosine deaminase activity, attenuated heparin binding, increased half-life, altered pH optimum, increased thermal stability, reduced receptor binding, or hyperglycosylation compared to the corresponding form of the ADA2 not containing the modifications (i.e. the unmodified ADA2).

For example, provided herein are variant ADA2 proteins that, when in active form such as dimer form, exhibit increased adenosine deaminase activity. For example, the variant ADA2 protein, when in active form such as dimer form, can exhibit at least 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 225%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800%, 900%, 1000% or more activity of the corresponding form of the unmodified ADA2, wherein adenosine deaminase activity is assessed under the same conditions. The catalytic efficiency (k_{cat}/K_M) of a variant ADA2 that exhibits increased adenosine deaminase activity is at least or at least about 1.2-fold, 1.3-fold, 1.4-fold, 1.5-fold, 1.6-fold, 1.7-fold, 1.8-fold, 1.9-fold, 2.0-fold, 2.2-fold, 2.5-fold, 3.0-fold, 3.5-fold, 4-fold, 4.5-fold, 5.0-fold, 6.0-fold, 7.0-fold, 8.0-fold, 9.0-fold, 10.0-fold greater or more, or 11.0-fold, 12.0-fold, 13.0-fold, 14.0-fold, 15.0-fold greater or more compared to the catalytic

efficiency (k_{cat}/K_M) of the corresponding form of the unmodified ADA2, wherein catalytic efficiency of adenosine deaminase activity is assessed under the same conditions. For example, when in dimer form, the variant ADA2 provided herein exhibits a catalytic efficiency (k_{cat}/K_M) that is at least 2 x 10^4 M^{-1} s^{-1}, 3 x 10^4 M^{-1} s^{-1}, 4 x 10^4 M^{-1} s^{-1}, 5 x 10^4 M^{-1} s^{-1}, 6 x 10^4 M^{-1} s^{-1}, 7 x 10^4 M^{-1} s^{-1}, 8 x 10^4 M^{-1} s^{-1}, 9 x 10^4 M^{-1} s^{-1}, 1 x 10^5 M^{-1} s^{-1}, 2 x 10^5 M^{-1} s^{-1}, 3 x 10^5 M^{-1} s^{-1}, 4 x 10^5 M^{-1} s^{-1}, 5 x 10^5 $M^{-1}s^{-1}$ or greater, or 6 x 10^5 $M^{-1}s^{-1}$, 7 x 10^5 $M^{-1}s^{-1}$, 8 x 10^5 $M^{-1}s^{-1}$, 9 x 10^5 $M^{-1}s^{-1}$, 1 x 10^6 $M^{-1}s^{-1}$, 2 x 10^6 $M^{-1}s^{-1}$, 3 x 10^6 $M^{-1}s^{-1}$, 4 x 10^6 $M^{-1}s^{-1}$, 5 x 10^6 $M^{-1}s^{-1}$ or greater.

In examples herein, provided herein are variant ADA2 proteins that, when in active form such as dimer form, exhibit reduced binding to any one or more adenosine receptor (ADR) selected from among A_1, A_{2A}, A_{2B} and A_3, and typically one or both of A_{2A} or A_{2B}. Without being bound by theory, it is contemplated herein that the activity of the adenosine deaminase activity provided herein for converting adenosine to inosine is greater or more efficient if binding of the ADA2 to an ADR is reduced. For example, provided herein are variant ADA2, when in active form such as dimer form, in which binding to one or more ADR is reduced at least or at least about 0.5-fold, 1-fold, 1.5-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold or more compared to the corresponding form of the unmodified ADA2.

In examples herein, provided herein are variant ADA2 proteins that, when in active form such as dimer form, exhibit reduced or attenuated heparin binding. ADA2 binds glycosaminoglycans (GAGs), including heparin and its analogs, such as heparan sulfate, and chondroitin sulfate. High-affinity binding to heparin/GAGs is mediated by a large, highly positively charged surface at the interface of dimer, and dimerization of ADA2 forms the heparin binding site. Because glycosaminoglycan is widely present throughout the body, it could interact with the administered ADA2 and act as a peripheral sink. Therefore, an ADA2 with reduced heparin binding can increase the bioavailability and pharmacokinetics of the administered ADA2. For example, ADA2 variants with attenuated heparin binding provided herein, result in improved bioavailability and pharmacokinetics, such as increased half-life, when administered, because the administered ADA2 molecules will not be sequestered in the peripheral sink by binding to the GAGs. In particular, provided herein are variant ADA2 proteins that, when in active form such as dimer form, exhibit no more than

1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the heparin binding of the corresponding form of the unmodified ADA2, wherein heparin binding is assessed under the same conditions.

In examples herein, provided are variant ADA2 proteins that, when in active form such as dimer form, exhibit an increased or longer plasma or serum half-life ($t_{1/2}$). For example, variant ADA2 provided herein, when in active form such as dimer form, exhibit a half-life that is at least or at least about 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 225%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800% or more longer, or 900%, 1000%, 1100%, 1200%, 1300%, 1400%, 1500%, 1600%, 1700%, 1800%, 1900%, 2000%, 3000%, 4000%, 5000%, 6000%, 7000%, 8000%, 9000%, 10000%, or more longer than the half-life of the corresponding form of the unmodified ADA2, wherein half-life is assessed under the same conditions.

In examples herein, provided are variant ADA2 proteins that, when in active form such as dimer form, exhibit an increased thermal stability. For example, when in active form such as dimer form, variant ADA2 provided herein exhibit thermal stability with a melting temperature (Tm) that is increased at least or at least about 0.5°C, 1.0°C, 2.0°C, 3.0°C, 4.0°C, 5.0°C, 6.0°C, 7.0°C, 8.0°C, 9.0°C, 10.0°C or more compared to the Tm of the corresponding form of the unmodified ADA2, wherein Tm is assessed under the same conditions. The melting temperature (Tm) of variant ADA2, when in active form such as dimer form, provided herein can be at least or at least about 67.6°C, 67.8°C, 68.0°C, 68.2°C, 68.4°C, 68.6°C, 68.8°C, 69.0°C, 69.2°C, 69.4°C, 69.6°C, 69.8°C, 70.0°C, 70.2°C, 70.4°C, 70.6°C, 70.8°C, 71.0°C, 71.2°C, 71.4°C, 71.6°C, 71.8°C or higher.

In examples herein, the adenosine deaminase activity of ADA2 or variants can be exhibited at a pH optima of from or from about pH 6.0 to pH 7.6, such as a pH of at least pH 6, 6.25, 6.5, 6.75, 7, 7.25 or 7.5. For example, ADA2 has a pH optima of at or about pH 6.5 ± 0.2. Variant ADA2 proteins provided herein can exhibit a pH optima for adenosine deaminase activity of from or from about pH 6.0 to 6.8, such as at or about pH 6.5 ± 0.2. In some cases, the variant ADA2 exhibits an altered pH optimum and the catalytic activity can be exhibited at a higher pH that is from or from

about pH 6.8 to pH 7.6, such as from or from about pH 7.0 to pH 7.5 or pH 7.2 to pH 7.4, each inclusive. Since proliferating tissue near blood vessels in the TME can have a more neutral pH, such variants could be more active in particular tumor environments. For example, ADA2 variant can exhibit a pH optima for adenosine deaminase activity of at least pH 6.8, 6.9, 7.0, 7.1, 7.2, 7.3, 7.4, 7.5, 7.6 or higher.

Based on this description, it is within the level of one of skill in the art to generate a variant ADA2 containing any one or more of the described modification(s), and test each for adenosine deaminase activity and/or one or more of properties from among heparin binding, half-life, pH optimum, thermal stability, receptor binding and/or glycosylation as described herein.

a. Exemplary Modifications

i. Amino Acid Replacements

In one example, the modification(s) can be an amino acid replacement(s). Provided herein are variant ADA2 polypeptides that contain one or more amino acid replacement in an ADA2 polypeptide at an amino acid position corresponding to amino acid residue 11, 13, 20, 22, 26, 86, 109, 118, 119, 124, 133, 139, 179, 183, 191, 217, 219, 221, 224, 258, 262, 264, 266, 267, 277, 283, 296, 309, 317, 321, 352, 366, 371, 372, 373, 374, 403, 404, 405, 406, 441, 444, 452, 461, 469 or 470, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5. For example, the amino replacement can be at an amino acid position corresponding to amino acid residue K11, K13, R20, V22, K26, D86, F109, R118, F119, P124, W133, Y139, E179, F183, Y191, R217, R219, L221, Y224, K258, S262, H264, S266, K267, R277, R283, V296, K309, K317, K321, R352, R366, K371, K372, D373, I374, T403, G404, H405, P406, R441, K444, K452, K461, K469 or K470, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

For example, provided herein are variant ADA2 polypeptides that contain one or more amino acid replacement in an ADA2 polypeptide that is any one or more of: K11A, K11D, K11E, K13A, K13D, K13E, R20A, R20D, R20E, R20N, V22S, K26A, K26D, K26E, D86A, D86C, D86E, D86F, D86G, D86H, D86I, D86K, D86L, D86M, D86N, D86P, D86Q, D86R, D86S, D86T, D86V, D86W, D86Y, F109S, F109A, R118D, R118A, F119S, F119K, P124A, P124S, W133S, W133T, Y139T, Y139A, E179A, E179C, E179D, E179F, E179G, E179H, E179I, E179K, E179L, E179M,

E179N, E179P, E179Q, E179R, E179S, E179T, E179V, E179W, E179Y, F183K, Y191S, Y191D, R217A, R217D, R217E, R219A, R219C, R219D, R219E, R219F, R219G, R219H, R219I, R219K, R219L, R219M, R219N, R219P, R219Q, R219S, R219T, R219V, R219W, R219Y, L221A, L221C, L221D, L221E, L221F, L221G, L221H, L221I, L221K, L221M, L221N, L221P, L221Q, L221R, L221S, L221T, L221V, L221W, L221Y, Y224R, Y224N, K258A, K258D, K258E, S262A, S262C, S262D, S262E, S262F, S262G, S262H, S262I, S262K, S262L, S262M, S262N, S262P, S262Q, S262R, S262T, S262V, S262W, S262Y, H264A, H264C, H264D, H264E, H264F, H264G, H264I, H264K, H264L, H264M, H264N, H264P, H264Q, H264R, H264S, H264T, H264V, H264W, H264Y, S266A, S266C, S266D, S266E, S266F, S266G, S266H, S266I, S266K, S266L, S266M, S266N, S266P, S266Q, S266R, S266T, S266V, S266W, S266Y, K267A, K267C, K267D, K267E, K267F, K267G, K267H, K267I, K267L, K267M, K267N, K267P, K267Q, K267R, K267S, K267T, K267V, K267W, K267Y, R277A, R277D, R277E, R283A, R283D, R283E, V296A, V296C, V296D, V296E, V296F, V296G, V296H, V296I, V296K, V296L, V296M, V296N, V296P, V296Q, V296R, V296S, V296T, V296W, V296Y, K309A, K309D, K309E, K317A, K317D, K317E, K321A, K321D, K321E, R352A, R352D, R352E, R366A, R366D, R366E, K371A, K371D, K371E, K371N, K372A, K372D, K372E, K372N, D373S, I374S, T403N, G404N, H405S, P406S, R441A, R441D, R441E, K444A, K444D, K444E, K452A, K452D, K452E, K461A, K461D, K461E, K469A, K469D, K469E, K470A, K470D, and K470E, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

In particular, provided herein are variant ADA2 polypeptides that contain one or more amino acid replacements in an ADA2 polypeptide that is any one or more of: K11A, K11E, R20A, R20D, R20E, R219K, R219Q, L221A, L221V, L221G, S262N, H264Q, H264G, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E and K452E, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5. For example, provided herein are variant ADA2 polypeptides that contain one or more amino acid replacements in an ADA2 polypeptide that is any one or more of: K11A, K11E, R20A, R20E, R219K, R219Q, L221A, L221V, L221G, S262N, H264Q, H264G, R366E, K371A, K371D, K371E, K372D, K372E, K452D and K452E, by mature numbering, with reference to amino acid residues set forth in

SEQ ID NO:5. In another example, provided herein are variant ADA2 polypeptides that contain one or more amino acid replacements in an ADA2 polypeptide that is any one or more of R20A, R20D, R20E, S262N, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E and K452E, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5. In examples, provided herein are variant ADA2 polypeptides that contain one or more amino acid replacements in an ADA2 polypeptide that is any one or more of K11A, R20A, R20E, R219Q, S262N, K371A, K371D or K371E, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

Also provided herein are variant ADA2 polypeptides that contain 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid replacements compared to the reference ADA2 polypeptide not containing the modifications (i.e. the unmodified ADA2). Variant ADA2 polypeptides can contain any two or more amino acid replacements provided above, so long as the resulting ADA2 variant exhibits or retains adenosine deaminase activity. The two or more amino acid replacements can confer the same altered activity or a different altered activity. For example, one amino acid replacement can confer altered heparin binding and the other can confer increased adenosine deaminase activity. Hence, the resulting ADA2 polypeptide variants exhibit two or more altered activities or properties.

For example, provided herein are variant ADA2 polypeptides that contain amino acid replacements K11A/R20A; K11A/R20A/K371A; R20A/K371A; K11A/K371A; S262N/K371D; S262N/K371E; S262N/R20E; S262N/R20E/K371D; S262N/R20E/K371E; R219Q/K371E; R219Q/K371D; R219Q/R20E; R219Q/K371E/R20E; R219Q/K371D/R20E; R219Q/S262N/K371E; R219Q/S262N/K371D; R219Q/S262N/R20E; R219Q/S262N/K371E/R20E; R219Q/S262N/K371D/R20E; or R219Q/S262N, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

For example, provided herein are variant ADA2 polypeptides that contain amino acid replacements K11A/R20A; K11A/R20A/K371A; R20A/K371A; K11A/K371A; S262N/K371D; S262N/K371E; S262N/R20E; S262N/R20E/K371D; S262N/R20E/K371E; R219Q/K371E; R219Q/K371D; R219Q/R20E; R219Q/K371E/R20E; R219Q/K371D/R20E; R219Q/S262N/K371E;

Date Reçue/Date Received 2021-07-28

R219Q/S262N/K371D; R219Q/S262N/R20E; R219Q/S262N/K371E/R20E; R219Q/S262N/K371D/R20E; or R219Q/S262N, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

Exemplary of such variant ADA2 polypeptides are any set forth in any of SEQ ID NOS:13-63 or 71-273, or a catalytically active portion thereof.

In other examples, also provided herein are variant ADA2 polypeptides that contain amino acid replacements R219Q/S262N/K11A; R219Q/S262N/K11D; R219Q/S262N/K11E; R219Q/S262N/K13A; R219Q/S262N/K13D; R219Q/S262N/K13E; R219Q/S262N/K371A; R219Q/S262N/K372A; R219Q/S262N/K372D; R219Q/S262N/K372E; R219Q/S262N/K452A; R219Q/S262N/K452D; R219Q/S262N/K452E; R219Q/S262N/R20A; R219Q/S262N/R20D; R219Q/S262N/R366A; R219Q/S262N/R366D; R219Q/S262N/R366E; R219Q/S262N/H264A; R219Q/S262N/H264Q; R219Q/S262N/H264N; R219Q/S262N/H264G; R219K/S262N; R219N/S262N; R219A/S262N; R219Q/S262N/L221A; R219Q/S262N/L221V; R219Q/S262N/L221G; R219Q/S262N/E179D; R219Q/S262N/E179A; R219Q/S262N/E179S; R219Q/S262N/E179T; R219Q/S262N/E179V; R219Q/S262N/E179G; R219Q/S262A; R219Q/S262V; R219Q/S262M; R219Q/S262N/K11A/R20A; R219Q/S262N/K11A/R20A/K371A; R219Q/S262N/R20A/K371A; R219Q/S262N/K11A/K371A; R219Q/S262N/K26A; R219Q/S262N/K26D; R219Q/S262N/K26E; R219Q/S262N/R217A; R219Q/S262N/R217D; R219Q/S262N/R217E; R219Q/S262N/K258A; R219Q/S262N/K258D; R219Q/S262N/K258E; R219Q/S262N/R277A; R219Q/S262N/R277D; R219Q/S262N/R277E; R219Q/S262N/R283A; R219Q/S262N/R283D; R219Q/S262N/R283E; R219Q/S262N/K309A; R219Q/S262N/K309D; R219Q/S262N/K309E; R219Q/S262N/K317A; R219Q/S262N/K317D; R219Q/S262N/K317E; R219Q/S262N/K321A; R219Q/S262N/K321D; R219Q/S262N/K321E; R219Q/S262N/R352A; R219Q/S262N/R352D; R219Q/S262N/R352E; R219Q/S262N/R441A; R219Q/S262N/R441D; R219Q/S262N/R441E; R219Q/S262N/K444A; R219Q/S262N/K444D; R219Q/S262N/K444E; R219Q/S262N/K461A; R219Q/S262N/K461D; R219Q/S262N/K461E; R219Q/S262N/K469A;

R219Q/S262N/K469D; R219Q/S262N/K469E; R219Q/S262N/K470A; R219Q/S262N/K470D; R219Q/S262N/K470E; R219Q/S262N/D86A; R219Q/S262N/D86C; R219Q/S262N/D86E; R219Q/S262N/D86F; R219Q/S262N/D86G; R219Q/S262N/D86H; R219Q/S262N/D86I; R219Q/S262N/D86K; R219Q/S262N/D86L; R219Q/S262N/D86M; R219Q/S262N/D86N; R219Q/S262N/D86P; R219Q/S262N/D86Q; R219Q/S262N/D86R; R219Q/S262N/D86S; R219Q/S262N/D86T; R219Q/S262N/D86V; R219Q/S262N/D86W; R219Q/S262N/D86Y; R219Q/S262N/E179C; R219Q/S262N/E179F; R219Q/S262N/E179H; R219Q/S262N/E179I; R219Q/S262N/E179K; R219Q/S262N/E179L; R219Q/S262N/E179M; R219Q/S262N/E179N; R219Q/S262N/E179P; R219Q/S262N/E179Q; R219Q/S262N/E179R; R219Q/S262N/E179W; R219Q/S262N/E179Y; R219C/S262N; R219D/S262N; R219E/S262N; R219F/S262N; R219G/S262N; R219H/S262N; R219I/S262N; R219L/S262N; R219M/S262N; R219P/S262N; R219S/S262N; R219T/S262N; R219V/S262N; R219W/S262N; R219Y/S262N; R219Q/S262N/L221C; R219Q/S262N/L221D; R219Q/S262N/L221E; R219Q/S262N/L221F; R219Q/S262N/L221H; R219Q/S262N/L221I; R219Q/S262N/L221K; R219Q/S262N/L221M; R219Q/S262N/L221N; R219Q/S262N/L221P; R219Q/S262N/L221Q; R219Q/S262N/L221R; R219Q/S262N/L221S; R219Q/S262N/L221T; R219Q/S262N/L221W; R219Q/S262N/L221Y; R219Q/S262C; R219Q/S262D; R219Q/S262E; R219Q/S262F; R219Q/S262G; R219Q/S262H; R219Q/S262I; R219Q/S262K; R219Q/S262L; R219Q/S262P; R219Q/S262Q; R219Q/S262R; R219Q/S262T; R219Q/S262W; R219Q/S262Y; R219Q/S262N/H264C; R219Q/S262N/H264D; R219Q/S262N/H264E; R219Q/S262N/H264F; R219Q/S262N/H264I; R219Q/S262N/H264K; R219Q/S262N/H264L; R219Q/S262N/H264M; R219Q/S262N/H264P; R219Q/S262N/H264R; R219Q/S262N/H264S; R219Q/S262N/H264T; R219Q/S262N/H264V; R219Q/S262N/H264W; R219Q/S262N/H264Y; R219Q/S262N/S266A; R219Q/S262N/S266C; R219Q/S262N/S266D; R219Q/S262N/S266E; R219Q/S262N/S266F; R219Q/S262N/S266G; R219Q/S262N/S266H; R219Q/S262N/S266I; R219Q/S262N/S266K; R219Q/S262N/S266L;

R219Q/S262N/S266M; R219Q/S262N/S266N; R219Q/S262N/S266P; R219Q/S262N/S266Q; R219Q/S262N/S266R; R219Q/S262N/S266T; R219Q/S262N/S266V; R219Q/S262N/S266W; R219Q/S262N/S266Y; R219Q/S262N/K267A; R219Q/S262N/K267C; R219Q/S262N/K267D; R219Q/S262N/K267E; R219Q/S262N/K267F; R219Q/S262N/K267G; R219Q/S262N/K267H; R219Q/S262N/K267I; R219Q/S262N/K267L; R219Q/S262N/K267M; R219Q/S262N/K267N; R219Q/S262N/K267P; R219Q/S262N/K267Q; R219Q/S262N/K267R; R219Q/S262N/K267S; R219Q/S262N/K267T; R219Q/S262N/K267V; R219Q/S262N/K267W; R219Q/S262N/K267Y; R219Q/S262N/V296A; R219Q/S262N/V296C; R219Q/S262N/V296D; R219Q/S262N/V296E; R219Q/S262N/V296F; R219Q/S262N/V296G; R219Q/S262N/V296H; R219Q/S262N/V296I; R219Q/S262N/V296K; R219Q/S262N/V296L; R219Q/S262N/V296M; R219Q/S262N/V296N; R219Q/S262N/V296P; R219Q/S262N/V296Q; R219Q/S262N/V296R; R219Q/S262N/V296S; R219Q/S262N/V296T; R219Q/S262N/V296W; R219Q/S262N/V296Y; R219Q/K11A/R20A; R219Q/K11A/R20A/K371A; R219Q/R20A/K371A; R219Q/K11A/K371A; S262N/K11A/R20A; S262N/K11A/R20A/K371A; S262N/R20A/K371A; or S262N/K11A/K371A, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

Exemplary of such variant ADA2 polypeptides are any set forth in any of SEQ ID NOS:659-663 or 682-917, or a catalytically active portion thereof.

ii. Modification(s) of PRB Domain

In other examples, also provided herein are modified ADA2 polypeptides that contain a modified PRB domain. The PRB domain is not required for catalytic activity, and, hence, as shown herein can be removed so that the ADA2 variant proteins activities other than deaminase activity, mediated by ADA2, are reduced or eliminated. According to the reported domain organization of ADA2, the PRB domain corresponds to residues 98-156 or 105-148 of mature ADA2 set forth in SEQ ID NO:5. The modifications of the PRB domain can include deletion of all or a portion of the PRB domain (i.e. deletion of one or more residues of the PRB domain), insertion of one or more amino acid residues into the PRB domain, amino acid

Date Reçue/Date Received 2021-07-28

replacement of one or more residues of the PRB domain or a combination thereof to thereby reduce or inhibit binding of the domain to a receptor or other activity thereof. For example, the PRB domain can contain up to or about or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, or 59 modified positions, such as generally up to or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44 modified positions.

In one example, as described in more detail below, all or a portion of the PRB domain can be deleted, such as by deletion of one or more contiguous amino acid residues of the PRB domain. For example, provided herein are variant ADA2 in which one or more contiguous amino acid residues between or about between amino acid residues 98 and 156 or amino acid residues 105 and 148 or amino acid residues 105 and 147 or amino acid residues 99 and 144, inclusive, with reference to residues set forth in SEQ ID NO:5, are deleted. Exemplary of such ADA2 polypeptides are deletion of contiguous amino acid residues corresponding to contiguous residues 98-156, 105-148, 105-147, 102-147 or 108-150, by mature numbering, with reference to the sequence of amino acids set forth in SEQ ID NO:5. For example, exemplary of such ADA2 polypeptides include polypeptides ADA2_del98-156 (98-156del; SEQ ID NO:548); ADA2_del105-148 (105-148del; SEQ ID NO:549); ADA2_del105-147 (105-147del; SEQ ID NO:550); and ADA2_del99-144 (99-144del; SEQ ID NO:579), by mature numbering, with reference to the sequence of amino acids set forth in SEQ ID NO:5.

In some examples, the variant ADA2 that contains a modification in the PRB domain, such as a deletion of contiguous residues, also contains a substitution of the modified or deleted region with a peptide linker. As a result, all or a portion of the PRB domain can be replaced with a sterically acceptable peptide linker sequence. In such examples, at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59 or all more contiguous amino acids from the PRB domain can be substituted or replaced with amino acids of a peptide linker that generally does not exceed 60 amino acids, and generally does not

exceed 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, or 50 amino acids. Any suitable linker can be selected so long as the resulting variant ADA2 exhibits adenosine deaminase activity.

Examples of peptide linkers include, but are not limited to: (Gly)n, where n is 2 to 20 (SEQ ID NO:368); —Gly-Gly—; GGG (SEQ ID NO:369); GGGG (SEQ ID NO:362); GGGGG (SEQ ID NO:360); GGGGGGG (SEQ ID NO:370); GGGGGGGGGG (SEQ ID NO:371); GGGGGGGGGGGGGGG (SEQ ID NO:372); GGGGS or (GGGGS)n (SEQ ID NO:343); GGGGSGGGGS (SEQ ID NO:580); GGGGSGGGGSGGGGS (SEQ ID NO:367); SSSSG or (SSSSG)n (SEQ ID NO:344); GKSSGSGSESKS (SEQ ID NO:345); GGSTSGSGKSSEGKG (SEQ ID NO:346); GSTSGSGKSSSEGSGSTKG (SEQ ID NO:347); GSTSGSGKPGSGEGSTKG (SEQ ID NO:348); EGKSSGSGSESKEF (SEQ ID NO:349); or AlaAlaProAla or (AlaAlaProAla)n (SEQ ID NO:350), where n is 1 to 6, such as 1, 2, 3, or 4. In particular examples, the peptide linker is GGG (SEQ ID NO:369); GGGGG (SEQ ID NO:360); GGGGGGG (SEQ ID NO:370); GGGGGGGGGG (SEQ ID NO:371); GGGGGGGGGGGGGGG (SEQ ID NO:372); GGGGS (SEQ ID NO:343); GGGGSGGGGS (SEQ ID NO:580); or GGGGSGGGGSGGGGS (SEQ ID NO:367).

Exemplary of such a modification is a variant ADA2 designated C105-T147del→$(Gly)_n$ (SEQ ID NO:280), where n is 2 to 20, whereby the PRB domain in the region corresponding to residues 105-147 with reference to numbering in SEQ ID NO:5 is replaced with a glycine linker of 2 to 20 amino acid residues in length. For example, the variant ADA2 can be C105-T147del→$(Gly)_{15}$ (SEQ ID NO:281); C105-T147del→$(Gly)_{10}$ (SEQ ID NO:282); C105-T147del→$(Gly)_7$ (SEQ ID NO:283); C105-T147del→$(Gly)_5$ (SEQ ID NO:284) or C105-T147del→$(Gly)_3$ (SEQ ID NO:285). Further examples of such a modification is a variant ADA designated V99-Q144del→$(GGGGS)_n$ (SEQ ID NO:581), where n is 1 to 5, whereby the PRB domain in the region corresponding to residues 99-144 with reference to numbering in SEQ ID NO:5 is replaced with a $(GGGGS)_n$ linker where the sequence of amino acids in the linker is repeated 1 to 5 times such that the linker is 5, 10, 15, 20 or 25 amino acid residues in length. For example, the variant ADA2 can be V99-Q144del→$(GGGGS)_1$ (SEQ ID NO:583); V99-Q144del→$(GGGGS)_2$ (SEQ ID NO:584); or V99-

Q144del→$(GGGGS)_3$ (SEQ ID NO:585). Further examples of such a modification is a variant ADA designated C105-T147del→$(GGGGS)_n$ (SEQ ID NO:582), where n is 1 to 5, whereby the PRB domain in the region corresponding to residues 105-147 with reference to numbering in SEQ ID NO:5 is replaced with a $(GGGGS)_n$ linker where the sequence of amino acids in the linker is repeated 1 to 5 times such that the linker is 5, 10, 15, 20 or 25 amino acid residues in length. For example, the variant ADA2 can be C105-T147del→$(GGGGS)_1$ (SEQ ID NO:586); C105-T147del→$(GGGGS)_2$ (SEQ ID NO:587); or C105-T147del→$(GGGGS)_3$ (SEQ ID NO:588), by mature numbering, with reference to the sequence of amino acids set forth in SEQ ID NO:5. Exemplary of such variant ADA2 polypeptides are any set forth in any of SEQ ID NOS:281-285 and 583-588, or a catalytically active portion thereof.

Also provided herein are variant ADA2 polypeptides that contain deletions, insertions, substitutions and/or amino acid replacements in the PRB domain, combined with other deletions, insertions, substitutions and/or amino acid replacements provided herein. For example, provided are variant ADA2 polypeptides that contain a deletion of all or a portion of the PRB domain, such as by deletion of one or more contiguous amino acid residues of the PRB domain, combined with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 additional amino acid replacements compared to the unmodified reference ADA2. Also provided herein are variant ADA2 polypeptides that contain a modification in the PRB domain, such as a deletion of contiguous residues and also contains a substitution of the modified or deleted region with a peptide linker, combined with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 additional amino acid replacements compared to the unmodified reference ADA2. For example, the variant ADA2 polypeptides that contain both a deletion of all or portion of the PRB domain and any one or more amino acid replacements provided above, so long as the resulting ADA2 variant exhibits or retains adenosine deaminase activity. The deletion and/or amino acid replacements can confer the same altered activity or a different altered activity. For example, deletion and/or substitution of the PRB domain can confer one altered activity, e.g., reduction in binding to a receptor, and amino acid replacement(s) can confer increased adenosine deaminase activity. Hence, the resulting ADA2 polypeptide variants exhibits two or more altered activities or properties.

For example, provided herein are variant ADA2 polypeptides that contain the following combination of deletions and/or substitutions and/or amino acid replacements: K371D/V99-Q144del→$(GGGGS)_1$; K371D/V99-Q144del→$(GGGGS)_2$; K371D/V99-Q144del→$(GGGGS)_3$; K371D/C105-T147del→$(GGGGS)_1$; K371D/C105-T147del→$(GGGGS)_2$; K371D/C105-T147del→$(GGGGS)_3$; R219Q/S262N/C105-T147del→$(Gly)_{15}$; R219Q/S262N/C105-T147del→$(Gly)_{10}$; R219Q/S262N/C105-T147del→$(Gly)_7$; R219Q/S262N/C105-T147del→$(Gly)_5$; R219Q/S262N/C105-T147del→$(Gly)_3$; R219Q/S262N/V99-Q144del→$(GGGGS)_1$; R219Q/S262N/V99-Q144del→$(GGGGS)_2$; R219Q/S262N/V99-Q144del→$(GGGGS)_3$; R219Q/S262N/C105-T147del→$(GGGGS)_1$; R219Q/S262N/C105-T147del→$(GGGGS)_2$; R219Q/S262N/C105-T147del→$(GGGGS)_3$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_1$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_2$; R219Q/S262N/K371D/V99-Q144del→$(GGGGS)_3$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_1$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_2$; R219Q/S262N/K371D/C105-T147del→$(GGGGS)_3$; K371D/C105-T147del→(Gly)n (where n is 2 to 20); K371D/C105-T147del→$(Gly)_{15}$; K371D/C105-T147del→$(Gly)_{10}$; K371D/C105-T147del→$(Gly)_7$; K371D/C105-T147del→$(Gly)_5$; K371D/C105-T147del→$(Gly)_3$; K371D/V99-Q144del→(GGGGS)n (where n is 1 to 5); K371D/C105-T147del→(GGGGS)n (where n is 1 to 5); K371D/N98-N156del; K371D/C105-E148del; K371D/C105-T147del; K371D/V99-Q144del; R219Q/S262N/C105-T147del→(Gly)n (where n is 2 to 20); R219Q/S262N/V99-Q144del→(GGGGS)n (where n is 1 to 5); R219Q/S262N/C105-T147del→(GGGGS)n (where n is 1 to 5); R219Q/S262N/N98-N156del; R219Q/S262N/C105-E148del; R219Q/S262N/C105-T147del; R219Q/S262N/V99-Q144del; R219Q/S262N/K371D/C105-T147del→(Gly)n (where n is 2 to 20); R219Q/S262N/K371D/C105-T147del→$(Gly)_{15}$; R219Q/S262N/K371D/C105-T147del→$(Gly)_{10}$; R219Q/S262N/K371D/C105-T147del→$(Gly)_7$; R219Q/S262N/K371D/C105-T147del→$(Gly)_5$; R219Q/S262N/K371D/C105-T147del→$(Gly)_3$; R219Q/S262N/K371D/V99-Q144del→(GGGGS)n (where n is 1 to 5); R219Q/S262N/K371D/C105-T147del→(GGGGS)n (where n is 1 to 5); R219Q/S262N/K371D/N98-N156del; R219Q/S262N/K371D/C105-E148del;

R219Q/S262N/K371D/C105-T147del; R219Q/S262N/K371D/V99-Q144del; R219Q/C105-T147del→(Gly)n (where n is 2 to 20); R219Q/V99-Q144del→(GGGGS)n (where n is 1 to 5); R219Q/C105-T147del→(GGGGS)n (where n is 1 to 5); R219Q/N98-N156del; R219Q/C105-E148del; R219Q/C105-T147del; R219Q/V99-Q144del; S262N/C105-T147del→(Gly)n (where n is 2 to 20); S262N/V99-Q144del→(GGGGS)n (where n is 1 to 5); S262N/C105-T147del→(GGGGS)n (where n is 1 to 5); S262N/N98-N156del; S262N/C105-E148del; S262N/C105-T147del; and S262N/V99-Q144del, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

Exemplary of such variant ADA2 polypeptides are any set forth in any of SEQ ID NOS:589-594, 602-606, 634-658, 664-681, 918-931, or a catalytically active portion thereof.

iii. Amino Acid Replacement(s) with Altered Interaction between the PRB Domain and Other regions of ADA2

In yet other examples, also provided herein are modified ADA2 polypeptides that contain amino acid replacements that confer altered interaction between PRB domain and the rest of ADA2 (e.g., the adenosine deaminase (ADA) domain). For example, according to the reported domain organization of ADA2, the PRB domain corresponds to residues 98-156 or 105-148 of mature ADA2 set forth in SEQ ID NO:5. Provided herein are variant ADA2 polypeptides that contain one or more amino acid replacement in an ADA2 polypeptide at an amino acid position corresponding to amino acid residue 109, 118, 119, 124, 133, 139, 183, 191 or 224, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5. For example, the amino replacement can be at an amino acid position corresponding to amino acid residue F109, R118, F119, P124, W133, Y139, F183, Y191 or Y224 with reference to amino acid residues set forth in SEQ ID NO:5. Modifications at each position, or combination thereof, can alter the interaction between the PRB domain and other domains in ADA2, such as the ADA domain.

For example, provided herein are variant ADA2 polypeptides that contain one or more amino acid replacement in an ADA2 polypeptide that is any one or more of: F109S, F109A, R118D, R118A, F119S, F119K, P124A, P124S, W133S, W133T,

Y139T, Y139A, F183K, Y191S, Y191D, Y224R or Y224N, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

Also provided herein are variant ADA2 polypeptides that contain amino acid replacements that confer altered interaction between the PRB domain and the rest of ADA2, combined with other deletions, insertions, substitutions and/or amino acid replacements provided herein. For example, provided are variant ADA2 polypeptides that contain 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid replacements compared to the reference ADA2 polypeptide not containing the modifications (i.e. the unmodified ADA2). Variant ADA2 polypeptides can contain any two or more amino acid replacements provided above, so long as the resulting ADA2 variant exhibits or retains adenosine deaminase activity. The two or more amino acid replacements can confer the same altered activity or a different altered activity. For example, one amino acid replacement can confer altered interaction between the PRB domain the ADA domain, and the other can confer increased adenosine deaminase activity. Hence, the resulting ADA2 polypeptide variants exhibits two or more altered activities or properties.

For example, provided herein are variant ADA2 polypeptides that contain amino acid replacements Y191D/Y224R; R219Q/S262N/F119S; R219Q/S262N/F119K; R219Q/S262N/Y224R; R219Q/S262N/Y224N; R219Q/S262N/Y191S; R219Q/S262N/Y191D; R219Q/S262N/F183K; R219Q/S262N/Y191D/Y224R; R219Q/S262N/F109S; R219Q/S262N/F109A; R219Q/S262N/R118D; R219Q/S262N/R118A; R219Q/S262N/Y139T; R219Q/S262N/Y139A; R219Q/S262N/W133S; R219Q/S262N/W133T; R219Q/S262N/P124A; or R219Q/S262N/P124S, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

Exemplary of such variant ADA2 polypeptides are any set forth in any of SEQ ID NOS:561-578 or 616-633, or a catalytically active portion thereof.

iv. Hyperglycosylation

Included among the variant ADA2 provided herein are those that have been modified by altering the level and/or type of glycosylation compared to an unmodified ADA2. Glycosylation can be increased or decreased compared to the unmodified ADA2 polypeptide. In some instances, the level or extent of glycosylation is

increased, resulting in a hyperglycosylated ADA2 polypeptide or protein. This can be achieved, for example, by incorporation of at least one non-native glycosylation site not found in the unmodified ADA2 polypeptide or protein to which a carbohydrate is linked. Hyperglycosylated ADA2 polypeptides also can be generated by linkage of a carbohydrate moiety to at least one native glycosylation site found but not glycosylated in the unmodified ADA2 polypeptide.

The variant ADA2 proteins provided herein can contain altered, such as new, O-linked glycosylation, N-linked glycosylation or O-linked and N-linked glycosylation. In some examples, a variant ADA2 includes 1, 2, 3, 4, 5 or more carbohydrate moieties, each linked to different glycosylation sites. The glycosylation site(s) can be a native glycosylation site(s) and/or a non-native glycosylation site(s). In some examples, the variant ADA2 is glycosylated at more than one non-native glycosylation site. For example, a variant ADA2 can be modified to introduce 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more non-native glycosylation sites.

Non-native glycosylation sites can be introduced by amino acid replacement. O-glycosylation sites can be created, for example, by amino acid replacement of a native residue with a serine or threonine. N-linked glycosylation sites can be created by creating the motif Asn-Xaa-Ser/Thr/Cys, where Xaa is not proline. Creation of this consensus sequence by amino acid modification can involve replacement of a native amino acid residue with an asparagine, replacement of a native amino acid residue with a serine, threonine or cysteine, or replacement of a native amino acid residue with an asparagine and amino acid replacement of native residue with a serine, threonine or cysteine. Non-native glycosylation sites can be created in any region in an ADA2 polypeptide. The level of glycosylation (*e.g.* the number of introduced non-native glycosylation sites) can be increased by at least about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, or more compared to the level of glycosylation of the corresponding form of the unmodified or wild-type ADA2.

Exemplary modifications provided herein include introducing a non-native glycosylation site by modification with one or more amino acid replacement(s) that include, but are not limited to, replacement with: N at a position corresponding to position 20 and S at a position corresponding to position 22; N at a position

corresponding to position 371 and S at a position corresponding to position 373; N at a position corresponding to position 372 and S at a position corresponding to position 374; N at a position corresponding to position 403 and S at a position corresponding to position 405; and N at a position corresponding to position 404 and S at a position corresponding to position 406, each by mature numbering, with reference to positions set forth in SEQ ID NO:5. For example, amino acid replacement(s) to introduce a non-native glycosylation site can include: R20N/V22S; K371N/D373S; K372N/I374S; T403N/H405S; or G404N/P406S, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

In other examples, modifications provided herein include introducing a non-native glycosylation site by modification with one or more amino acid replacement(s) in or near the PRB domain. Exemplary modifications provided herein include introducing a non-native glycosylation site by modification with one or more amino acid replacement(s) that include, but are not limited to, replacement with: N at a position corresponding to position 125 and A at a position corresponding to position 126; N at a position corresponding to position 127 and S at a position corresponding to position 129; N at a position corresponding to position 126 and T at a position corresponding to position 128; N at a position corresponding to position 112 and T at a position corresponding to position 114; N at a position corresponding to position 134, C at a position corresponding to position 135 and T at a position corresponding to position 136; N at a position corresponding to position 134, S at a position corresponding to position 135 and T at a position corresponding to position 136; N at a position corresponding to position 142 and S at a position corresponding to position 144; N at a position corresponding to position 137 and T at a position corresponding to position 139; N at a position corresponding to position 111 and S at a position corresponding to position 113, each by mature numbering, with reference to positions set forth in SEQ ID NO:5. For example, amino acid replacement(s) to introduce a non-native glycosylation site in or near the PRB domain can include: R125N/P126A; S127N/K129S; P126N/E128T; R112N/I114T; I134N/L135C/L136T; I134N/L135S/L136T; R142N/Q144S; E137N/Y139T; or P111N/G113S, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

In other examples, also provided herein are modified ADA2 polypeptides that contain addition (i.e. insertion) of one or more contiguous residues at the N-terminus or the C-terminus. Such replacements can introduce a non-native glycosylation site. The modified ADA2 polypeptides can contain insertion of up to or about or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more amino acid residues at one or both of the N-terminus or C-terminus. For example, addition or insertion of amino acids can provide for altered glycosylation sites in the encoded protein. Exemplary of a modification is insertion ---→N1/---→A2/---→S3 at the N-terminus, by mature numbering, with reference to the amino acid positions set forth in SEQ ID NO:5.

Exemplary of such variant ADA2 polypeptides are any set forth in any of SEQ ID NOS:274-279 and 552-560.

Also provided herein are variant ADA2 polypeptides that contain one or more amino acid replacement(s) that introduce a non-native glycosylation site, combined with other deletions, insertions, substitutions and/or amino acid replacements provided herein. For example, provided are variant ADA2 polypeptides that contain 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid replacements compared to the reference ADA2 polypeptide not containing the modifications (i.e. the unmodified ADA2). Variant ADA2 polypeptides can contain any two or more amino acid replacements provided above, so long as the resulting ADA2 variant exhibits or retains adenosine deaminase activity. The two or more amino acid replacements can confer the same altered activity or a different altered activity. For example, one or more amino acid replacement(s) can introduce a non-native glycosylation site, and another amino acid replacement(s) can confer increased adenosine deaminase activity. Hence, the resulting ADA2 polypeptide variants exhibits two or more altered activities or properties.

For example, provided herein are variant ADA2 polypeptides that contain amino acid replacements R219Q/S262N/---→N1/---→A2/---→S3; R219Q/S262N/R20N/V22S; R219Q/S262N/K371N/D373S; R219Q/S262N/K372N/I374S; R219Q/S262N/T403N/H405S; R219Q/S262N/G404N/P406S; R219Q/S262N/R125N/P126A; R219Q/S262N/S127N/K129S; R219Q/S262N/P126N/E128T;

R219Q/S262N/R112N/I114T; R219Q/S262N/I134N/L135C/L136T; R219Q/S262N/I134N/L135S/L136T; R219Q/S262N/R142N/Q144S; R219Q/S262N/E137N/Y139T; or R219Q/S262N/P111N/G113S, by mature numbering, with reference to amino acid residues set forth in SEQ ID NO:5.

Exemplary of such variant ADA2 polypeptides are any set forth in any of SEQ ID NOS:596-601 or 607-615, or a catalytically active portion thereof.

b. Nucleic Acid Molecules

Also provided herein are nucleic acid molecules that encode any of the variant ADA2 polypeptides provided herein. A modified nucleic acid molecule that encodes any of the variant ADA2 polypeptides provided herein includes codon changes corresponding to modifications provided herein (*e.g.* replacement or substitution, insertion or addition, or deletion of one or more nucleotides). It is within the level of a skilled artisan, who is familiar with codons that correspond to various amino acids, to identify such codon changes based on exemplification of the modified amino acids herein. In particular examples, the nucleic acid sequence can be codon optimized, for example, to increase expression levels of the encoded sequence. The particular codon usage is dependent on the host organism in which the modified polypeptide is expressed. One of skill in the art is familiar with optimal codons for expression in mammalian or human cells, bacteria or yeast, including for example *Escherichia coli* or *Saccharomyces cerevisiae*. For example, codon usage information is available from the Codon Usage Database available at kazusa.or.jp.codon (*see e.g.* Richmond (2000) *Genome Biology*, 1:241 for a description of the database). See also, Forsburg (2004) *Yeast*, 10:1045-1047; Brown *et al.* (1991) *Nucleic Acids Research*, 19:4298; Sharp *et al.* (1988) *Nucleic Acids Res.*, 12:8207-8211; Sharp *et al.* (1991) *Yeast*, 657-78). Vectors contain the nucleic acid molecules for expression and production of the ADA2 polypeptides are provided.

c. Production of Variant ADA2 Proteins

The variant ADA2 polypeptides and encoding nucleic acid molecules provided herein can be produced by standard recombinant DNA techniques known to one of skill in the art. Any method known in the art to effect mutation of any one or more amino acids in a target protein can be employed. Methods include standard site-directed or random mutagenesis of encoding nucleic acid molecules, or solid phase

polypeptide synthesis methods. In particular, total chemical synthesis methods, including peptide synthesis followed by peptide ligation can be employed. Nucleic acid molecules encoding an ADA2 polypeptide can be subjected to mutagenesis, such as random mutagenesis of the encoding nucleic acid, error-prone PCR, site-directed mutagenesis (using *e.g.*, a kit, such as kit such as QuikChange available from Stratagene), overlap PCR, gene shuffling, or other recombinant methods. The nucleic acid encoding the polypeptides can then be introduced into a host cell to be expressed heterologously. In some examples, the variant ADA2 polypeptides are produced synthetically, such as using total chemical synthesis, solid phase or solutions phase peptide synthesis.

Exemplary methods for producing and expressing a nucleic acid molecule encoding an ADA2 polypeptide, including any variant ADA2 polypeptide, are described in Section E. Depending on how the variant ADA2 molecule is produced, or the particular nature of the modification(s), the variant ADA2 polypeptides provided herein can be produced as a monomer, dimer, or other multimer. For example, the variant ADA2 is a heterodimer or homodimer.

In particular, ADA2 normally exists as a homodimer that is composed of two identical polypeptide chains. As described above, nonpolar interactions between residues of two identical polypeptide subunits mediate formation of the homodimer upon secretion of ADA2 from cells. Since wildtype ADA2 is a homodimer, it is understood that mention of an amino acid sequence of the reference or unmodified ADA2 polypeptide refers to the sequence of amino acids of a single ADA2 polypeptide subunit. The variant ADA2 can contain one or more ADA2 polypeptide subunits, that are the same (i.e. homodimer) or different (i.e. heterodimer). For example, a variant ADA2 homodimer is readily produced and secreted by cells transformed with a nucleic acid molecule encoding a variant ADA2 polypeptide, such as nucleic acid encoding a polypeptide that has the sequence of amino acids set forth in any of SEQ ID NOS:13-63, 71-285 or 552-931, or a catalytically active fragment thereof. If cells are encoded with two or more different nucleic acid molecules, each encoding a different ADA2 polypeptide, a heterodimer can be produced.

In one example, the variant ADA2 polypeptide provided herein is a dimer. For example, the resulting variant ADA2 polypeptide is a homodimer that contains a first

and second polypeptide subunit that are the same, i.e. each has the same amino acid sequence containing the identical modification(s) with respect to the amino acid sequence of the reference or unmodified ADA2 polypeptide. The homodimer can be formed by transforming a nucleic acid molecule encoding the variant polypeptide into a cell, which, upon secretion, results in nonpolar interaction between residues of two variant polypeptide subunits to mediate formation of the dimer.

In another example, the resulting ADA2 polypeptide is a heterodimer that contains a first and second polypeptide subunit that are different. In such an example, one or both of the first or second polypeptide subunit contains a sequence of amino acids containing a modification(s) with respect to the amino acid sequence of the reference or unmodified ADA2 polypeptide. In some cases, both the first and second polypeptide subunit can contain a sequence of amino acids containing a modification(s) compared to the reference of unmodified ADA2 polypeptide, but the nature of the modification(s) are different. The heterodimer can be formed by transforming into a cell both a first nucleic acid molecule encoding a first variant polypeptide subunit and a second nucleic acid molecule encoding a second different polypeptide subunit. The second nucleic acid molecule can encode a polypeptide subunit containing the sequence of amino acids of the reference or wildtype ADA2, or can encode a variant polypeptide subunit containing a sequence of amino acids containing modification(s) with respect to the amino acid sequence of the reference or unmodified ADA2. The heterodimer is produced upon expression and secretion from a cell as a result of nonpolar interaction between residues of the two polypeptide subunits to mediate formation of the dimer. In such processes, generally a mixture of dimeric molecules is formed, including homodimers and heterodimers. For the generation of heterodimers, additional steps for purification can be necessary. For example, the first and second polypeptide can be engineered to include a tag with metal chelates or other epitope, where the tags are different. The tagged domains can be used for rapid purification by metal-chelate chromatography, and/or by antibodies, to allow for detection by western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

In other examples, the variant ADA2 polypeptide is a monomer. A monomer can be produced by mutation of one or more residues that are involved in protein

Date Reçue/Date Received 2021-07-28

dimerization, so long as the adenosine deaminase activity is retained. Exemplary of residues that can be targeted for mutagenesis include, but are not limited to, amino acid residues 1, 4, 5, 8, 9, 11, 12, 15, 344, 362 or 366, with reference to amino acid residues set forth in SEQ ID NO:5. The residues can be replaced with one of the other nineteen amino acid residues at the position. It is within the level of a skilled artisan to generate and assess monomer formation of a polypeptide. For example, monomer formation can be assessed, and monomers purified, by size-exclusion chromatography (SEC). Adenosine deaminase activity also can be assessed, such as using any of the assays described herein or known in the art.

In some examples, dimeric or other multimeric molecules of a variant ADA2 can be formed by conjugation or fusion of the encoded variant ADA2 polypeptide to any moiety or other polypeptide that are themselves able to interact to form a stable structure. For example, separate encoded ADA2 polypeptides, where at least one is a variant ADA2 polypeptide, can be joined by multimerization, whereby multimerization of the polypeptides is mediated by a multimerization domain. The variant ADA2 dimers or multimers can be formed by generation of a chimeric molecule where a variant ADA2 is linked, directly or indirectly, to a multimerization domain. A nucleic acid molecule encoding a variant ADA2 can be joined (directly or indirectly) with a nucleic acid encoding a multimerization domain. For example, a variant ADA2 dimer provided herein can contain a first ADA2 polypeptide subunit linked directly or indirectly via a linker to a multimerization domain and a second ADA2 polypeptide subunit linked directly or indirectly via a linker to a multimerization domain, wherein one or both of the first and second polypeptide are a variant ADA2 polypeptide. The first and second ADA2 polypeptide can be the same or different. Exemplary of a multimerization domain is an Fc domain, which is described further below.

Homo- or heteromultimeric polypeptides can be generated from co-expression of separate nucleic acid molecules encoding ADA2 polypeptides. Chimeric ADA2 polypeptides can be readily produced and secreted by cells, such as mammalian cells, transformed with the appropriate nucleic acid molecule. For example, a cell can be transformed with a first nucleic acid molecule encoding a variant ADA2 and a second nucleic acid molecule encoding the same or different ADA2. The second nucleic acid

molecule can encode a polypeptide subunit containing the sequence of amino acids of the reference or wildtype ADA2, or can encode a variant polypeptide subunit containing a sequence of amino acids containing modification(s) with respect to the amino acid sequence of the reference or unmodified ADA2. The secreted forms of the ADA2 polypeptide include those where the variant ADA2 is a homodimer of the first encoded variant ADA2 polypeptide, a homodimer of the second encoded ADA2 polypeptide, such as wildtype or second variant ADA2 polypeptide, and ADA2 heterodimers containing two polypeptide subunits that are different. In some cases, higher ordered multimers can form.

Multimerization domains are well known to a skilled artisan. Generally, a multimerization domain includes any capable of forming a stable protein-protein interaction. The multimerization domains can interact via an immunoglobulin sequence (*e.g.* Fc domain; see *e.g.*, International Patent Pub. Nos. WO 93/10151 and WO 2005/063816; U.S. Pub. No. 2006/0024298; U.S. Patent No. 5,457,035), leucine zipper (*e.g.* from nuclear transforming proteins fos and jun or the proto-oncogene c-myc or from General Control of Nitrogen (GCN4)), a hydrophobic region, a hydrophilic region, or a free thiol which forms an intermolecular disulfide bond between the chimeric molecules of a homo- or heteromultimer. In addition, a multimerization domain can include an amino acid sequence containing a protuberance complementary to an amino acid sequence comprising a hole, such as is described, for example, in U.S. Patent No. 5, 731,168; International Patent Pub. Nos. WO 98/50431 and WO 2005/063816; Ridgway *et al.* (1996) *Protein Engineering*, 9:617-621. Such a multimerization region can be engineered such that steric interactions not only promote stable interaction, but further promote the formation of heterodimers over homodimers from a mixture of chimeric monomers. Generally, protuberances are constructed by replacing small amino acid side chains from the interface of the first polypeptide with larger side chains (*e.g.*, tyrosine or tryptophan). Compensatory cavities of identical or similar size to the protuberances are optionally created on the interface of the second polypeptide by replacing large amino acid side chains with smaller ones (*e.g.*, alanine or threonine).

An ADA2 polypeptide, such as any variant ADA2 polypeptide provided herein, can be joined anywhere, but typically via its N- or C- terminus, to the N- or C-

terminus of a multimerization domain to form a chimeric polypeptide. The linkage can be direct or indirect via a linker. Also, the chimeric polypeptide can be a fusion protein or can be formed by chemical linkage, such as through covalent or non-covalent interactions. For example, when preparing a chimeric polypeptide containing a multimerization domain, nucleic acid encoding an ADA2 polypeptide can be operably linked to nucleic acid encoding the multimerization domain sequence, directly or indirectly or optionally via a linker domain. The construct can encode a chimeric protein where the C-terminus of the ADA2 polypeptide is joined to the N-terminus of the multimerization domain. In some instances, a construct can encode a chimeric protein where the N-terminus of the ADA2 polypeptide is joined to the N- or C-terminus of the multimerization domain.

In examples where the multimerization domain is a polypeptide, a gene fusion encoding the ADA2-multimerization domain chimeric polypeptide is inserted into an appropriate expression vector. The resulting ADA2-multimerization domain chimeric proteins can be expressed in host cells transformed with the recombinant expression vector, and allowed to assemble into multimers, where the multimerization domains interact to form multivalent polypeptides. Chemical linkage of multimerization domains to ADA2 polypeptides can also be effected using heterobifunctional linkers.

The resulting chimeric polypeptides, and multimers formed therefrom, can be purified by any suitable method such as, for example, by affinity chromatography over Protein A or Protein G columns. Where two nucleic acid molecules encoding different ADA2 chimeric polypeptides are transformed into cells, formation of homo- and heterodimers will occur. Conditions for expression can be adjusted so that heterodimer formation is favored over homodimer formation. For example, for multimers formed by interaction of disulfide-linkage of an Fc multimerization domain, homodimers can be reduced under conditions that favor the disruption of inter-chain disulfides, but do not affect intra-chain disulfides. Alternatively, the formation of this type of heterodimer can be biased by genetically engineering and expressing ADA2 fusion molecules using a multimerization domain that promotes formation of heterodimers, such as using a *c-jun* and *c-fos* leucine zipper combination. Since the leucine zippers form predominantly heterodimers, they can be used to drive the formation of the heterodimers when desired. The ADA2 polypeptides contain an

Fc region or other multimerization domain also can be engineered to include a tag to permit purification of desired heterodimers. The products of the nuclear oncogenes *fos* and *jun* contain leucine zipper domains that preferentially form a heterodimer (O'Shea *et al.* (1989) *Science*, 245:646; Turner and Tijian (1989) *Science*, 243:1689). For example, the leucine zipper domains of the human transcription factors *c-jun* and *c-fos* have been shown to form stable heterodimers with a 1:1 stoichiometry (see *e.g.*, Busch and Sassone-Corsi (1990) *Trends Genetics*, 6:36-40; Gentz *et al.* (1989) *Science*, 243:1695-1699). Although *jun-jun* homodimers also have been shown to form, they are about 1000-fold less stable than *jun-fos* heterodimers.

D. ADA2 CONJUGATES AND FUSION PROTEINS

Any ADA2 molecule, including any provided herein, can be conjugated, directly or indirectly, to one or more heterologous moiety. The ADA2 can be a wildtype ADA2, including allelic and species variants, or can be any variant described herein in Section C.2. above. The ADA2 molecule in the conjugate can be a monomer or a dimer, for example, a heterodimer or a homodimer. Typically, the ADA2 in the conjugate is a homodimer. The heterologous moiety can be conjugated to one or both polypeptide subunits of the dimer.

For example, the ADA2 can be any that contains a polypeptide having the sequence of amino acids set forth in any of SEQ ID NOS:5 or 326-336, 338-342, 375 or 380-383, or a sequence of amino acids that exhibits at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to any of SEQ ID NOS:5 or 326-336, 338-342, 375 or 380-383, or a catalytically active fragment thereof. In one example, the ADA2 in the conjugate provided herein can contain a polypeptide having the sequence of amino acids set forth in any of SEQ ID NOS:5 or 326-336, 338-342, 375 or 380-383 or a catalytically active fragment thereof, such as any of SEQ ID NOS:5, 326-334, 340, 375 or 380-383, or a catalytically active fragment thereof. For example, the ADA2 in the conjugate provided herein can contain a polypeptide having the sequence of amino acids set forth in SEQ ID NO:5, or a catalytically active portion thereof. The catalytically active portion can be one that lacks all or a portion of the PRB domain, such as those set forth in any of SEQ ID NOS:548-550 or 579.

In other examples of conjugates provided herein, the conjugate contains a variant ADA2 polypeptide, such as any described herein. For example, conjugates provided herein can be an ADA2 that contains the variant polypeptide set forth in any of SEQ ID NOS:13-63, 71-285 or 552-931.

The heterologous moiety can include protein or polypeptide moieties or non-polypeptide moieties. For example, the heterologous moiety can be, but is not limited to, a peptide, small molecule, nucleic acid, carbohydrate and polymer. The heterologous moiety can be linked, directly or indirectly, to the ADA2 protein molecule. For example, the heterologous moiety can be a protein or polypeptide moiety, which can be directly or indirectly conjugated to the ADA2 polypeptide, or produced as fusion proteins that are directly or indirectly fused. In other cases, the heterologous moiety is a non-peptide moiety that is conjugated to the ADA2 molecules.

The ADA2 protein can be conjugated to one or more heterologous moieties, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more heterologous moieties. A heterologous moiety can be a heterologous polypeptide moiety, or a heterologous non-polypeptide moiety, or both. In other examples, the heterologous moieties can include a combination of a heterologous polypeptide and a non-polypeptide moiety. In some examples, all the heterologous moieties are identical. In some examples, at least one heterologous moiety is different from the other heterologous moieties. In some examples, any ADA2 provided herein can be conjugated to two, three or more than three heterologous moieties in tandem. In other examples, any ADA2 provided herein can be conjugated to two, three, or more than three heterologous moieties wherein at least an additional moiety is interposed between two heterologous moieties (*e.g.*, an ADA2 polypeptide, a linker, a protease-cleavable substrate, a self-immolative spacer, or combinations thereof).

Conjugation with heterologous moieties can confer beneficial properties compared to an ADA2 molecule that is not conjugated with the heterologous moiety. Exemplary heterologous moieties are moieties that increase the *in vivo* half-life of the molecule. Other exemplary beneficial properties provided by a heterologous moiety include, but are not limited to, increased protein expression in mammalian expression systems, improved biophysical properties such as stability and solubility, improved

protein purification and detection, visualization and localization and/or increased enzymatic activity. For example, a heterologous moiety can be one that facilitates detection, visualization or localization of an ADA2 protein molecule or a fragment thereof containing the heterologous moiety. Detection, visualization and/or location of any ADA2 fragment thereof can be *in vivo, in vitro, ex vivo*, or combinations thereof.

In some cases, when conjugated to an ADA2 or fragment thereof, the heterologous moiety increases stability of the ADA2 or a fragment thereof. For example, the presence of the heterologous moiety can maintain one or more physical properties of an ADA2 in response to an environmental condition (*e.g.*, an elevated temperature or low or high pH conditions) compared to the physical property in the absence of the heterologous moiety. In some examples, the physical property can include maintenance of the covalent structure of an ADA2 (*e.g.*, the absence of proteolytic cleavage, unwanted oxidation or deamidation). In other examples, the physical property can be the maintenance of a properly folded state (*e.g.*, the absence of soluble or insoluble aggregates or precipitates). The stability of any ADA2 or ADA2 conjugate can be measured by assaying a biophysical property of the protein, for example thermal stability, pH unfolding profile, stable removal of glycosylation, solubility, biochemical function (*e.g.* adenosine deaminase activity or heparin binding activity) and/or combinations thereof. Stability can be measured using methods known in the art, such as, HPLC (high performance liquid chromatography), SEC (size exclusion chromatography), DLS (dynamic light scattering). Methods to measure thermal stability include, but are not limited to differential scanning calorimetry (DSC), differential scanning fluorimetry (DSF), circular dichroism (CD), and thermal challenge assay. Exemplary methods to assess the stability of any ADA2 or conjugate are described below in Section F.

In some examples, when conjugated to an ADA2 or fragment thereof, the presence of the heterologous moiety reduces or attenuates binding of ADA2 to heparin and other glycosaminoglycans (GAGs) compared to the ADA2 protein not containing the heterologous moiety (i.e. the free or non-conjugated ADA2). For example, ADA2 conjugates provided herein include those that exhibit no more than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%,

45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% of the heparin binding of the ADA2 protein not containing the heterologous moiety (i.e. the free or non-conjugated ADA2). For example, it is shown herein that PEGylated ADA2 exhibits reduced heparin binding compared to the corresponding non-PEGylated ADA2 (see *e.g.* Example 8). Typically, the heparin binding is exhibited when the ADA2 is in dimer form, and the ADA2 conjugate is a dimer. It also is understood the comparison of binding between the conjugated and non-conjugated form is assessed under the same or substantially the same conditions. In particular, the reduction in binding in the presence of the heterologous moiety in the conjugate can be due to steric blockage and/or alteration of electrostatic charges on the surface.

In examples of conjugates provided herein, the heterologous moiety improves one or more properties of the ADA2 (*e.g.* half-life) without substantially affecting the biological activity or function of the ADA2 protein (*e.g.*, adenosine deaminase activity). For example, ADA2 conjugates provided herein exhibit about 50% to 500%, such as about 50% to 400%, 50% to 300%, 50% to 200%, 50% to 150%, 50% to 100%, 50% to 80%, 80% to 400%, 80% to 300%, 80% to 200%, 80% to 150%, 80% to 100%, 100% to 400%, 100% to 300%, 100% to 200% or 100% to 150% of the adenosine deaminase activity compared to the ADA2 protein not containing the heterologous moiety (i.e. the free or non-conjugated ADA2). For example, the ADA2 conjugate can exhibit at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 100%, 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 210%, 220%, 230%, 240%, 250%, 260%, 270%, 280%, 290%, 300%, 350%, 400%, 450%, 500% or more of the adenosine deaminase activity compared to the ADA2 polypeptide not containing the heterologous moiety (i.e. the free or non-conjugated ADA2). In some cases, ADA2 conjugates provided herein exhibit increased or improved adenosine deaminase activity compared to the ADA2 polypeptide not containing the heterologous moiety (i.e. the free or non-conjugated ADA2), such as greater than 100% or more adenosine deaminase activity. Typically, the adenosine deaminase activity is exhibited when the ADA2 is in dimer form, and the ADA2 conjugate is a dimer. It also is understood the comparison of adenosine binding between the conjugated and non-conjugated form is assessed under the same or substantially the same conditions.

Date Reçue/Date Received 2021-07-28

1. Half-Life Extending Moieties

Non-limiting examples of heterologous moieties include any that, when conjugated or linked (directly or indirectly) to the ADA2 molecule, confers an increase in the *in vivo* and/or *in vitro* half-life compared to the free- or non-conjugated ADA2. Half-life of any ADA2 provided herein can be determined by any method known to those of skill in the art and/or described herein, *e.g.*, adenosine deaminase activity assays. Exemplary of such half-life extending moieties are described in the following subsections.

For example, the heterologous moieties are peptides and polypeptides with either unstructured or structured characteristics that are associated with the prolongation of *in vivo* half-life when conjugated to an ADA2. Non-limiting examples include albumin, albumin fragments, Fc fragments of immunoglobulins, the β subunit of the C-terminal peptide (CTP) of the β subunit of human chorionic gonadotropin, HAP sequences, XTEN sequences, a transferrin or a fragment thereof, a PAS polypeptide, polyglycine linkers, polyserine linkers, albumin-binding moieties, non-natural amino acid based conjugation or half-life extension, or any fragments, derivatives, variants, or combinations of these polypeptides.

The heterologous moiety can be a half-life extending moieity, *i.e.*, a heterologous moiety that increases the *in vivo* half-life of any ADA2 provided herein compared to the *in vivo* half-life of the ADA2 lacking such heterologous moiety. *In vivo* half-life of any ADA2 provided herein can be determined by any method known to those of skill in the art and/or described herein, *e.g.*, adenosine deaminase activity assays.

Exemplary half-life extending moieties that can be conjugated, directly or indirectly, to any ADA2 provided herein include: biocompatible fatty acids and derivatives thereof, hydroxy alkyl starch (HAS) (*e.g.* hydroxy ethyl starch (HES)), polyethylene glycol (PEG), Poly $(Gly_x\text{- }Ser_y)_n$, homo-amino-acid polymers (HAP), hyaluronic acid (HA), heparosan polymers (HEP), phosphorylcholine-based polymers (PC polymer), Fleximers, dextran, polysialic acids (PSA), Fc domain, Transferrin, Albumin, elastin-like peptides, XTEN sequences, albumin binding peptides, a CTP peptide, a non-natural amino acid or non-natural amino acid conjugate, and any combination thereof.

In some example, when conjugated to an ADA2 or fragment thereof, the presence of one or more half-life extending moieties results in the half-life of any ADA2 provided herein to be increased compared to the half-life of the ADA2 lacking such one or more half-life extending moieties (i.e. the free or non-conjugated ADA2). For example, ADA2 conjugates provided herein exhibit a half-life that is at least about 110%, 120%, 130%, 140%, 150%, 160%, 170%, 180%, 190%, 200%, 225%, 250%, 300%, 350%, 400%, 450%, 500%, 600%, 700%, 800% or more longer than the half-life of ADA2 polypeptide not containing the heterologous moiety (i.e. the free or non-conjugated ADA2), or 900%, 1000%, 1100%, 1200%, 1300%, 1400%, 1500%, 1600%, 1700%, 1800%, 1900%, 2000%, 3000%, 4000%, 5000%, 6000%, 7000%, 8000%, 9000%, 10000%, or more longer than the half-life of ADA2 polypeptide not containing the heterologous moiety (i.e. the free or non-conjugated ADA2). In some examples, the half-life of any ADA2 conjugate provided herein that is linked, directly or indirectly, to a half- life extending moiety exhibits a half-life that is about 1.5-fold to about 20-fold, about 1.5-fold to about 15-fold, about 1.5-fold to about 10-fold longer, about 2-fold to about 10-fold, about 2-fold to about 9-fold, about 2-fold to about 8-fold, about 2-fold to about 7-fold, about 2-fold to about 6-fold, about 2-fold to about 5- fold, about 2-fold to about 4-fold, about 2-fold to about 3-fold, about 2.5-fold to about 10- fold, about 2.5-fold to about 9-fold, about 2.5-fold to about 8-fold, about 2.5-fold to about 7-fold, about 2.5-fold to about 6-fold, about 2.5-fold to about 5-fold, about 2.5-fold to about 4-fold, about 2.5-fold to about 3-fold, about 3-fold to about 10-fold, about 3-fold to about 9-fold, about 3-fold to about 8-fold, about 3-fold to about 7-fold, about 3-fold to about 6-fold, about 3-fold to about 5-fold, about 3-fold to about 4-fold, about 4-fold to about 6 fold, about 5-fold to about 7-fold, or about 6-fold to about 8-fold longer than the half-life of the corresponding ADA2 lacking such half-life extending moiety. Typically, the half-life for activity *in vivo* is exhibited when the ADA2 is in dimer form, and the ADA2 conjugate is a dimer. It also is understood the comparison of half-life between the conjugated and non-conjugated form is assessed under the same or substantially the same conditions.

In some examples, the half-life of any ADA2 conjugate provided herein that is linked, directly or indirectly, to a half- life extending moiety can be or is at least or at least about 10 hours, 11 hours, 12 hours, 13 hours, 14 hours, 15 hours, 16 hours, 17

hours, 18 hours, 19 hours, 20 hours, 21 hours, 22 hours, 23 hours, 24 hours, 25 hours, 26 hours, 27 hours, 28 hours, 29 hours, 30 hours, 32 hours, 34 hours, 36 hours, 38 hours, 40 hours, 42 hours, 46 hours, 48 hours, 50 hours, 55 hours, 60 hours, 65 hours, 70 hours, 75 hours, 80 hours or more. For example, the half-life of any ADA2 conjugate provided herein can be 10 hours to 60 hours, such as 12 hours to 48 hours or 13 hours to 36 hours. For example, Example 9 shows that an ADA2 conjugate that is a PEGylated ADA2 exhibits a half-life of about or approximately 12 to 14 hours, and PEGylated variant ADA2 molecules (*e.g.* R20E or K371D) exhibit an even greater half-life of about or approximately 16 hours to 24 hours. Example 14 shows that other PEGylated variant ADA2 molecules (*e.g.* R219Q/S262N) exhibit an even greater half-life of about or approximately 39 hours to 47 hours.

The following sub-sections describe exemplary half-life extending moieties in the ADA2 conjugates provided herein.

a. Low Complexity Polypeptides

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to at least one heterologous moiety that is a polypeptide with low compositional and/or structural complexity (*e.g.*, a disordered polypeptide with no secondary or tertiary structure in solution under physiologic conditions). In one example, the low complexity polypeptide sequences are made of unstructured, hydrophilic amino acid polymers. The low complexity polypeptides can provide beneficial properties, for example, if the protein is subjected to higher temperature or harsh conditions, such as HPLC purification.

b. C-terminal peptide (CTP) of the β Subunit of Human Chorionic Gonadotropin

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to a heterologous moiety that includes one C-terminal peptide (CTP) of the β subunit of human chorionic gonadotropin, or fragment, variant, or derivative thereof. One or more CTP peptides inserted into a recombinant protein is known to increase the *in vivo* half-life of that protein (see, *e.g.*, U.S. Patent No. 5,712,122). Exemplary CTP peptides include DPRFQDSSSSKAPPPSLPSPSRLPGPSDTPIL (SEQ ID NO:303) or

Date Reçue/Date Received 2021-07-28

SSSSKAPPPSLPSPSRLPGPSDTPILPQ. (SEQ ID NO:304) (See, *e.g.*, U.S. Patent Publication No. US 2009/0087411).

c. Immunoglobulin Constant Region (Fc) or Portions Thereof

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to an Fc domain or variant thereof. Fc domains, fragments, variants, and derivatives are known to one of skill in the art and are described, *e.g.*, in U.S. Patent No. 5,457,035; U.S. Patent Publication No. US 2006/0024298, International PCT Publication Nos. WO 2011/069164, WO 2012/006623, WO 2012/006635, or WO 2012/006633. Preparations of fusion proteins containing polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, see *e.g.*, Ashkenazi *et al.* (1991) *PNAS* 88: 10535; Byrn *et al.* (1990) *Nature*, 344:667; and Hollenbaugh and Aruffo, (2002) "Construction of Immunoglobulin Fusion Proteins," in *Current Protocols in Immunology*, Ch. 10, pp. 10.19.1-10.19.11.

An Fc region has domains denoted C_H (constant heavy) domains (C_H1, C_H2, C_H3 (optionally C_H4)). Depending on the isotype, (*i.e.* IgG, IgM, IgA IgD or IgE), the Fc region can have three or four CH domains. Some isotypes (*e.g.* IgG) Fc regions also contain a hinge region (see Janeway *et al.* 2001, Immunobiology, Garland Publishing, N.Y., N.Y). In humans, there are five antibody isotypes classified based on their heavy chains denoted as delta (δ), gamma (γ), mu (μ), and alpha (α) and epsilon (ε), giving rise to the IgD, IgG, IgM, IgA, and IgE classes of antibodies, respectively. The IgA and IgG classes contain the subclasses IgA1, IgA2, IgG1, IgG2, IgG3, and IgG4. Sequence differences between immunoglobulin heavy chains cause the various isotypes to differ in, for example, the number of C domains, the presence of a hinge region, and the number and location of interchain disulfide bonds. For example, IgM and IgE heavy chains contain an extra C domain (C_H4), that replaces the hinge region. The Fc regions of IgG, IgD, and IgA pair with each other through their Cγ3, Cδ3, and Cα3 domains, whereas the Fc regions of IgM and IgE dimerize through their Cμ4 and Cε4 domains. IgM and IgA form multimeric structures with ten and four antigen-binding sites, respectively.

Fc regions are known to a skilled artisan, and any can be used in the conjugates provided herein so long as the resulting conjugate retains adenosine deaminase activity. An Fc region or a portion thereof for producing any ADA2 provided herein can be obtained from a number of different sources. In some examples, an Fc region or a portion thereof is derived from a human immunoglobulin. The Fc region or a portion thereof can also be derived from an immunoglobulin of another mammalian species, including for example, a rodent (*e.g.* a mouse, rat, rabbit, guinea pig) or non-human primate (*e.g.* chimpanzee, macaque) species. Moreover, the Fc region or a portion thereof can be derived from any immunoglobulin class, including IgG (including human subclasses IgG1, IgG2, IgG3, or IgG4), IgA (including human subclasses IgA1 and IgA2), IgD, IgE, and IgM. In one example, the human isotype IgG1 is used. An ADA2 provided herein that is conjugated to an Fc region of an immunoglobulin can confer several desirable properties including increased stability, increased serum half-life (see Capon *et al.* (1989) Nature 337:525) as well as binding to Fc receptors such as the neonatal Fc receptor (FcRn) (U.S. Pat. Nos. 6,086,875, 6,485,726, 6,030,613; WO 03/077834; US 2003/0235536). In other examples, where effector functions mediated by the Fc-Fc receptor (FcR) interactions are to be minimized, fusion with IgG isotypes that poorly recruit complement or effector cells, such as for example, the Fc of IgG2 or IgG4, is contemplated. Further, linkers can be used to covalently link Fc to another polypeptide to generate a Fc chimera.

Exemplary sequences of heavy chain constant regions for human IgG sub-types are set forth in SEQ ID NO:355 (IgG1), SEQ ID NO:356 (IgG2), SEQ ID NO:357 (IgG3), and SEQ ID NO:358 (IgG4). For example, for the exemplary heavy chain constant region set forth in SEQ ID NO:355, the C_H1 domain corresponds to amino acids 1-98, the hinge region corresponds to amino acids 99-110, the C_H2 domain corresponds to amino acids 111-223, and the C_H3 domain corresponds to amino acids 224-330.

Modified Fc domains also are contemplated herein for conjugation to any ADA2 provided herein. In some examples, the Fc region is modified such that it exhibits altered binding to an FcR to result in altered (*i.e.* more or less) effector function compared to the effector function of an Fc region of a wild-type immunoglobulin

heavy chain. Thus, a modified Fc domain can have altered affinity, including but not limited to, increased or low or no affinity for the Fc receptor. For example, the different IgG subclasses have different affinities for the Fcγ receptors (FcγRs), with IgG1 and IgG3 typically binding substantially better to the receptors than IgG2 and IgG4. In addition, different FcγRs mediate different effector functions. FcγR1, FcγRIIa/c, and FcγRIIIa are positive regulators of immune complex triggered activation, characterized by having an intracellular domain that has an immunoreceptor tyrosine-based activation motif (ITAM). FcγRIIb, however, has an immunoreceptor tyrosine-based inhibition motif (ITIM) and is therefore inhibitory. In some instances, an ADA2 conjugate including an Fc domain provided herein can be modified to enhance binding to the complement protein C1q.

In certain examples, Fc region for conjugation to any ADA2 provided herein can include one or more truncated Fc regions that are nonetheless sufficient to confer Fc receptor (FcR) binding properties to the Fc region. For example, the portion of an Fc region that binds to FcRn (*i.e.*, the FcRn binding portion) can include from about amino acids 282-438 of IgG1, with the primary contact sites being amino acids 248, 250-257, 272, 285, 288, 290-291, 308-311, and 314 of the C_H2 domain and amino acid residues 385-387, 428, and 433-436 of the C_H3 domain (amino acid numbering based on the EU numbering system; see Edelman *et al.* (1969) PNAS 63:78-85 and Kabat *et al.* (1991) *Sequences of Proteins of Immunological Interest*, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Thus, an Fc region in any ADA2 provided herein can include an FcRn binding portion. FcRn binding portions can be derived from heavy chains of any isotype, including IgG1, IgG2, IgG3 and IgG4. Altering the affinity of an Fc region for a receptor can modulate the effector functions and/or pharmacokinetic properties associated by the Fc domain. Modified Fc domains are known to one of skill in the art and described in the literature, see *e.g.* U.S. Patent No. 5,457,035; U.S. Patent Publication No. US 2006/0024298; and International Patent Publication No. WO 2005/063816 for exemplary modifications.

In certain examples, an Fc region for conjugation to any ADA2 provided herein can include at least one of: a hinge (*e.g.*, upper, middle, and/or lower hinge region) domain (about amino acids 216-230 of an antibody Fc region based on EU

numbering), a C_H2 domain (about amino acids 231-340 of an antibody Fc region based on EU numbering), a C_H3 domain (about amino acids 341-438 of an antibody Fc region based on EU numbering), a C_H4 domain, or a variant, portion, or fragment thereof. In other examples, an Fc region can include a complete Fc domain (*i.e.*, a hinge domain, a C_H2 domain, and a C_H3 domain). In some examples, an Fc region can include, a hinge domain (or a portion thereof) fused to a C_H3 domain (or a portion thereof), a hinge domain (or a portion thereof) fused to a C_H2 domain (or a portion thereof), a C_H2 domain (or a portion thereof) fused to a C_H3 domain (or a portion thereof), a C_H2 domain (or a portion thereof) fused to both a hinge domain (or a portion thereof) and a C_H domain (or a portion thereof). In still other examples, an Fc region lacks at least a portion of a C_H2 domain (*e.g.*, all or part of a C_H2 domain). In a particular example, an Fc region can include amino acids corresponding 221 to 447 (based on the EU numbering system; see Edelman *et al.* (1969) PNAS 63:78-85 and Kabat *et al.* (1991) *Sequences of Proteins of Immunological Interest*, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242)

An Fc region for conjugation to any ADA2 provided herein can include, for example, a modification (*e.g.,* an amino acid substitution) at one or more of the amino acid positions disclosed in International. PCT Pub. Nos. WO88/07089, W096/14339, WO98/05787, W098/23289, W099/51642, W099/58572, WO00/09560, WO00/32767, WO00/42072, WO02/44215, WO02/060919, WO03/074569, WO04/016750, WO04/029207, WO04/035752, WO04/063351, WO04/074455, WO04/099249, WO05/040217, WO04/044859, WO05/070963, WO05/077981, WO05/092925, WO05/123780, WO06/019447, WO06/047350, and WO06/085967; U.S. Pat. Publ. Nos. US 2007/0231329, US2007/0231329, US2007/0237765, US2007/0237766, US2007/0237767, US2007/0243188, US2007/0248603, US2007/0286859, US2008/0057056; or U.S. Pat. Nos. 5,648,260; 5,739,277; 5,834,250; 5,869,046; 6,096,871 ; 6,121,022; 6,194,551; 6,242,195; 6,277,375; 6,528,624; 6,538,124; 6,737,056; 6,821,505; 6,998,253; 7,083,784; 7,404,956; and 7,317,091. In one example, the specific modification (*e.g.*, the specific substitution of one or more amino acids disclosed in the art) can be made at one or more of the disclosed amino acid positions. In another example, a different change at one or more of the disclosed

amino acid positions (*e.g.*, the different substitution of one or more amino acid position disclosed in the art) can be made.

In some examples, any ADA2 provided herein can be conjugated to at least one Fc region as a fusion protein. Typically, such a fusion retains at least a functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. For example, a full-length Fc sequence of IgG1 includes amino acids 99-330 of the sequence set forth in SEQ ID NO:355. An exemplary Fc sequence for hIgG1 is set forth in SEQ ID NO:359, and contains almost all of the hinge sequence, and the complete sequence for the C_H2 and C_H3 domain as set forth in SEQ ID NO:355. Another exemplary Fc polypeptide is the Fc polypeptide set forth in SEQ ID NO:361. Another exemplary Fc polypeptide is set forth in PCT Pub. No. WO 93/10151, and is a single chain polypeptide extending from the N-terminal hinge region to the native C-terminus of the Fc region of a human IgG1 antibody (SEQ ID NO:359). The precise site at which the linkage is made is not critical: particular sites are well known and can be selected in order to optimize the biological activity, secretion, or binding characteristics of the ADA2 protein molecule. For example, other exemplary Fc polypeptide sequences begin at amino acid C109 or P113 of the sequence set forth in SEQ ID NO:355 (see *e.g.*, U.S. Pub. No. 2006/0024298).

An Fc region for conjugation to any ADA2 provided herein can also contain amino acid substitution which alters the glycosylation of the chimeric protein known in the art. For example, the Fc region of any ADA2 provided herein can be conjugated to an Fc region having a mutation leading to reduced glycosylation (*e.g.*, N- or O-linked glycosylation) or to an altered glycoform of the wild-type Fc moiety (*e.g.*, a low fucose or fucose-free glycan).

An Fc region for conjugation to any ADA2 provided herein also can be engineered to include a tag with metal chelates or other epitope. The tagged domain can be used for rapid purification by metal-chelate chromatography, and/or by antibodies, to allow for detection by western blots, immunoprecipitation, or activity depletion/blocking in bioassays.

Date Reçue/Date Received 2021-07-28

d. Albumin or Fragment, or Variant Thereof

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to a heterologous moiety that includes albumin or a functional fragment thereof. Human serum albumin (HSA, or HA), a protein of 609 amino acids in its full-length form (exemplary sequence set forth in SEQ ID NO:305), is responsible for a significant proportion of the osmotic pressure of serum and also functions as a carrier of endogenous and exogenous ligands. Albumin can be a full-length albumin or a functional fragment, variant, derivative, or analog thereof. Examples of albumin or the fragments or variants thereof are disclosed in US Pat. Publ. Nos. 2008/0194481, 2008/0004206, 2008/0161243, 2008/0261877, or 2008/0153751 or PCT Publ. Nos. 2008/033413, 2009/058322, or 2007/021494.

In some examples, any ADA2 provided herein can include albumin, a fragment, or a variant thereof which is further linked to a heterologous moiety selected from an immunoglobulin constant region or portion thereof (*e.g.*, an Fc region), a PAS sequence, HES, XTEN sequences, PEG, or any combinations thereof.

e. Albumin Binding Moiety

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to a heterologous moiety that is an albumin binding moiety, for example, an albumin binding peptide, a bacterial albumin binding domain, an albumin-binding antibody fragment, a fatty acid, or any combinations thereof.

For example, the albumin binding protein can be a bacterial albumin binding protein, an antibody or an antibody fragment including domain antibodies (see U.S. Pat. No. 6,696,245). An albumin binding protein, for example, can be a bacterial albumin binding domain, such as the one of Streptococcal protein G (Konig, T. and A. Skerra, A. (1998) J Immunol. Methods 218:73-83). Other examples of albumin binding peptides that can be used to conjugate to any ADA2 provided herein are, for instance, those having a Cys-Xaa_1-Xaa_2-Xaa_3 -Xaa_4-Cys consensus sequence (SEQ ID NO:306), wherein Xaa_1 is Asp, Asn, Ser, Thr, or Trp; Xaa_2 is Asn, Gln, His, Ile, Leu, or Lys; Xaa_3 is Ala, Asp, Phe, Trp, or Tyr; and Xaa_4 is Asp, Gly, Leu, Phe, Ser, or Thr (US Patent Pub. No. 2003/0069395; Dennis *et al.* (2002) J. Biol. Chem. 277: 35035-35043).

Domain 3 from Streptococcal protein G (Kraulis *et al*, (1996) FEBS Lett. 378:190-194; Linhult *et al.* (2002) Protein Sci. 11:206-213) is an example of a bacterial albumin-binding domain. Examples of albumin-binding peptides include a series of peptides having the core sequence DICLPRWGCLW (SEQ ID NO:307). (See, *e.g.*, Dennis *et al.* (2002) J. Biol. Chem. 277: 35035-35043). Other examples of albumin-binding peptides include: RLIEDICLPRWGCLWEDD (SEQ ID NO:308); QRLMEDICLPRWGCLWEDDF (SEQ ID NO:309); QGLIGDICLPRWGCLWGDSVK (SEQ ID NO:310), and GEWWEDICLPRWGCLWEEED (SEQ ID NO:311).

Examples of albumin-binding antibody fragments that can be conjugated to any ADA2 provided herein include those disclosed in Muller and Kontermann, Curr. Opin. Mol. Ther. (2007) 9:319-326; Roovers *et al.* (2007), Cancer Immunol. Immunother. 56:303-317; Holt *et al.* (2008) Prot. Eng. Design Sci., 21 :283-288. An example of such albumin binding moiety is the 2-(3-maleimidopropanamido)-6-(4-(4-iodophenyl)butanamido) hexanoate ("Albu" tag) (Trussel *et al.* (2009) Bioconjugate Chem. 20:2286-2292).

Fatty acids, in particular long chain fatty acids (LCFA) and long chain fatty acid-like albumin-binding compounds can be used to extend the *in vivo* half-life of any ADA2 provided herein. An example of an LCFA-like albumin-binding compound is 16-(l-(3-(9-(((2,5-dioxopyrrolidin-l-yloxy) carbonyloxy)-methyi)-7-sulfo-9H-fluoren-2-ylamino)-3-oxopropyl)-2,5-dioxopyrrolidin-3-ylthio) hexadecanoic acid (see, *e.g.*, WO 2010/140148).

f. PAS Sequences

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to at least one heterologous moiety that is a PAS sequence, which is an amino acid sequence that includes mainly alanine and serine residues or that includes mainly alanine, serine, and proline residues. The amino acid sequences form random coil conformation under physiological conditions. Accordingly, the PAS sequence is a building block, an amino acid polymer, or a sequence cassette made of alanine, serine, and proline, which can be used as a part of the heterologous moiety conjugated to any ADA2 provided herein.

One of skilled in the art is aware that an amino acid polymer also can form a random coil conformation when residues other than alanine, serine, and proline are added as a minor constituent in the PAS sequence. Minor constituents include amino acids other than alanine, serine, and proline that can be added in the PAS sequence to a certain degree, *e.g.*, up to about 12%, *i.e.*, about 12 of 100 amino acids of the PAS sequence, up to about 10%, *i.e.* about 10 of 100 amino acids of the PAS sequence, up to about 9%, *i.e.*, about 9 of 100 amino acids, up to about 8%, *i.e.*, about 8 of 100 amino acids, about 6%, *i.e.*, about 6 of 100 amino acids, about 5%, *i.e.*, about 5 of 100 amino acids, about 4%, *i.e.*, about 4 of 100 amino acids, about 3%, *i.e.*, about 3 of 100 amino acids, about 2%, *i.e.*, about 2 of 100 amino acids, or about 1%, *i.e.*, about 1 of 100 of the amino acids. The amino acids different from alanine, serine and proline can be selected from Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Tyr, or Val.

Under physiological conditions, the PAS sequence stretch forms a random coil conformation and thereby can mediate an increased *in vivo* and/or in vitro stability to any ADA2 provided herein. Since the random coil domain does not adopt a stable structure or function by itself, the biological activity mediated by any ADA2 provided herein is essentially preserved. In other examples, the PAS sequences that form random coil domains are biologically inert, especially with respect to proteolysis in blood plasma, immunogenicity, isoelectric point/electrostatic behavior, binding to cell surface receptors or internalization, but are still biodegradable, which provides clear advantages over synthetic polymers such as PEG.

Non-limiting examples of the PAS sequences forming random coil conformation include an amino acid sequence such as ASPAAPAPASPAAPAPSAPA (SEQ ID NO:312), AAPASPAPAAPSAPAPAAPS (SEQ ID NO:313), APSSPSPSAPSSPSPASPSS (SEQ ID NO:314), APSSPSPSAPSSPSPASPS (SEQ ID NO:315), SSPSAPSPSSPASPSPSSPA (SEQ ID NO:316), AASPAAPSAPPAAASPAAPSAPPA (SEQ ID NO:317), ASAAAPAAASAAASAPSAAA (SEQ ID NO:318) or any combinations thereof. Additional examples of PAS sequences are known in the art (see, *e.g.*, US Pat. Publ. No. 2010/0292130 and International PCT Publ. No. WO 2008/155134)

Date Reçue/Date Received 2021-07-28

g. HAP Sequences

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to at least one heterologous moiety that is a glycine-rich homo-amino-acid polymer (HAP). The HAP sequence can include a repetitive sequence of glycine, which has at least 50 amino acids, at least 100 amino acids, 120 amino acids, 140 amino acids, 160 amino acids, 180 amino acids, 200 amino acids, 250 amino acids, 300 amino acids, 350 amino acids, 400 amino acids, 450 amino acids, or 500 amino acids in length. In one example, the HAP sequence is capable of extending half-life of a moiety fused to or linked to the HAP sequence. Non-limiting examples of the HAP sequence includes, but are not limited to $(Gly)_n$ (SEQ ID NO:368), $(Gly_4Ser)_n$ (SEQ ID NO:343) or $Ser(Gly_4Ser)_n$ (SEQ ID NO:595), wherein n is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. In one example, n is 20, 21, 22, 23, 24, 25, 26, 26, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40. In another example, n is 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, or 200.

h. XTEN Sequences

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to at least one heterologous moiety that includes an XTEN sequence, polypeptide or fragment, variant, or derivative thereof. XTEN sequence is an extended length polypeptide sequence with non-naturally occurring, substantially non-repetitive sequences that are composed mainly of small hydrophilic amino acids, with the sequence having a low degree or no secondary or tertiary structure under physiologic conditions (Schellenberger *et al.* (2009) Nat Biotechnol. 27(12):1186-1190). An exemplary XTEN sequence is an unstructured recombinant polypeptide of 864 amino acids (SEQ ID NO:373), which extends the plasma half-life of the protein fused to the moiety. As a heterologous moiety, XTEN sequences can function as a half-life extension moiety. In addition, XTEN sequences can provide desirable properties including, but are not limited to, enhanced pharmacokinetic parameters and solubility characteristics. For example, conjugation of XTEN sequences to any ADA2 provided herein can confer one or more of the following advantageous properties: conformational flexibility, enhanced aqueous solubility, high degree of protease resistance, low immunogenicity, low binding to mammalian receptors, or

increased hydrodynamic (or Stokes) radii. In some examples, an XTEN sequence can increase pharmacokinetic properties such as longer *in vivo* half-life or increased area under the curve (AUC), such that any ADA2 provided herein stays *in vivo* and retains adenosine deaminase activity for an increased period of time compared to the same ADA2 without the XTEN heterologous moiety.

Examples of XTEN sequences that can be used as heterologous moieties conjugated to any ADA2 provided herein include any of those described in U.S. Pat Nos. 7,855,279 and 7,846,445, U.S. Patent Publication Nos. 2009/0092582, 2010/0239554, 2010/0323956, 2011/0046060, 2011/0046061, 2011/0077199, 2011/0172146, 2012/0178691, 2013/0017997, or 2012/0263701, or International Patent Publication Nos. WO 2010091122, WO 2010144502, WO 2010144508, WO 2011028228, WO 2011028229, or WO 2011028344.

i. Transferrin or Fragment thereof

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to at least one heterologous moiety that is a transferrin or a fragment thereof. Any transferrin can be conjugated to any ADA2 provided herein. For example, wildtype human Tf (Tf) is a 679 amino acid protein (amino acid sequence set forth in SEQ ID NOS:320 and 324; GenBank Acc. Nos. NP_001054.1 and AAB22049.1; nucleic acid sequence set forth in SEQ ID NOS:319 and 322-323, GenBank Acc. Nos. NM001063, M12530, XM039845, and S95936), of approximately 75 kDa (excluding glycosylation), with two main domains, the N terminal domain (about 330 amino acids) and the C terminal domain (about 340 amino acids), which appear to originate from a gene duplication. The N domain includes two subdomains, N1 domain and N2 domain, and the C domain includes two subdomains, C1 domain and C2 domain.

In one example, the transferrin heterologous moiety includes a transferrin splice variant. In one example, a transferrin splice variant can be a splice variant of human transferrin (SEQ ID NO:325; Genbank Acc. No. AAA61140). In another example, the transferrin portion of the chimeric protein includes one or more domains of the transferrin sequence, *e.g.*, N domain, C domain, N1 domain, N2 domain, C1 domain, C2 domain or any combinations thereof.

j. Polymer conjugation

An ADA2 conjugate provided herein can include an ADA2 that is linked, directly or indirectly, to at least one heterologous moiety that is a polymeric molecule (polymer). Exemplary of polymers are such as polyols (*i.e.*,poly-OH), polyamines (*i.e.*,poly-NH_2) and polycarboxyl acids (*i.e.*,poly-COOH), and further heteropolymers *i.e.*,polymers containing one or more different coupling groups *e.g.*, a hydroxyl group and amine groups. Examples of suitable polymeric molecules include polymeric molecules selected from among polyalkylene oxides (PAO), such as polyalkylene glycols (PAG), including polyethylene glycols (PEG), ethylene glycol/propylene glycol copolymers, methoxypolyethylene glycols (mPEG) and polypropylene glycols, PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole (CDI-PEG) branched polyethylene glycols (PEGs), polyvinyl alcohol (PVA), polycarboxylates, polyvinylpyrrolidone, polyoxazoline, polyacryloylmorpholine, poly-D,L-amino acids, polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextrans including carboxymethyl-dextrans, heparin, homologous albumin, celluloses, including methylcellulose, carboxymethylcellulose, ethylcellulose, hydroxyethylcellulose carboxyethylcellulose and hydroxypropylcellulose, hydrolysates of chitosan, starches such as hydroxyethyl-starches and hydroxypropyl-starches, glycogen, agaroses and derivatives thereof, guar gum, pullulan, inulin, xanthan gum, carrageenan, pectin, alginic acid hydrolysates, bio-polymer, and those disclosed in the art, for example, in U.S. Patent No. 8,741,283 and International PCT Publication No. WO 2007/149686.

For example, polymer conjugated to any ADA2 provided herein can generally correspond to the following formula:

$[R—NH]_z$-(ADA2)

wherein (ADA2) represents any ADA2 described herein, such as wildtype, variants or modified forms thereof;

NH— is an amino group of an amino acid found on the ADA2 provided herein for the attachment to the polymer;

z is a positive integer, such as from about 1 to about 32, or 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-1 1, 10-12, 11-13, 12-14, 13-15, 14-16, 15-17, 16-18, 17-19, 18-

20, 19-21, 20-22, 21-23, 22-24, 23-25, 24-26, 25-27, 26-28, 27-29, 28-30, 29-31 or 30-32;

R is a substantially non-antigenic polymer molecule that is attached to the ADA2 provided herein in a releasable or non-releasable form. Exemplary non-antigenic polymeric molecule can be any described herein and those disclosed in the art, for example, in U.S. Patent No. 8,741,283 and International PCT Publication No. WO 2007/149686.

For example, any ADA2 described herein can be conjugated to least one polyethylene glycol (PEG) molecule. In some examples, the polymer can be water-soluble. In some examples, any ADA2 provided herein is conjugated to a PEG heterologous moiety and further includes a heterologous moiety selected from an immunoglobulin constant region or portion thereof (*e.g.*, an Fc region), a PAS sequence, hydroxyethyl starch (HES) and albumin or fragment or variant thereof, an XTEN sequence, or any combinations thereof.

Covalent or other stable attachment (conjugation) of polymeric molecules, such as polyethylene glycol (PEGylation moiety (PEG)), to any ADA2 polypeptide, including variant ADA2 polypeptides, impart beneficial properties to the resulting ADA2-polymer composition. Such properties include improved biocompatibility, extension of protein (and enzymatic activity) half-life in the plasma, cells and/or in other tissues within a subject, effective shielding of the protein from proteases and hydrolysis, improved biodistribution, enhanced pharmacokinetics and/or pharmacodynamics, increased stability, decreased immunogenicity, prolonged/sustained treatment effects in a subject, and increased water solubility (see U.S. Pat. No. 4,179,337).

i. Polyethylene Glycol (PEG)

Polyethylene glycol (PEG) has been widely used in biomaterials, biotechnology and medicine primarily because PEG is a biocompatible, nontoxic, water-soluble polymer that is typically nonimmunogenic (Zhao and Harris, *ACS Symposium Series* 680: 458-72, 1997). In the area of drug delivery, PEG derivatives have been widely used in covalent attachment (i. e., "PEGylation") to proteins to reduce immunogenicity, proteolysis and kidney clearance and to enhance solubility (Zalipsky, Adv. Drug Del. Rev. 16:157-82, 1995). Similarly, PEG has been attached

to low molecular weight, relatively hydrophobic drugs to enhance solubility, reduce toxicity and alter biodistribution. Typically, PEGylated drugs are injected as solutions.

A closely related application is synthesis of crosslinked degradable PEG networks or formulations for use in drug delivery since much of the same chemistry used in design of degradable, soluble drug carriers can also be used in design of degradable gels (Sawhney *et al.*, *Macromolecules* 26: 581-87, 1993). It also is known that intermacromolecular complexes can be formed by mixing solutions of two complementary polymers. Such complexes are generally stabilized by electrostatic interactions (polyanion-polycation) and/or hydrogen bonds (polyacid-polybase) between the polymers involved, and/or by hydrophobic interactions between the polymers in an aqueous surrounding (Krupers *et al.*, *Eur. Polym J.* 32:785-790, 1996). For example, mixing solutions of polyacrylic acid (PAAc) and polyethylene oxide (PEO) under the proper conditions results in the formation of complexes based mostly on hydrogen bonding. Dissociation of these complexes at physiologic conditions has been used for delivery of free drugs (*i.e.*, non-PEGylated). In addition, complexes of complementary polymers have been formed from both homopolymers and copolymers.

Numerous reagents for PEGylation have been described in the art. Such reagents include, but are not limited to, N-hydroxysuccinimidyl (NHS) activated PEG, succinimidyl mPEG, $mPEG_2$-N-hydroxysuccinimide, mPEG succinimidyl alpha-methylbutanoate, mPEG succinimidyl propionate, mPEG succinimidyl butanoate, mPEG carboxymethyl 3-hydroxybutanoic acid succinimidyl ester, homobifunctional PEG-succinimidyl propionate, homobifunctional PEG propionaldehyde, homobifunctional PEG butyraldehyde, PEG maleimide, PEG hydrazide, p-nitrophenyl-carbonate PEG, mPEG-benzotriazole carbonate, propionaldehyde PEG, mPEG butryaldehyde, branched $mPEG_2$ butyraldehyde, mPEG acetyl, mPEG piperidone, mPEG methylketone, mPEG "linkerless" maleimide, mPEG vinyl sulfone, mPEG thiol, mPEG orthopyridylthioester, mPEG orthopyridyl disulfide, Fmoc-PEG-NHS, Boc-PEG-NHS, vinylsulfone PEG-NHS, acrylate PEG-NHS, fluorescein PEG-NHS, and biotin PEG-NHS (see *e.g.*, Monfardini *et al.*, *Bioconjugate Chem.* 6:62-69, 1995; Veronese *et al.*, *J. Bioactive Compatible Polymers* 12:197-207,

1997; US 5,672,662; US 5,932,462; US 6,495,659; US 6,737,505; US 4,002,531; US 4,179,337; US 5,122,614; US 5,324,844; US 5,446,090; US 5,612,460; US 5,643,575; US 5,766,581; US 5,795,569; US 5,808,096; US 5,900,461; US 5,919,455; US 5,985,263; US 5,990,237; US 6,113,906; US 6,214,966; US 6,258,351; US 6,340,742; US 6,413,507; US 6,420,339; US 6,437,025; US 6,448,369; US 6,461,802; US 6,828,401; US 6,858,736; US 8,741,283; US 2001/0021763; US 2001/0044526; US 2001/0046481; US 2002/0052430; US 2002/0072573; US 2002/0156047; US 2003/0114647; US 2003/0143596; US 2003/0158333; US 2003/0220447; US 2004/0013637; US 2004/0235734; WO 05000360; US 2005/0114037; US 2005/0171328; US 2005/0209416; EP 1064951; EP 0822199; WO 01076640; WO 0002017; WO 0249673; WO 94/28024; and WO 01/87925).

In particular, the polymer is a polyethylene glycol (PEG). Suitable polymeric molecules for attachment to any ADA2 polypeptide, including variant ADA2 polypeptides, include, but are not limited to, polyethylene glycol (PEG) and PEG derivatives such as methoxy-polyethylene glycols (mPEG), PEG-glycidyl ethers (Epox-PEG), PEG-oxycarbonylimidazole (CDI-PEG), branched PEGs, and polyethylene oxide (PEO) (see *e.g.,* Roberts *et al.*, *Advanced Drug Delivery Review* (2002) 54: 459-476; Harris and Zalipsky, S (eds.) "Poly(ethylene glycol), Chemistry and Biological Applications" ACS Symposium Series 680, 1997; Mehvar *et al.*, *J. Pharm. Pharmaceut. Sci.*, 3(1):125-136, 2000; Harris, (2003) *Nature Reviews Drug Discovery* 2:214-221; and Tsubery, (2004) *J Biol. Chem* 279(37):38118-24).

The polymeric moiety, such as the PEG moiety, can be of a molecular weight typically ranging from about 1 kDa to about 100 kDa. In some embodiments the polymeric molecule that is conjugated to a protein, such as any ADA2 provided herein, has a molecular weight of at least or at least about or 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 kDa or more than 1000 kDa. Other sizes can be used, depending on the desired profile (*e.g.*, the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a protein or analog).

The PEG moiety can be of any molecular weight, and can be branched or unbranched. In some examples, the heterologous polymer is a PEG with a branched

structure. Branched polyethylene glycols are described, for example, in U.S. Pat. No. 5,643,575; Morpurgo *et al.* (1996) Appl. Biochem. Biotechnol. 56:59-72; Vorobjev *et al.* (1999) Nucleosides Nucleotides 18:2745-2750; and Caliceti *et al.* (1999) Bioconjug. Chem. 10:638-646.

While numerous reactions have been described for PEGylation, those that are most generally applicable confer directionality, utilize mild reaction conditions, and do not necessitate extensive downstream processing to remove toxic catalysts or byproducts. For instance, monomethoxy PEG (mPEG) has only one reactive terminal hydroxyl, and thus its use limits some of the heterogeneity of the resulting PEG-protein product mixture. Activation of the hydroxyl group at the end of the polymer opposite to the terminal methoxy group is generally necessary to accomplish efficient protein PEGylation, with the aim being to make the derivatised PEG more susceptible to nucleophilic attack. The attacking nucleophile is usually the epsilon-amino group of a lysine residue, but other amines also can react (*e.g.* the N-terminal alpha-amine or the ring amines of histidine) if local conditions are favorable.

A more directed attachment is possible in proteins containing a single lysine or cysteine. The latter residue can be targeted by PEG-maleimide for thiol-specific modification. Alternatively, PEG hydrazide can be reacted with a periodate oxidized protein and reduced in the presence of $NaCNBH_3$. More specifically, PEGylated CMP sugars can be reacted with a protein in the presence of appropriate glycosyl-transferases. Alternatively, pegyaltion of ADA2 can occur in variants containing substitutions with non-natural amino acids that allow for site-specific chemical conjugation at optimized positions within the protein. PEGylation techniques can allow where a number of polymeric molecules are coupled to the polypeptide in question. When using this technique the immune system has difficulties in recognizing the epitopes on the polypeptide's surface responsible for the formation of antibodies, thereby reducing the immune response. For polypeptides introduced directly into the circulatory system of the human body to give a particular physiological effect (*i.e.* pharmaceuticals) the typical potential immune response is an IgG and/or IgM response, while polypeptides which are inhaled through the respiratory system (*i.e.* industrial polypeptide) potentially can cause an IgE response

(*i.e.* allergic response). One of the theories explaining the reduced immune response is that the polymeric molecule(s) shield(s) epitope(s) on the surface of the polypeptide responsible for the immune response leading to antibody formation. Another theory or at least a partial factor is that the heavier the conjugate is, the more reduced immune response is obtained.

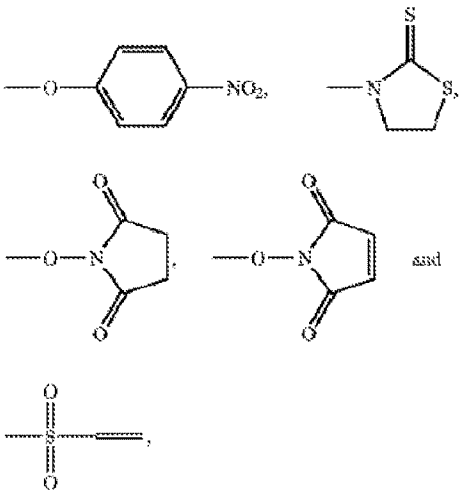
Typically, to make the PEGylated ADA2 polypeptides provided herein, including variant ADA2 polypeptides, PEG moieties are conjugated, via covalent attachment, to the polypeptides. Techniques for PEGylation include, but are not limited to, specialized linkers and coupling chemistries (see *e.g.*, Roberts *et al.*, *Adv. Drug Deliv. Rev.* 54:459-476, 2002), attachment of multiple PEG moieties to a single conjugation site (such as via use of branched PEGs; see *e.g.*, Guiotto *et al.*, *Bioorg. Med. Chem. Lett.* 12:177-180, 2002), site-specific PEGylation and/or mono-PEGylation (see *e.g.*, Chapman *et al.*, *Nature Biotech.* 17:780-783, 1999), and site-directed enzymatic PEGylation (see *e.g.*, Sato, *Adv. Drug Deliv. Rev.*, 54:487-504, 2002). Methods and techniques described in the art can produce proteins having at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more than 10 PEG or PEG derivatives attached to a single protein molecule (see *e.g.*, U.S. 2006/0104968).

The number of polyethylene glycol moieties attached to each ADA2 molecule can also vary. For example, any ADA2 provided herein can be conjugated to, on average, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15, 17, 20, 25, 30 or more polyethylene glycol molecules. For example, the PEGylated ADA2 polypeptides, including variant ADA2 polypeptides generally contains at least 5 PEG moieties per molecule. In other examples, the range of number of PEG molecules per protein molecule can be 1-3, 2-4, 3-5, 4-6, 5-7, 6-8, 7-9, 8-10, 9-1 1, 10-12, 11-13, 12-14, 13-15, 14-16, 15-17, 16-18, 17-19, 18-20, 19-21, 20-22, 21-23, 22-24, 23-25, 24-26, 25-27, 26-28, 27-29, 28-30, 29-31 or 30-32. For example, the ADA2 polypeptides, including variant ADA2 polypeptides can have a PEG to protein molar ratio between 32:1 and 1:1, such as about or up to 30:1, 20:1, 15:1, 10:1 and 5:1. The number of PEG molecules per protein can be varied in order to modify the physical and kinetic properties of the combined conjugate to fit any particular clinical situation, as determined by one of skill in the art. Methods for determining the PEG to protein molar ratio is disclosed in

the art, for example, in Delgado *et al.* (1992) Crit. Rev. Thera. Drug Carrier Sys. 9:249-304).

Covalent attachment of the PEG to the drug (known as "PEGylation") can be accomplished by known chemical synthesis techniques. For example, the PEGylation of protein can be accomplished by reacting NHS-activated PEG with the protein under suitable reaction conditions. Various methods of modifying polypeptides by covalently attaching (conjugating) a PEG or PEG derivative (*i.e.,* "PEGylation") are known in the art (see *e.g.*, U.S. 5,672,662; U.S. 6,737,505; U.S. 2004/0235734; U.S. 2006/0104968). Covalent attachment of a variety of polymers, such as PEG or PEG derivatives, is described in U.S. 8,741,283.

Activated polymers and derivatives can be employed to facilitate the conjugation of the polymer to any ADA2 provided herein. Activated polymers and derivatives have a leaving or activating group, which facilitates the attachment of the polymer system to an amine group found on the polypeptide, such as an ADA2 provided herein. For example, activated groups are those groups which are capable of reacting with an amine group (nucleophile) found on any ADA2 provided herein, such as the epsilone amine group of lysine. Exemplary activating groups include:



or other suitable leaving or activating groups such as N-hydroxybenzotriazolyl, halogen, N-hydroxyphthalimidyl, imidazolyl, O-acyl ureas, pentafluorophenol, 2,4,6-tri-chlorophenol or other suitable leaving groups apparent to one of skill in the art.

Exemplary activated PEGs include, for example, those disclosed in the art, such as in U.S. Pat. Nos. 5,122,614, 5,324,844, 5,612,460 and 5,808,096 (succinimidyl carbonate-activated polyethylene glycol (SC-PEG) and related activated PEGs), and U.S. Pat. No. 5,349,001 (cyclic imide thione activated PEGs). Conjugation reactions typically are carried out in a suitable buffer using a several-fold molar excess of activated PEG. In some examples, conjugates made with linear PEGs, such as SC-PEG, can contain, on average, from about 1 to about 32 PEG molecules per protein molecule. Consequently, for these, molar excesses of several hundred fold, *e.g.*, about 200 to about 1000-fold can be employed. The molar excess used for branched polymers and polymers attached to the enzyme will be lower and can be determined using the techniques known in the art.

In some examples, the activated polymer linkers of the polymeric systems based on benzyl elimination or trimethyl lock lactonization, as described in U.S. Pat. Nos. 6,180,095, 6,720,306, 5,965,119, 6,624,142 and 6,303,569. In other examples, polymer conjugation of any ADA2 provided herein can be achieved using bicine polymer residues, as described in the art, for example, in U.S. Pat. Nos. 7,122,189, 7,087,229 and 8,741,283. In other examples, polymer conjugation of any ADA2 provided herein can be achieved using branched polymer residues, such as those described in U.S. Pat. Nos. 5,681,567, 5,756,593, 5,643,575; 5,919,455, 6,113,906, 6,153,655, 6,395,266 and 6,638,499, 6,251,382, 6,824,766, and 8,741,283. In other examples, polymer conjugation of any ADA2 provided herein can be achieved using a hindered ester-based linker, such as those described in International PCT Pub. No. WO 2008/034119. In some examples, the activated polyethylene glycol is one which provides a urethane linkage or amide-linkage with the protein such as any ADA2 provided herein.

Methods of preparing polymers having terminal carboxylic acids in high purity are described in the art, for example in U.S. Pat. Pub. No. 2007/0173615. The methods include first preparing a tertiary alkyl ester of a polyalkylene oxide followed by conversion to the carboxylic acid derivative thereof. The first step of the preparation of the PAO carboxylic acids of the process includes forming an intermediate such as t-butyl ester of polyalkylene oxide carboxylic acid. This intermediate is formed by reacting a PAO with a t-butyl haloacetate in the presence of

a base such as potassium t-butoxide. Once the t-butyl ester intermediate has been formed, the carboxylic acid derivative of the polyalkylene oxide can be readily provided in purities exceeding 92%, such as exceeding 97%, 99%, or 99.5%.

In other examples, polymers having terminal amine groups can be employed to make conjugates to ADA2 provided herein. The methods of preparing polymers containing terminal amines in high purity are described in the art, for example in U.S. Pat. Nos. 7,868,131 and 7,569,657. For example, polymers having azides react with phosphine-based reducing agent such as triphenylphosphine or an alkali metal borohydride reducing agent such as $NaBH_4$. Alternatively, polymers including leaving groups react with protected amine salts such as potassium salt of methyl-tert-butyl imidodicarbonate (KNMeBoc) or the potassium salt of di-tert-butyl imidodicarbonate ($KNBoc_2$) followed by deprotecting the protected amine group. The purity of the polymers containing the terminal amines formed by these processes is greater than about 95%, such as greater than 99%.

In some examples, the PEG portion of the polymer conjugate of ADA2 provided herein can be selected from among:

J-O—$(CH_2CH_2O)_u$—

J-O—$(CH_2CH_2O)_u$—$CH_2C(O)$—O—,

J-O—$(CH_2CH_2O)_u$—CH_2CH_2NR—, and

J-O—$(CH_2CH_2O)_u$—CH_2CH_2SH—,

wherein u is the degree of polymerization, *i.e.* from about 10 to about 2,300;

R is selected from among hydrogen, C_{1-6} alkyls, C_{2-6} alkenyls, C_{2-6} alkynyls, C_{3-12} branched alkyls, C_{3-8} cycloalkyls, C_{1-6} substituted alkyls, C_{2-6} substituted alkenyls, C_{2-6} substituted alkynyls, C_{3-8} substituted cycloalkyls, aryls substituted aryls, aralkyls, C_{1-6} heteroalkyls, substituted C_{1-6} heteroalkyls, C_{1-6} alkoxy, phenoxy and C_{1-6} heteroalkoxy, and

J is a capping group, *i.e.*, a group which is found on the terminal of the polymer and, in some aspects, can be selected from among NH_2 (or $CH_2CH_2NH_2$), H, SH (or CH_2CH_2SH), CO_2H (or CH_2CO_2H), C_{1-6} alkyls, such as a methyl, or other PEG terminal activating groups known in the art.

For example, the PEG portion of the polymer conjugate can be selected from among

CH_3—O—$(CH_2CH_2O)_u$—, CH_3—O—$(CH_2CH_2O)_u$—$CH_2C(O)$—O—, CH_3—O—$(CH_2CH_2O)_u$—CH_2CH_2NH— and CH_3—O—$(CH_2CH_2O)_u$—CH_2CH_2SH—,

where u is a positive integer, such that the average total molecular weight of the polymer portion ranges from about 2 kDa to about 100 kDa.

In other examples, the PEG portion of the polymer conjugate of ADA2 provided herein can be selected from among:

—Y_1—$(CH_2CH_2O)_u$—$CH_2CH_2Y_1$—,

—Y_1—$(CH_2CH_2O)_u$—$CH_2C(=Y_2)$—Y_1—,

—Y_1—$C(=Y_2)$—$(CH_2)a_1$—Y_3—$(CH_2CH_2O)_u$—CH_2CH_2—Y_3—$(CH_2)a_1$—$C(=Y_2)$—Y_1—,

—Y_1—$(CR_2R_3)a_2$—Y_3—$(CH_2)b_1$—O—$(CH_2CH_2O)b_1$—$(CH_2)b_1$—Y_3—$(CR_2R_3)a_2$—Y_1—,

—Y_1—$(CH_2CH_2O)_u$—CH_2CH_2—,

—Y_1—$(CH_2CH_2O)_u$—$CH_2C(=Y_2)$—,

—$C(=Y_2)$—$(CH_2)a_1$—Y_3—$(CH_2CH_2O)_u$—CH_2CH_2—Y_3—$(CH_2)a_1$—$C(=Y_2)$—, and

—$(CR_2R_3)a_2$—Y_3—$(CH_2)b_1$—O—$(CH_2CH_2O)_u$—$(CH_2)b_1$—Y_3—$(CR_2R_3)a_2$—,

wherein: Y_1 and Y_3 are independently O, S, SO, SO_2, NR_4 or a bond;

Y_2 is O, S, or NR_5;

R_2-R_5 are independently selected from among hydrogen, C_{1-6} alkyl, C_{2-6} alkenyl, C_{2-6} alkynyl, C_{3-19} branched alkyl, C_{3-8} cycloalkyl, C_{1-6} substituted alkyl, C_{2-6} substituted alkenyl, C_{2-6} substituted alkynyl, C_{3-8} substituted cycloalkyl, aryl, substituted aryl, heteroaryl, substituted heteroaryl, C_{1-6} heteroalkyl, substituted C_{1-6} heteroalkyl, C_{1-6} alkoxy, aryloxy, C_{1-6} heteroalkoxy, heteroaryloxy, C_{2-6} alkanoyl, arylcarbonyl, C_{2-6} alkoxycarbonyl, aryloxycarbonyl, C_{2-6} alkanoyloxy, arylcarbonyloxy, C_{2-6} substituted alkanoyl, substituted arylcarbonyl, C_{2-6} substituted alkanoyloxy, substituted aryloxycarbonyl, C_{2-6} substituted alkanoyloxy and substituted arylcarbonyloxy;

a_1, a_2, and b_1 are independently zero or a positive integer from 1 to 6, for example, 0, 1 or 2; and

u is an integer from about 10 to about 2300.

In other examples, the PEG portion of the polymer conjugate of ADA_2 provided herein

can be functionalized, for example, in the following manner:

$—C(=Y_4)—(CH_2)_m—(CH_2CH_2O)_u—$,

$—C(=Y_4)—Y—(CH_2)_m—(CH_2CH_2O)_u—$,

$—C(=Y_4)—NR_2—(CH_2)_m—(CH_2CH_2O)_u—$,

$—CR_6R_7—(CH_2)_m—(CH_2CH_2O)_u—$

wherein: R_2, R_6 and R_7 are independently selected from among H, C_{1-6} alkyls, aryls, substituted aryls, aralkyls, heteroalkyls, substituted heteroalkyls and substituted C_{1-6} alkyls;

m is zero or is a positive integer, such as 1 or 2,

Y_4 is O or S; and

u represents the degree of polymerization.

In some examples, the polymer conjugates of ADA2 provided herein can be made by methods which include converting the multi-arm PEG-OH or "star-PEG" products, such made by NOF Corp, Tokyo, Japan, into a suitably activated polymer, using the activation techniques described in U.S. Pat. Nos. 5,122,614 or 5,808,096. In one example, the multi-arm polymers can contain four or more polymer arms and preferably four or eight polymer arms. In some examples, four of the PEG arms are converted to suitable functional groups, such as succinimidyl carbonate (SC), for facilitating attachment to the polypeptide, such as any ADA2 provided herein.

The polymeric conjugates provided herein can be water-soluble at room temperature. A non-limiting list of such polymers include polyalkylene oxide homopolymers such as polyethylene glycol (PEG) or polypropylene glycols, polyoxyethylenated polyols, copolymers thereof and block copolymers thereof.

As an exemplary illustrative method for making PEGylated ADA2 polypeptides, including variant ADA2 polypeptides, PEG aldehydes, succinimides and carbonates have each been applied to conjugate PEG moieties, typically succinimidyl PEGs. Exemplary succinimidyl monoPEG (mPEG) reagents including mPEG-Succinimidyl Propionates (mPEG-SPA), mPEG Succinimidyl Carboxymethyl Ester (mPEG-SCM), mPEG-Succinimidyl Butanoates (mPEG-SBA), and (for

attaching "branched" PEGs) mPEG2-N-Hydroxylsuccinimide. These PEGylated succinimidyl esters contain different length carbon backbones between the PEG group and the activated cross-linker, and either a single or branched PEG group. These differences can be used, for example, to provide for different reaction kinetics and to potentially restrict sites available for PEG attachment to ADA2 during the conjugation process. Such PEGylated ADA2 compositions can be readily purified to yield compositions having at least about 90% to about 100% PEGylated ADA2 molecules, and being substantially free of non-PEGylated ADA2 (less than 5% non-PEGylated).

In one example, the PEGylation includes conjugation of mPEG-SCM, for example, mPEG-SCM-20K (having a molecular weight of about 20 kDa) or another succinimidyl carboxymethyl esters of PEG derivative, to any ADA2 polypeptide, including variant ADA2 polypeptides. Succinimidyl carboxymethyl esters of PEG derivatives, such as mPEG-SCM-20K readily couple to amino groups of lysines in proteins or the N-terminal amine in biological active molecules. For example, covalent conjugation of m-PEG-SCM-20K and ADA2 (which is approximately 59 kDa in size as a monomer) provides stable amide bonds between ADA2 and mPEG. Typically, the mPEG-SCM-20K or other PEG is added to any ADA2 polypeptide, including variant ADA2 polypeptides, at a PEG:polypeptide molar ratio of 15:1 in a suitable buffer, followed by sterilization, *e.g.,* sterile filtration, and continued conjugation, for example, with stirring, overnight at 4°C in a cold room.

Other methods of coupling succinimidyl esters of PEG, including butanoic acid derivatives such as mPEG-SBA-30K, to a polypeptide are known in the art (see *e.g.*, U.S. 5,672,662; U.S. 6,737,505; U.S. 8,784,791; U.S. 2004/0235734 and U.S. 2005/0158273). For example, a polypeptide, such as any ADA2 provided herein, can be coupled to an NHS activated PEG derivative by reaction in a borate buffer (0.1 M, pH 8.0) for one hour at 4°C. The resulting PEGylated protein can be purified by ultrafiltration. Alternatively, PEGylation of a bovine alkaline phosphatase can be accomplished by mixing the phosphatase with mPEG-SBA in a buffer containing 0.2 M sodium phosphate and 0.5 M NaCl (pH 7.5) at 4°C for 30 minutes. Unreacted PEG can be removed by ultrafiltration or using resin columns such as Capto Phenyl resin columns (GE Healthcare). Another method reacts polypeptide with mPEG-SBA in deionized water to which triethylamine is added to raise the pH to 7.2-9. The

resulting mixture is stirred at room temperature for several hours to complete the PEGylation.

As shown herein, PEGylation of variant ADA2 confers reduction in heparin binding property to the ADA2. The reduction in heparin binding can be in addition to any attenuated heparin binding resulting from amino acid replacement(s) that reduce heparin binding. Thus, PEGylation of ADA2 also improves pharmacokinetic properties of the ADA2, and PEGylation can be used in place of or in addition to amino acid replacement(s) to attenuate heparin binding.

ii. Hydroxyethyl Starch (HES)

In some examples, at least one heterologous moiety is a polymer, *e.g.*, hydroxyethyl starch (HES) or a derivative thereof. Hydroxyethyl starch (HES) is a derivative of naturally occurring amylopectin and is degraded by alpha-amylase in the body. HES is a substituted derivative of the carbohydrate polymer amylopectin, which is present in corn starch at a concentration of up to 95% by weight. HES exhibits advantageous biological properties and is used as a blood volume replacement agent and in hemodilution therapy in the clinics.

Amylopectin contains glucose moieties, wherein in the main chain α-1,4-glycosidic bonds are present and at the branching sites α-1,6-glycosidic bonds are found. The physical-chemical properties of this molecule are mainly determined by the type of glycosidic bonds. Due to the nicked α-1,4-glycosidic bond, helical structures with about six glucose-monomers per turn are produced. The physicochemical as well as the biochemical properties of the polymer can be modified via substitution. The introduction of a hydroxyethyl group can be achieved via alkaline hydroxyethylation. By adapting the reaction conditions it is possible to exploit the different reactivity of the respective hydroxy group in the unsubstituted glucose monomer with respect to hydroxyethylation. One of skill in the art can determine the substitution pattern. HES is mainly characterized by the molecular weight distribution and the degree of substitution (DS), which refers to the molar ratio of the substitution.

In one example, hydroxyethyl starch has a mean molecular weight (weight mean) of from 1 to 300 kD, from 2 to 200 kD, from 3 to 100 kD, or from 4 to 70 kD. Hydroxyethyl starch can further exhibit a molar degree of substitution (DS) of from

0.1 to 3, preferably 0.1 to 2, more preferred, 0.1 to 0.9, preferably 0.1 to 0.8, and a ratio between C2:C6 substitution in the range of from 2 to 20 with respect to the hydroxyethyl groups. A non-limiting example of HES having a mean molecular weight of about 130 kD is a HES with a DS of 0.2 to 0.8, such as 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, or 0.8, and in particular, a DS of 0.4 to 0.7 such as 0.4, 0.5, 0.6, or 0.7.

In one example, the HES can have a mean molecular weight of about 130 kD, and is VOLUVEN® (Fresenius Kabi, Germany). VOLUVEN® is an artificial colloid, employed, *e.g.*, for volume replacement used in the therapeutic indication for therapy and prophylaxis of hypovolemia. The characteristics of VOLUVEN® are a mean molecular weight of 130 ± 20 kDa, with a molar substitution of 0.4 and a C2:C6 ratio of about 9:1. In other examples, ranges of the mean molecular weight of hydroxyethyl starch are, *e.g.*, 4 to 70 kDa or 10 to 70 kDa or 12 to 70 kDa or 18 to 70 kDa or 50 to 70 kDa or 4 to 50 kDa or 10 to 50 kDa or 12 to 50 kDa or 18 to 50 kDa or 4 to 18 kDa or 10 to 18 kDa or 12 to 18 kDa or 4 to 12 kDa or 10 to 12 kDa or 4 to 10 kDa. In other examples, the mean molecular weight of hydroxyethyl starch employed is in the range of from more than 4 kDa and below 70 kDa, such as about 10 kDa, or in the range of from 9 to 10 kDa or from 10 to 1 1 kDa or from 9 to 11 kDa, or about 12 kDa, or in the range of from 1 1 to 12 kDa) or from 12 to 13 kDa or from 1 1 to 13 kDa, or about 18 kDa, or in the range of from 17 to 18 kDa or from 18 to 19 kDa or from 17 to 19 kDa, or about 30 kDa, or in the range of from 29 to 30, or from 30 to 31 kDa, or about 50 kDa, or in the range of from 49 to 50 kDa or from 50 to 51 kDa or from 49 to 51 kDa.

In some examples, the heterologous moiety can be a mixture of hydroxyethyl starches having different mean molecular weights and/or different degrees of substitution and/or different ratios of C2:C6 substitution. Therefore, mixtures of hydroxyethyl starches can be employed having different mean molecular weights and different degrees of substitution and different ratios of C2:C6 substitution, or having different mean molecular weights and different degrees of substitution and the same or about the same ratio of C2:C6 substitution, or having different mean molecular weights and the same or about the same degree of substitution and different ratios of C2:C6 substitution, or having the same or about the same mean molecular weight and different degrees of substitution and different ratios of C2:C6 substitution, or having

different mean molecular weights and the same or about the same degree of substitution and the same or about the same ratio of C2:C6 substitution, or having the same or about the same mean molecular weights and different degrees of substitution and the same or about the same ratio of C2:C6 substitution, or having the same or about the same mean molecular weight and the same or about the same degree of substitution and different ratios of C2:C6 substitution, or having about the same mean, molecular weight and about the same degree of substitution and about the same ratio of C2:C6 substitution.

iii. Polysialic Acids (PSA)

In certain examples, at least one heterologous moiety is a polymer, *e.g.*, polysialic acids (PSAs) or a derivative thereof. Polysialic acids (PSAs) are naturally occurring unbranched polymers of sialic acid produced by certain bacterial strains and in mammals in certain cells (Roth J., *et al.* (1993) in Polysialic Acid: From Microbes to Man, eds Roth J., Rutishauser U., Troy F. A. (Birkhauser Verlag, Basel, Switzerland), pp 335-348). They can be produced in various degrees of polymerization from about 80 or more sialic acid residues to about 2, by limited acid hydrolysis or by digestion with neuraminidases, or by fractionation of the natural, bacterially derived forms of the polymer.

The composition of different polysialic acids also varies such that there are homopolymeric forms *i.e.* the α-2,8-linked polysialic acid of the capsular polysaccharide of *E. coli* strain K1 and the group-B meningococci, which is also found on the embryonic form of the neuronal cell adhesion molecule (N-CAM). Heteropolymeric forms also exist, such as the alternating α-2,8 α-2,9 polysialic acid of *E. coli* strain K92 and group C polysaccharides of *N. meningitidis*. Sialic acid can also be found in alternating copolymers with monomers other than sialic acid such as group W135 or group Y of *N. meningitidis*. Polysialic acids have important biological functions including the evasion of the immune and complement systems by pathogenic bacteria and the regulation of glial adhesiveness of immature neurons during fetal development (wherein the polymer has an anti-adhesive function) (Cho and Troy, (1994) P.N.A.S. 91:11427-11431), although there are no known receptors for polysialic acids in mammals.

In other examples, the α-2,8-linked polysialic acid of *E. coli* strain K1, also known as colominic acid, are used (in various lengths). Various methods of attaching or conjugating polysialic acids to a polypeptide have been described (see *e.g.*, U.S. Pat. No. 5,846,951; WO 01/87922, and US 2007/0191597).

iv. Other polymers

In other examples, the polymer moiety for conjugation to any ADA2 provided herein can be selected from among one or more effectively non-antigenic materials such as dextran, polyvinyl alcohols, carbohydrate-based polymers, hydroxypropylmeth-acrylamide (HPMA), polyalkylene oxides, and/or copolymers thereof, including other polymers known in the art and/or described in U.S. Pat. No. 6,153,655. It is within the level of one of skill in the art to select the polymer based on the purpose of use, and to select the suitable conjugation methods.

2. Methods of Producing Conjugates or Fusion Proteins

Heterologous moieties can be conjugated directly or indirectly to any ADA2 provided herein. For example, the heterologous moieties can be conjugated in a post-translational manner, after the recombinant production of the ADA2 polypeptide, by direct chemical linkage or indirectly via a linker. In other examples, heterologous moieties that are protein or polypeptide moieties, can be directly or indirectly conjugated to any ADA2 provided herein. In one example, the protein or polypeptide moieties can be directly linked, for example, as a fusion protein. In other examples, the heterologous moiety is conjugated indirectly, via a linker. In other examples, the heterologous moiety can be linked by disulfide bonds formed between a thiol group in the heterologous moiety and the cysteine residues in the ADA2 provided herein.

Linkers

Linkers, or spacers, can be used to connect heterologous moieties and polypeptides, such as any ADA2 provided herein. A linker refers to a peptide or polypeptide sequence (*e.g.* a synthetic peptide or polypeptide sequence), or a non-peptide linker for which its main function is to connect two moieties, such as an ADA2 provided herein and the heterologous moiety. Linkers can be used to maintain the structural flexibility and other conformational characteristics of the individual residues or at the secondary, tertiary, or quaternary structural levels of domains or

moieties of the polypeptide conjugate or fusion protein, in order to maintain functional properties of the moieties. Linkers can also provide additional beneficial properties to the polypeptide conjugate or fusion protein, such as increased protein expression in mammalian expression systems, improved biophysical properties such as stability and solubility, improved protein purification and detection and/or increased enzymatic activity. In some examples, two or more linkers can be linked in tandem. Linkers can be peptide linkers that link a protein or polypeptide moiety to the ADA2 polypeptide. Other exemplary linkers include chemical linking agents and heterobifunctional linking agents.

When multiple linkers are present between the ADA2 and the heterologous moiety, each of the linkers can be the same or different. Generally, linkers provide flexibility to the polypeptide molecule. Linkers are not typically cleaved; however in certain examples, such cleavage can be desirable. Accordingly, in some embodiments a linker can contain one or more protease-cleavable sites, which can be located within the sequence of the linker or flanking the linker at either end of the linker sequence.

Linkers can be introduced into polypeptide sequences, such as any ADA2 provided herein, using techniques known in the art (*e.g.*, chemical conjugation, recombinant techniques, or peptide synthesis). Modifications can be confirmed by DNA sequence analysis. In some examples, the linkers can be introduced using recombinant techniques. In other examples, the linkers can be introduced using solid phase peptide synthesis. In other examples, the polypeptide, such as any ADA2 provided herein, can contain simultaneously one or more linkers that have been introduced using recombinant techniques and one or more linkers that have been introduced using solid phase peptide synthesis or methods of chemical conjugation known in the art.

i. Peptide Linkers

Peptide linkers can be used to link the heterologous moiety to the ADA2 polypeptide provided herein. In one example, peptide linkers can be fused to the C-terminal end of a first polypeptide (*e.g.*, the ADA2 polypeptide) and the N-terminal end of a second polypeptide (*e.g.*, a protein or polypeptide heterologous moiety). This structure can be repeated multiple times such that at least one, preferably 2, 3, 4, or

more polypeptides are linked to one another via peptide linkers at their respective termini.

For example, two molecules (*e.g.*, the ADA2 polypeptide and the heterologous moiety) can be adjacent in the construct or separated by a linker polypeptide that contains, 1, 2, 3, or more, amino acids. In some examples, the peptide linker can contain at least two amino acids, at least three, at least four, at least five, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100 amino acids. In other examples, the peptide linker can contain at least 200, at least 300, at least 400, at least 500, at least 600, at least 700, at least 800, at least 900, or at least 1,000 amino acids. In some examples, the peptide linker can contain at least about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1 100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, or 2000 amino acids. The peptide linker can contain 1-5 amino acids, 1-10 amino acids, 1 -20 amino acids, 10-50 amino acids, 50-100 amino acids, 100-200 amino acids, 200-300 amino acids, 300-400 amino acids, 400-500 amino acids, 500-600 amino acids, 600-700 amino acids, 700-800 amino acids, 800-900 amino acids, or 900-1000 amino acids. The linker is of a length such that the two variable domains are bridged without substantial interference. For example, a linker polypeptide can have a sequence Z_1-X-Z_2, where Z_1 and Z_2 are the ADA2 polypeptide and the heterologous moiety, respectively, and where X is a sequence of a peptide linker. In another example, the polypeptide has a sequence of Z_1-X-Z_2(-X-Z)$_n$, where "n" is any integer, *i.e.* generally 1 or 2.

Typically, the peptide linker is of a sufficient length to allow both the ADA2 polypeptide and the heterologous moiety to retain their conformational structure and functions. Examples of peptide linkers include, but are not limited to: —Gly-Gly—, GGGG (SEQ ID NO:362), GGGGG (SEQ ID NO:360), GGGGS or (GGGGS)n (SEQ ID NO:343), SSSSG or (SSSSG)n (SEQ ID NO:344), GKSSGSGSESKS (SEQ ID NO:345), GGSTSGSGKSSEGKG (SEQ ID NO:346), GSTSGSGKSSSEGSGSTKG (SEQ ID NO:347), GSTSGSGKPGSGEGSTKG (SEQ ID NO:348), EGKSSGSGSESKEF (SEQ ID NO:349), AlaAlaProAla or (AlaAlaProAla)n (SEQ ID NO:350), SGGSGGS (SEQ ID NO:363), GGSGGSGGSGGSGGG (SEQ ID NO:364), GGSGGSGGGGSGGGGS (SEQ ID NO:365),

GGSGGSGGSGGSGGSGGS (SEQ ID NO:366), GGGGSGGGGSGGGGS (SEQ ID NO:367), $Ser(Gly_4Ser)_n$ (SEQ ID NO:595) or $(Gly\text{-}Ser)_n$ residues with some Glu or Lys residues dispersed throughout to increase solubility, where n can be an integer from 1 to 20, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20. Other exemplary linkers include peptide linkers with the formula $[(Gly)_x\text{-}Ser_y]_z$ where x is from 1 to 4, y is 0 or 1, and z is from 1 to 50. In other examples, the peptide linker includes the sequence G_n, where n can be an integer from 1 to 100. In another example, the sequence of the peptide linker can be $(GA)_n$ or $(GGS)_n$. Other exemplary linkers include:

(1) Gly_4Ser with NcoI ends (SEQ ID NO:351)

CCATGGGCGG CGGCGGCTCT GCCATGG

(2) $(Gly_4Ser)_2$ with NcoI ends (SEQ ID NO:352)

CCATGGGCGG CGGCGGCTCT GGCGGCGGCG GCTCTGCCAT GG

(3) $(Ser_4Gly)_4$ with NcoI ends (SEQ ID NO:353)

CCATGGCCTC GTCGTCGTCG GGCTCGTCGT CGTCGGGCTC GTCGTCGTCG GGCTCGTCGT CGTCGGGCGC CATGG

(4) $(Ser_4Gly)_2$ with NcoI ends (SEQ ID NO:354)

CCATGGCCTC GTCGTCGTCG GGCTCGTCGT CGTCGGGCGC CATGG

Linking moieties are described, for example, in Huston *et al.* (1988) *PNAS* 85:5879-5883, Whitlow *et al.* (1993) *Protein Engineering* 6:989-995, and Newton *et al.*, (1996) *Biochemistry* 35:545-553. Other suitable peptide linkers include any of those described in U.S. Patent Nos. 4,751,180 or 4,935,233. A polynucleotide encoding a desired peptide linker can be inserted between, and in the same reading frame as a polynucleotide encoding any ADA2 provided herein and the protein or polypeptide heterologous moiety, using any suitable conventional technique. Linking moieties can also include derivatives and analogs of the naturally occurring amino acids, as well as various non-naturally occurring amino acids (D- or L-), hydrophobic or non-hydrophobic, known in the art.

In some examples, a peptide linker includes peptides (or polypeptides) (*e.g.*, natural, or non-naturally occurring peptides) which includes an amino acid sequence that links or genetically fuses a first linear sequence of amino acids to a second linear sequence of amino acids to which it is not naturally linked or genetically fused in

nature. For example, the peptide linker can include non-naturally occurring polypeptides which are modified forms of naturally occurring polypeptides (*e.g.*, that includes a mutation such as an addition, substitution or deletion). In another example, the peptide linker can include non-naturally occurring amino acids. In another example, the peptide linker can include naturally occurring amino acids occurring in a linear sequence that does not occur in nature. In still another example, the peptide linker can include a naturally occurring polypeptide sequence.

ii. Heterobifunctional linking agents

Linkage of any ADA2 provided herein and a heterologous moiety can be direct or indirect. For example, the linkage can be achieved by chemical linkage or facilitated by bifunctional or heterobifunctional linkers, such as any known in the art or provided herein.

Numerous heterobifunctional cross-linking reagents that are used to form covalent bonds between amino groups and thiol groups and to introduce thiol groups into proteins, are known to those of skill in the art (see, *e.g.*, the PIERCE CATALOG, *ImmunoTechnology Catalog & Handbook,* 1992-1993, which describes the preparation of and use of such reagents and provides a commercial source for such reagents; see, also, *e.g.*, Cumber *et al.* (1992) *Bioconjugate Chem.* 3:397-401; Thorpe *et al.* (1987) *Cancer Res.* 47:5924-5931; Gordon *et al.* (1987) *Proc. Natl. Acad Sci.* 84:308-312; Walden *et al.* (1986) *J. Mol. Cell Immunol.* 2:191-197; Carlsson *et al.* (1978) *Biochem. J.* 173: 723-737; Mahan *et al.* (1987) *Anal. Biochem.* 162:163-170; Wawrzynczak *et al.* (1992) *Br. J. Cancer* 66:361-366; Fattom *et al.* (1992) *Infection & Immun.* 60:584-589). These reagents can be used to form covalent bonds between the N-terminal portion of the heterologous moiety and the C-terminal portion of the ADA2 provided herein, or between each of those portions and a linker. These reagents include, but are not limited to: N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; disulfide linker); sulfosuccinimidyl 6-[3-(2-pyridyldithio)propionamido]hexanoate (sulfo-LC-SPDP); succinimidyloxycarbonyl-α-methyl benzyl thiosulfate (SMBT, hindered disulfate linker); succinimidyl 6-[3-(2-pyridyldithio) propionamido]hexanoate (LC-SPDP); sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC); succinimidyl 3-(2-pyridyldithio)butyrate (SPDB; hindered disulfide bond linker); sulfosuccinimidyl 2-

(7-azido-4-methylcoumarin-3-acetamide) ethyl-1,3'-dithiopropionate (SAED); sulfo-succinimidyl 7-azido-4-methylcoumarin-3-acetate (SAMCA); sulfosuccinimidyl-6-[alpha-methyl-alpha-(2-pyridyldithio)toluamido]-hexanoate (sulfo-LC-SMPT); 1,4-di-[3'-(2'-pyridyldithio)propionamido]butane (DPDPB); 4-succinimidyloxycarbonyl-α-methyl-α-(2-pyridylthio)toluene (SMPT, hindered disulfate linker); sulfosuccinimidyl-6-[α-methyl-α-(2-pyrimiyldi-thio)toluamido]hexanoate (sulfo-LC-SMPT); m-maleimidobenzoyl-N-hydroxy-succinimide ester (MBS); m-maleimidobenzoyl-N-hydroxysulfo-succinimide ester (sulfo-MBS); N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB; thioether linker); sulfosuccinimidyl-(4-iodoacetyl)amino benzoate (sulfo-SIAB); succinimidyl-4-(p-malcimidophcnyl)butyratc (SMPB); sulfosuccinimidyl-4-(p-malcimido-phenyl)butyrate (sulfo-SMPB); azidobenzoyl hydrazide (ABH); maleimido caproyl (MC); maleimido propanoyl (MP); succinimidyl 4-(K-maleimidomethyl)-cyclohexane-l-carboxylate (SMCC); m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); N-succinimidyl(4- iodoacetyl)aminobenzonate (SIAB); and succinimidyl 6-[3-(2-pyridyldithio)- propionamide hexanoate (LC-SPDP) (see, *e.g.*, U.S. Pat. No. 7,375,078). Other exemplary linkers include, but are not limited to linkers with the formula:

—$C(O)CH_2OCH_2C(O)$—;

—$C(O)CH_2NHCH_2C(O)$—;

—$C(O)CH_2SCH_2C(O)$—;

—$C(O)CH_2CH_2CH_2C(O)$—, and

—$C(O)CH_2CH_2C(O)$—.

These linkers, for example, can be used in combination with peptide linkers, such as those that increase flexibility or solubility or that provide for or eliminate steric hindrance. Any other linkers known to those of skill in the art for linking a polypeptide molecule to another molecule can be employed. General properties are such that the resulting molecule retains the adenosine deaminase function and stability of the protein. For *in vivo* use of the ADA2 conjugate or fusion protein, generally the linker must be biocompatible for administration to animals, including humans.

E. METHODS OF PRODUCING NUCLEIC ACIDS ENCODING ADA2 AND POLYPEPTIDES THEREOF

Polypeptides of any ADA2 as described herein, including variants or modified forms thereof, can be obtained by methods well known in the art for protein purification and recombinant protein expression. Polypeptides also can be synthesized chemically. Modified or variant forms can be engineered from a wildtype polypeptide using standard recombinant DNA methods. For example, any ADA2, including variants or modified forms can be engineered from a wildtype polypeptide, such as by site-directed mutagenesis.

1. Isolation or Preparation of Nucleic Acids Encoding ADA2 Polypeptides

Polypeptides can be cloned or isolated using any available methods known in the art for cloning and isolating nucleic acid molecules. Such methods include PCR amplification of nucleic acids and screening of libraries, including nucleic acid hybridization screening, antibody-based screening and activity-based screening. For example, when the polypeptides are produced by recombinant means, any method known to those of skill in the art for identification of nucleic acids that encode desired genes can be used. Any method available in the art can be used to obtain a full length or partial (*i.e.*, encompassing the entire coding region) cDNA or genomic DNA clone encoding the ADA2 polypeptide, such as from a cell or tissue source.

Methods for amplification of nucleic acids can be used to isolate nucleic acid molecules encoding a desired polypeptide, including for example, polymerase chain reaction (PCR) methods. Examples of such methods include use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp, Applied Biosystems, Carlsbad, CA). A nucleic acid containing material can be used as a starting material from which a desired polypeptide-encoding nucleic acid molecule can be isolated. For example, DNA and mRNA preparations, cell extracts, tissue extracts, fluid samples (*e.g.*, blood, serum, saliva), samples from healthy and/or diseased subjects can be used in amplification methods. The source can be from any eukaryotic species including, but not limited to, vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, and other primate sources. Nucleic acid libraries also can be used as a source of starting material. Primers can be designed to amplify a desired polypeptide. For

example, primers can be designed based on expressed sequences from which a desired polypeptide is generated. Primers can be designed based on back-translation of a polypeptide amino acid sequence. If desired, degenerate primers can be used for amplification. Oligonucleotide primers that hybridize to sequences at the 3' and 5' termini of the desired sequence can be uses as primers to amplify by PCR sequences from a nucleic acid sample. Primers can be used to amplify the full-length ADA2. Nucleic acid molecules generated by amplification can be sequenced and confirmed to encode a desired polypeptide.

In addition, nucleic acid molecules encoding the ADA2 polypeptide can be chemically synthesized or produced in a semi-synthetic manner. The synthetically or semi-synthetically produced nucleic acid molecule can encode the amino acid sequence of any ADA2, such as any described herein in Section C above. For example, the synthesized or semi-synthetically produced nucleic acid molecule can be encoded by a nucleic acid molecule having a sequence of nucleotides of any as described herein. Chemically synthesized nucleic acid molecules can span the entire length of the wildtype ADA2 gene, or a truncated sequence thereof. Chemical gene synthesis can be achieved by any methods known in the art, such as annealing chemically synthesized oligonucleotides. Semi-synthetic gene assembly, such as the Gibson assembly method, can also be used to produce the nucleic acid molecule encoding any of the ADA2 polypeptides, including variants, as described herein.

The nucleic acid encoding any of the ADA2 polypeptides can be a codon-optimized nucleic acid molecule, where the codon is optimized for the expression system used to produce the polypeptide (i.e., codons that are preferred in the organism of the expression system are used more frequently in the synthesized nucleic acid). For example, for production of the polypeptide in an *Escherichia coli* expression system, the codons for each amino acid can be optimized such that the most preferred codon in *E. coli* are used for each amino acid.

Additional nucleotide sequences can be joined to a polypeptide-encoding nucleic acid molecule, including linker sequences containing restriction endonuclease sites for the purpose of cloning the synthetic gene into a vector, for example, a protein expression vector or a vector designed for the amplification of the core protein coding DNA sequences. Furthermore, additional nucleotide sequences specifying functional

DNA elements can be operatively linked to a polypeptide-encoding nucleic acid molecule. Examples of such sequences include, but are not limited to, promoter sequences designed to facilitate intracellular protein expression, and secretion sequences, for example heterologous signal sequences, designed to facilitate protein secretion. Such sequences are known to those of skill in the art. Additional nucleotide residue sequences such as sequences of bases specifying protein binding regions also can be linked to enzyme-encoding nucleic acid molecules. Such regions include, but are not limited to, sequences of residues that facilitate or encode proteins that facilitate uptake of an enzyme into specific target cells, or otherwise alter pharmacokinetics of a product of a synthetic gene.

In addition, tags or other moieties can be added, for example, to aid in detection or affinity purification of the polypeptide. For example, additional nucleotide residue sequences such as sequences of bases specifying an epitope tag or other detectable marker also can be linked to enzyme-encoding nucleic acid molecules. Examples of such sequences include nucleic acid sequences encoding the FLAG® tag or the Strep tag.

The identified and isolated nucleic acids can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art can be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pCMV4, pCMV-Script (Agilent Technologies, Santa Clara, CA), pBR322, pUC plasmid derivatives or pBluescript vectors (Stratagene, La Jolla, CA). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. Insertion can be effected using TOPO cloning vectors (Invitrogen, Carlsbad, CA).

If the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules can be enzymatically modified. Alternatively, any site desired can be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers can contain specific chemically synthesized oligonucleotides encoding restriction endonuclease

recognition sequences. In an alternative method, the cleaved vector and protein gene can be modified by homopolymeric tailing.

Recombinant molecules can be introduced into host cells via, for example, transformation, transfection, infection, electroporation and sonoporation, so that many copies of the gene sequence are generated. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated protein gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene can be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

In addition to recombinant production, any ADA2, including variants or modified forms thereof provided herein can be produced by direct peptide synthesis using solid-phase techniques (see *e.g.*, Stewart *et al.* (1969) *Solid-Phase Peptide Synthesis*, WH Freeman Co., San Francisco; Merrifield J (1963) *J Am Chem Soc.*, 85:2149-2154). *In vitro* protein synthesis can be performed using manual techniques or by automation. Automated synthesis can be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City CA) in accordance with the instructions provided by the manufacturer. Various fragments of a polypeptide can be chemically synthesized separately and combined using chemical methods.

2. Generation of Mutant or Modified Nucleic Acid and Encoding Polypeptides

The modifications provided herein can be made by standard recombinant DNA techniques such as are routine to one of skill in the art. Any method known in the art to effect mutation of any one or more amino acids in a target protein can be employed. Methods include standard site-directed mutagenesis (using *e.g.*, a kit, such as QuikChange available from Stratagene) of encoding nucleic acid molecules, or by solid phase polypeptide synthesis methods. Site-specific variations to the wildtype ADA2 or any of the ADA2 variants provided herein can also be introduced during the chemical gene synthesis or the semi-synthetic gene assembly if such methods are used to generate the nucleic acid sequence encoding the ADA2.

3. Vectors and Cells

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 3
CONTENANT LES PAGES 1 À 196

NOTE : Pour les tomes additionels, veuillez contacter le Bureau canadien des brevets

JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 3
CONTAINING PAGES 1 TO 196

NOTE: For additional volumes, please contact the Canadian Patent Office

NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS:

1. A variant Adenosine Deaminase 2 (ADA2) protein or a catalytically active portion thereof, comprising one or more modifications in the sequence of amino acids of an unmodified ADA2 protein or a catalytically active portion thereof, wherein:

i) the unmodified ADA2 protein or catalytically active portion thereof comprises a sequence of amino acids selected from the group consisting of:

a) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383;

b) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383;

c) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383 with all or a part of the ADA2 putative receptor binding domain (PRB domain) deleted, wherein the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5;

d) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383 with all or a part of the PRB domain deleted and a linker inserted in place of deleted residues, wherein:

the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; and

the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20;

e) the ADA2 catalytic domain, and, optionally, additional contiguous amino acids from the ADA2 protein contiguous to the catalytic domain, wherein the ADA2 catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5;

f) the sequence of amino acids selected from the group consisting of the catalytic domain of the polypeptides of any one of SEQ ID NOS: 5 and 380-383, wherein the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5;

g) the sequence of amino acids comprising the catalytic domain with all or a part of the PRB domain deleted, wherein:

the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and

the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5;

h) the sequence of amino acids selected from the group consisting of the catalytic domain of any one of SEQ ID NOS: 5 and 380-383 with all or a part of the PRB domain deleted, wherein:

the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and

the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5;

i) the ADA2 catalytic domain, and, optionally, additional contiguous amino acids from the ADA2 protein contiguous to the catalytic domain, with all or a part of the PRB domain deleted, and a linker in place of deleted residues, wherein:

the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and

the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; and

the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20; and

j) the sequence of amino acids selected from the group consisting of the catalytic domain of any one of SEQ ID NOS: 5 and 380-383 with all or a part of the PRB domain deleted, and a linker in place of deleted residues, wherein:

the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5;

the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; and

the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20;

ii) the variant ADA2 protein or catalytically active portion thereof has 1 to 10 amino acid modifications, in addition to any deletion of all or a portion of the PRB domain, as compared to the unmodified ADA2 protein or catalytically active portion thereof as set forth in i) parts a) to j), wherein modifications comprise insertions, deletions, and replacements of amino acid residues;

iii) the variant ADA2 protein or the catalytically active portion thereof, when in dimer form, exhibits reduced heparin binding compared to the corresponding dimer form of the unmodified ADA2 protein or catalytically active portion thereof as set forth in i) parts a) to j);

iv) the variant ADA2 protein or catalytically active portion thereof comprises amino acid replacements that confer reduced heparin binding selected from the group consisting of R20A, R20D, R20E, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E, K452E, K11A/R20A, K11A/R20A/K371A, R20A/K371A, and K11A/K371A, with reference to amino acid positions set forth in SEQ ID NO:5;

v) the variant ADA2 protein or the catalytically active portion thereof, when in dimer form, exhibits adenosine deaminase activity to convert adenosine to inosine; and

vi) the variant ADA2 protein or the catalytically active portion thereof does not comprise any of the amino acid replacements R219K, R219Q, R219N, S262M or S262N.

2. The variant ADA2 protein or catalytically active portion thereof of claim 1, further comprising amino acid modifications that confer, to the dimer form, one or more properties selected from the group consisting of longer serum half-life, increased thermal stability, altered receptor binding, and addition of one or more glycosylation sites, compared to the corresponding dimer form of the unmodified ADA2 protein of SEQ ID NO:5 or dimer form of the corresponding catalytically active portion thereof, wherein:

replacements that confer longer serum half-life are selected from R20E or K371D, with reference to amino acid positions set forth in SEQ ID NO:5;

the replacement that confers increased thermal stability is K371E, with reference to amino acid positions set forth in SEQ ID NO:5;

replacements that confer altered receptor binding are selected from the group consisting of F119S, F119K, Y224R, Y224N, Y191S, Y191D, F183K, Y191D/Y224R, F109S, F109A, R118D,

R118A, Y139T, Y139A, W133S, W133T, P124A, and P124S, with reference to amino acid positions set forth in SEQ ID NO:5; and

amino acid modifications that result in one or more additional glycosylation sites are selected from the group consisting of -->N1/-->A2/-->S3, R20N/V22S, K371N/D373S, K372N/I374S, T403N/H405S, G404N/P406S, R125N/P126A, S127N/K129S, P126N/E128T, R112N/I114T, I134N/L135C/L136T, I134N/L135S/L136T, R142N/Q144S, E137N/Y139T, and P111N/G113S, with reference to amino acid positions set forth in SEQ ID NO:5.

3. The variant ADA2 protein or catalytically active portion thereof of claim 1 or claim 2, wherein the unmodified ADA2 protein or catalytically active portion thereof is selected from the group consisting of:

a) the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383 with all or a part of the ADA2 putative receptor binding domain (PRB domain) deleted, wherein the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5;

b) the sequence of amino acids comprising the catalytic domain with all or a part of the PRB domain deleted, wherein:

the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and

the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5; and

c) the sequence of amino acids selected from the group consisting of the catalytic domain of SEQ ID NOS: 5 and 380-383 with all or a part of the PRB domain deleted, wherein:

the catalytic domain consists of residues corresponding to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5; and

the PRB domain consists of residues corresponding to residues 98-156 of SEQ ID NO:5.

4. The variant ADA2 protein or catalytically active portion thereof of claim 3, wherein the unmodified ADA2 protein or catalytically active portion thereof comprises:

a linker in place of the deleted portion of the PRB domain; and

the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20.

5. The variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-4, wherein the variant ADA2 protein or catalytically active portion thereof comprises one or more amino acid replacement(s) selected from the group consisting of R20A, R20D, R20E, R366A, R366D, R366E, K371A, K371D, K371E, K372A, K372D, K372E and K452E, with reference to amino acid positions set forth in SEQ ID NO:5.

6. The variant ADA2 protein or catalytically active portion thereof of any one of claims 1-3 and 5, wherein the variant ADA2 protein or catalytically active portion thereof lacks residues N98-N156, C105-E148, C105-T147, or V99-Q144, and optionally, includes a linker in place thereof, wherein the linker is $(GGGGS)_n$, where n = 1 to 5, or is $(Gly)_n$, where n is 2-20.

7. The variant ADA2 protein or catalytically active portion thereof of any one of claims 1-4, comprising the sequence of amino acids set forth in any one of SEQ ID NOS: 19-24, 27-33, 55-58, 589-594, and 646-657.

8. The variant ADA2 protein or catalytically active portion thereof of any one of claims 1-4 and 7, wherein:

the variant ADA2 protein or catalytically active portion thereof comprises one or more amino acid replacement(s) selected from the group consisting of R20E, R366D, R366E, R20A/K371A, K11A/K371A, K371D, K371E, K372A, K372D, K372E and K452E, with reference to amino acid positions set forth in SEQ ID NO:5; and

the variant ADA2 protein or catalytically active portion thereof, when in the dimer form, exhibits increased adenosine deaminase activity and reduced heparin binding, compared to the dimer form of the unmodified ADA2 protein of SEQ ID NO:5 or the dimer form of the catalytically active portion thereof.

9. The variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-8, wherein the unmodified ADA2 protein is a homodimer, and the monomer form of the unmodified ADA2 protein comprises the sequence of amino acid residues set forth in SEQ ID NO:5.

10. The variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-9, wherein the unmodified ADA2 protein comprises the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383, or is the catalytically active portion of the unmodified ADA2 protein.

11. The variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-10, wherein the unmodified ADA2 protein consists of the sequence of amino acids selected from the group consisting of SEQ ID NOS: 5 and 380-383 or is the catalytically active portion of the unmodified ADA2 protein.

12. The variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-11, wherein the unmodified ADA2 protein comprises the sequence of amino acids set forth in SEQ ID NO: 5 or is the catalytically active portion of the unmodified ADA2 protein.

13. The variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-12, wherein the variant ADA2 protein or the catalytically active portion thereof is PEGylated.

14. The variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-13, wherein the catalytically active portion of the variant ADA2 protein comprises the ADA domain that corresponds to residues 77-473 of the sequence of amino acids set forth in SEQ ID NO:5.

15. A variant ADA2 multimer, comprising a plurality of variant ADA2 proteins or the catalytically active portions thereof of any one of claims 1-14, wherein the variant ADA2 proteins or the catalytically active portions thereof are the same or different.

16. A variant ADA2 dimer, comprising the variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-14.

17. A conjugate, comprising the variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-14, or the multimer of claim 15, or the dimer of claim 16, linked directly or indirectly via a linker to a heterologous moiety.

18. A nucleic acid molecule encoding the variant ADA2 protein or the catalytically active portion thereof of any one of claim 1-14, or the variant ADA2 multimer of claim 15, or the variant ADA2 dimer of claim 16.

19. A vector, comprising the nucleic acid molecule of claim 18.

20. An isolated cell or a cell culture, comprising the vector of claim 19.

21. The cell or cell culture of claim 20, wherein the cell is a tumor-infiltrating lymphocyte (TIL), a cytotoxic T lymphocyte (CTL), a natural killer (NK) cell, or a lymphokine-activated killer (LAK) cell.

22. The cell or cell culture of claim 21, wherein the cell is a T cell that encodes and expresses:

a chimeric antigen receptor (CAR) that is specific for a tumor cell antigen; and

the variant ADA2 protein or the catalytically active portion thereof, or the variant ADA2 multimer, or the variant ADA2 dimer.

23. A method of producing a variant ADA2 protein or the catalytically active portion thereof, comprising culturing or expanding the cell of any one of claims 20-22, under conditions whereby the variant ADA2 protein or the catalytically active portion thereof is expressed.

24. The method of claim 23, wherein the variant ADA2 protein is a dimer.

25. The method of claim 23 or claim 24, comprising expanding cells to produce expanded cells.

26. Expanded cells produced by the method of claim 25.

27. Use of the expanded cells of claim 26 for treatment of a tumor.

28. A pharmaceutical composition, comprising the variant ADA2 protein or the catalytically active portion thereof, or the dimer or the multimer of the variant ADA2 protein or the

catalytically active portion thereof of any one of claims 1-16, or the conjugate of claim 17, in a pharmaceutically acceptable vehicle.

29. The pharmaceutical composition of claim 28, wherein the variant ADA2 protein or the catalytically active portion thereof is PEGylated.

30. The pharmaceutical composition of claim 28 or claim 29, wherein the variant ADA2 protein comprises the sequence of amino acid residues set forth in any of SEQ ID NOs: 19-24, 27-33, 55-58, 589-594, and 646-657, or the catalytically active portion thereof.

31. Use of the variant ADA2 protein or the catalytically active portion thereof, or the dimer or the multimer of the variant ADA2 protein or the catalytically active portion thereof of any one of claims 1-16, or the conjugate of claim 17, for treatment of a tumor, a cancer, a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy, or an immunodeficiency disease.

32. Use of the pharmaceutical composition of any one of claims 28-30 for treatment of a tumor, a cancer, a non-cancer hyperproliferative disease, a fibrotic disease, an infectious disease, a vasculopathy, or an immunodeficiency disease in a subject.

```
Human      MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
Chimpanzee MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
           ************************************************************

Human      ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Chimpanzee ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
           ************************************************************

Human      MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD 180
Chimpanzee MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRTSEKCSKWILLEDYRKRVQNVTEFDD 180
           ************************************.***********************

Human      SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Chimpanzee SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
           ************************************************************

Human      LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
Chimpanzee LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
           ************************************************************

Human      IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
Chimpanzee IAESIRTAMGLRTKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKVGVKLPYFFHAGET 360
           ****** ***** ********************************* ************

Human      DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
Chimpanzee DWQGTSIDRNILDALMLNTSRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
           **********************:*************************************

Human      LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Chimpanzee LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
           ************************************************************

Human      KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Chimpanzee KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
           ********************************
```

Figure 1A

```
Human     MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
Gorilla   MLVDGPSERPALRFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
          ************ ********************************************** 

Human     ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Gorilla   ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNVLRMMPKGAALHLHDIGIVT 120
          ************************************:********************** 

Human     MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD 180
Gorilla   MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRTSEKCSKWILLEDYRKQVQNVTEFDD 180
          ************************************.*********************:**** 

Human     SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Gorilla   SLLRNFTLVTQHPEVIYTNQNVVWSKFENIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
          ********************************.***************************** 

Human     LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
Gorilla   LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
          ************************************************************ 

Human     IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
Gorilla   IAESIRTAMGLRTKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
          ****** ***** ************************************************ 

Human     DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
Gorilla   DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
          ************************************************************ 

Human     LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Gorilla   LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
          ************************************************************ 

Human     KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Gorilla   KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
          ********************************
```

Figure 1B

```
Human       MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
Pygmy_chimp MLVDGPSERPALCFLLLAVAMSFFSSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
            ************************.*************************************

Human       ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Pygmy_chimp ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
            ************************************************************

Human       MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD 180
Pygmy_chimp MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRTSEKCSKWILLEDYRKRVQNVTEFDD 180
            ************************************.*********************** 

Human       SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Pygmy_chimp SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
            ************************************************************

Human       LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
Pygmy_chimp LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
            ************************************************************

Human       IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
Pygmy_chimp IAESIRTAMGLRTKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKVGVKLPYFFHAGET 360
            ****** ***** ************************************ ***********

Human       DWQGTSIDRNILDALMLNTRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
Pygmy_chimp DWQGTSIDRNILDALMLNASRIGHGFALSKHPAVRTYSWEKDIPIEVCPISNQVLKLVSD 420
            ************************::************************************

Human       LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Pygmy_chimp LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
            ************************************************************

Human       KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Pygmy_chimp KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
            ********************************
```

Figure 1C

```
Human       MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
Orangutan   MLVDGPSEWPALRFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEEQAN 60
            ******** *** ********************************************* **

Human       ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Orangutan   ERLMMLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
            **** ********************************************************

Human       MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD 180
Orangutan   MDWLVRNVTYRPHCHICFTPKGIMQFRFAHPTPRTSEKCSKWILLEDYRKRVQNVTEFDD 180
            ************************:*************************************

Human       SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Orangutan   SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFQSMQEFYEDNV 240
            ************************************************:*********** 

Human       LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
Orangutan   LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
            ************************************************************

Human       IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
Orangutan   IAESIRTAMGLRTKFPTVVAGFDLVGREDTGHSLQDYKEALMIPAKGGVKLPYFFHAGET 360
            ****** ***** ***************:*********:**************.********** 

Human       DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
Orangutan   DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRAYSWKKDIPIEVCPISNQVLKLVSD 420
            ************************************:*********************** 

Human       LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Orangutan   LRNHPVATLMATGHPMVISSDDPAIFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
            ************************:*********************************** 

Human       KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Orangutan   KYSALLEIEKNTFMEIWKKRWDKFIADVATK 511
            ***:*** ********************** 
```

Figure 1D

```
Human     MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
Gibbon    MLVDGPSEWPALRFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLSTKEEQAN 60
          ******** *** ********************************************.***** **

Human     ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Gibbon    ERLMTLKITEMKEAMKTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
          ********:*********************************************** ****

Human     MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD 180
Gibbon    MDWLVRNVTYRPHCHICFTPKGIMQFRFAHPTPRTSEKCSKWILLEDYRKRVQNVTEFDD 180
          ************************:*********.*********************** ***

Human     SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Gibbon    SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFX------DNV 233
          ************************************************       ***

Human     LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 300
Gibbon    LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDHRSKDVAV 293
          ************************************************************

Human     IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
Gibbon    IAESIRTAMGLRAKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPTKDGVKLPYFFHAGET 353
          ****** ***** ********************************:********************

Human     DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRTYSWKKDIPIEVCPISNQVLKLVSD 420
Gibbon    DWQGTSIDKNILDALMLNTTRIGHGFALSKHPAVRAYSWKKDIPIEVCPISNQVLKLVSD 413
          ********:*********************************:********************

Human     LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Gibbon    LRNHPVATLMATGHPMVISSDDPAIFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 473
          ************************************************************

Human     KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Gibbon    KYSTLLETEKNTFMEIWKKRWDKFIADVATK 504
          ********:*****************************
```

Figure 1E

```
Human      MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN  60
Macaque    MLVDGPSEWPALRFLLLAVAMSFFRSALSIDE--AHLLLKEKMMRLGGRLVLTTKEEQAN  58
           ********* *** ************ ********  ********************* ***.**** **

Human      ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT  120
Macaque    ERLMTLKIAEMKEAMKTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDTGIVT  118
           ************************:********************************** ****

Human      MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD  180
Macaque    MDWLVRNVTYRPHCHICFTSKGIMQFRFAHPTPRTSEKCSKWILLEDYRKRVQNVTEFDD  178
           ************************.:********************************** *****

Human      SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV  240
Macaque    SLLRNFTLVTQHPEVIYTNQNVVWSKFQTIFFTISGLIRYAPVFRDYVFRSMQEFYEDNV  238
           ************************:**********:*********************** *****

Human      LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDDRSKDVAV  300
Macaque    LYMEIRARLLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYSDDRSKDVTV  298
           ************************************************.****** *****:*

Human      IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET  360
Macaque    IAESIRTAMGLRTKFPTVVAGFDLVGHEDTGHSLHYYKEALMIPARDGGKLPYFFHAGET  358
           ****** ****** ************************ ********** ************

Human      DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVRTYSWEKDIPIEVCPISNQVLKLVSD  420
Macaque    DWQGTSIDKNILDALMLNTTRIGHGFALSKHPAARAYSWEKDIPIEVCPISNQVLKLVSD  418
           *********:********************.*:****:********************** *****

Human      LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI  480
Macaque    LRNHPVAALMAIGHPMVISSDDPAMFGAKGLSYDFYEAFMGIGGMKADLRTLKQLAMNSI  478
           *********:*** ********************************.********************

Human      KYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Macaque    RYSTLLETEKNTFMEIWKKRWDKFIADVATK 509
           :*******:*********************
```

Figure 1F

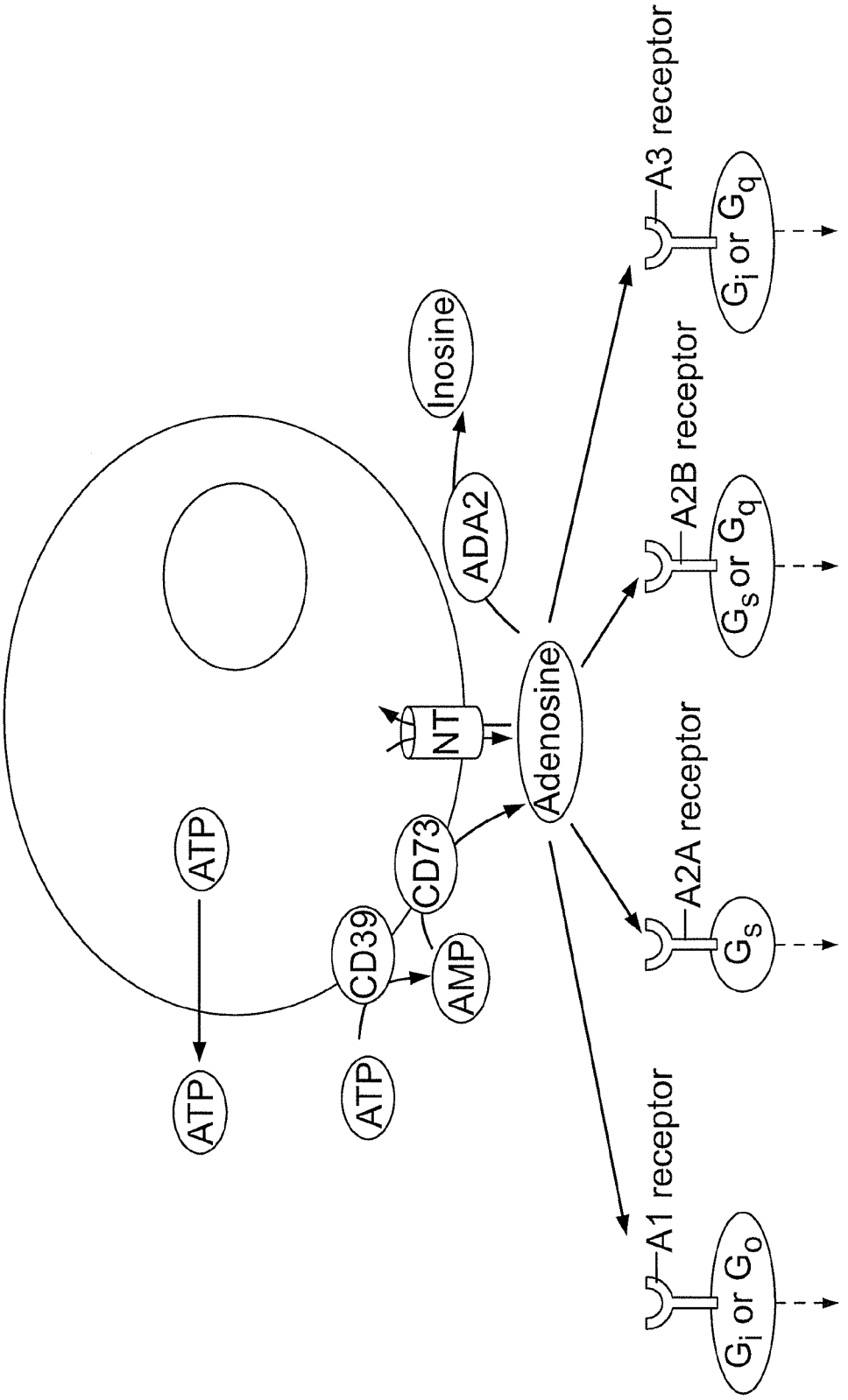
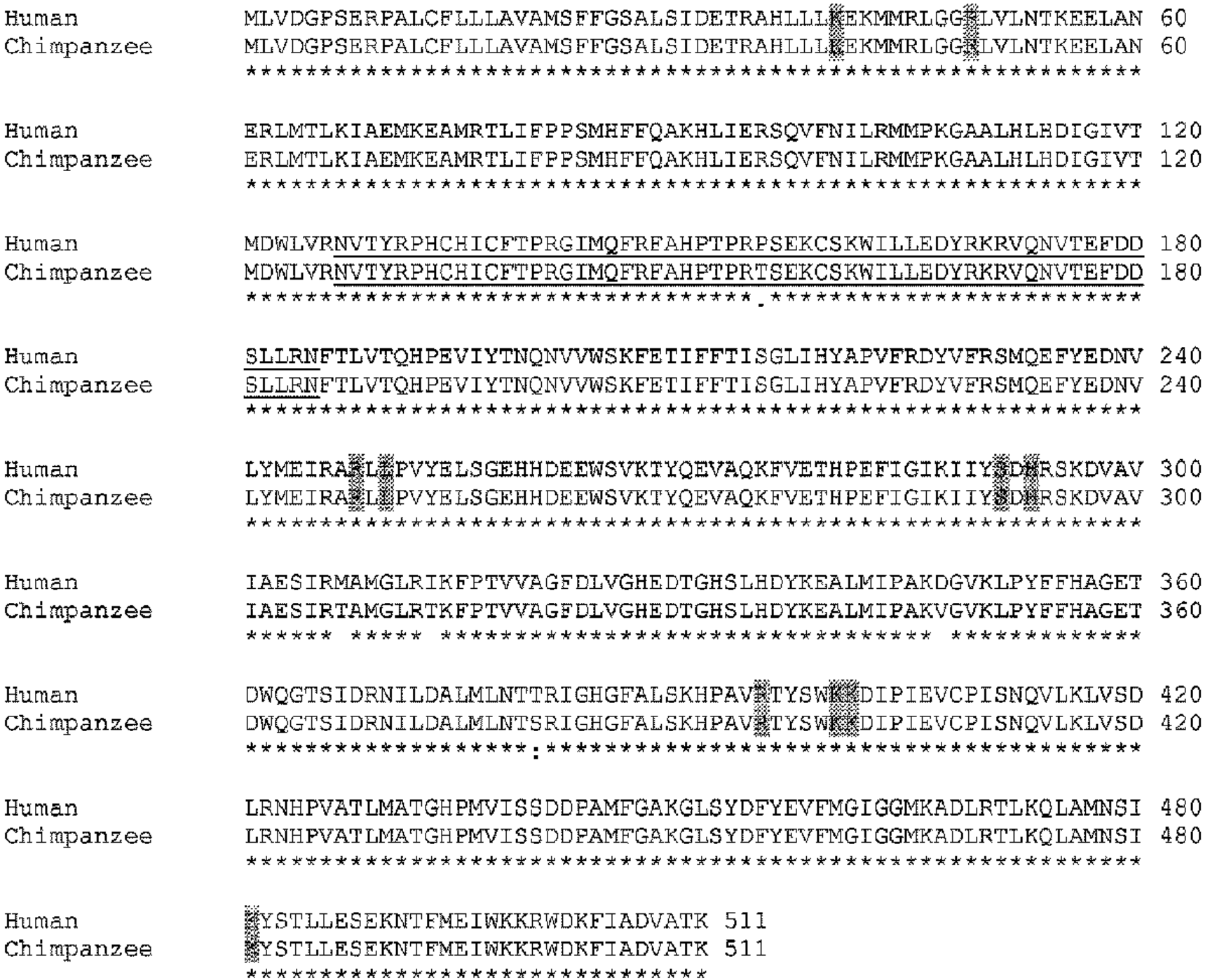


Figure 2

Date Reçue/Date Received 2021-07-28


Human MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
Chimpanzee MLVDGPSERPALCFLLLAVAMSFFGSALSIDETRAHLLLKEKMMRLGGRLVLNTKEELAN 60
Human ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Chimpanzee ERLMTLKIAEMKEAMRTLIFPPSMHFFQAKHLIERSQVFNILRMMPKGAALHLHDIGIVT 120
Human MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRPSEKCSKWILLEDYRKRVQNVTEFDD 180
Chimpanzee MDWLVRNVTYRPHCHICFTPRGIMQFRFAHPTPRTSEKCSKWILLEDYRKRVQNVTEFDD 180
Human SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Chimpanzee SLLRNFTLVTQHPEVIYTNQNVVWSKFETIFFTISGLIHYAPVFRDYVFRSMQEFYEDNV 240
Human LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYGDIRSKDVAV 300
Chimpanzee LYMEIRALLPVYELSGEHHDEEWSVKTYQEVAQKFVETHPEFIGIKIIYGDIRSKDVAV 300
Human IAESIRMAMGLRIKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKDGVKLPYFFHAGET 360
Chimpanzee IAESIRTAMGLRTKFPTVVAGFDLVGHEDTGHSLHDYKEALMIPAKVGVKLPYFFHAGET 360
Human DWQGTSIDRNILDALMLNTTRIGHGFALSKHPAVMTYSWKRDIPIEVCPISNQVLKLVSD 420
Chimpanzee DWQGTSIDRNILDALMLNTSRIGHGFALSKHPAVMTYSWKRDIPIEVCPISNQVLKLVSD 420
Human LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Chimpanzee LRNHPVATLMATGHPMVISSDDPAMFGAKGLSYDFYEVFMGIGGMKADLRTLKQLAMNSI 480
Human RYSTLLESEKNTFMEIWKKRWDKFIADVATK 511
Chimpanzee RYSTLLESEKNTFMEIWKKRWDKFIADVATK 511