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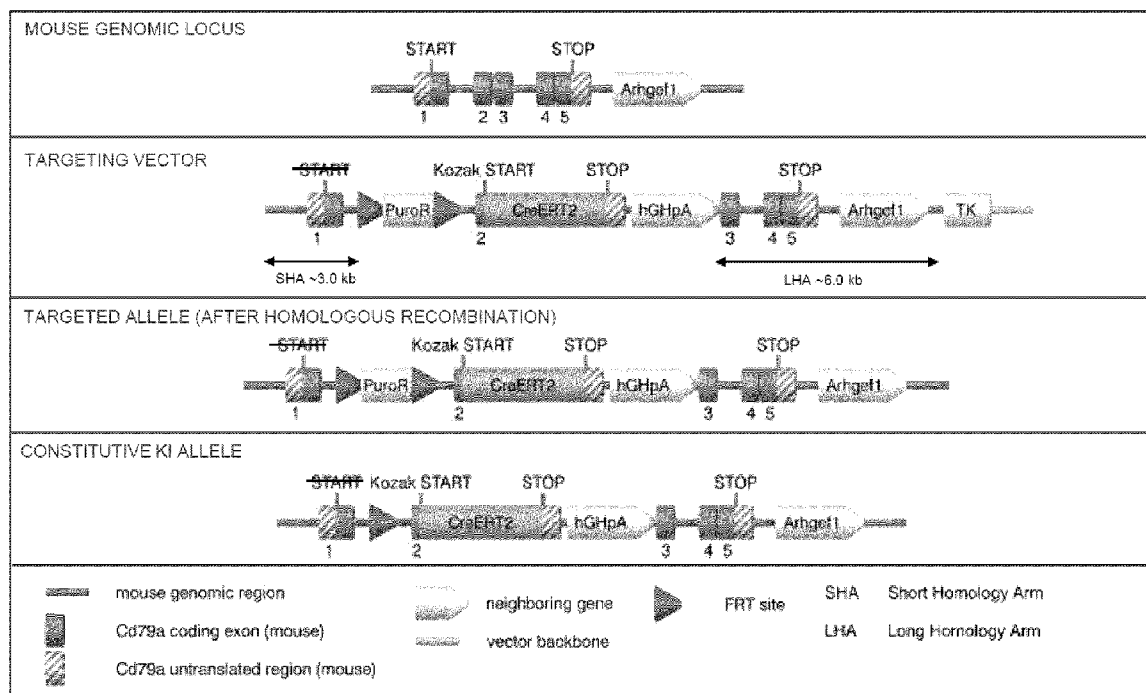


Figure 1A

(57) Abstract: The present disclosure relates to the production of antigen-binding molecules. Animals used in the production of antigen-binding molecules are disclosed, and methods, nucleic acids and cells. Also disclosed are animals comprising sequences providing for inducible inhibition of immune responses.

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Methods for Antibody Production

This application claims priority from US 63/513380 filed 13 July 2023 and US 63/609429 filed 13 December 2023, the contents and elements of which are herein incorporated by reference for all purposes.

Technical Field

The present disclosure relates to the fields of cellular and molecular biology, and immunology. More specifically, the present disclosure relates to the production of antigen-binding molecules, in particular in the context of therapeutic, prophylactic, diagnostic, imaging and research applications.

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Background

Animal immunization is widely used for the development of monoclonal antibodies for therapeutic and diagnostic use. Traditional mouse hybridoma techniques involve producing antibody-secreting hybridoma cell lines by fusing splenocytes harvested from immunized mice with immortalized myeloma cells. While mice are the most commonly used species for the production of antibodies by animal immunization, other species of animal are also used, such as rabbit and goat.

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A key limitation of classical full protein target immunization approaches to antibody development is that they can only produce antibodies against the target with limited control over the site of binding (*i.e.* the target epitope on the protein of interest), with the antibody response primarily directed to immunodominant epitopes. The alternative strategy of small antigen immunization, representing a desired binding region of the target protein, allows more control over the site of antibody binding, but at the expense of increased risk that the antibody will not bind to the protein *in vivo* (*i.e.*, that it will not recognize the protein in its "native" form), *e.g.* due to the antigenic sequence being unavailable, or presented differently, in the native, folded protein. As a result, there is a need for extensive screening to find antibodies that have high binding affinity to the target *in vivo*, with no guarantee of success.

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Existing approaches to antibody production by animal immunization do not provide for the efficient and reliable production of monoclonal antibodies having desired functional properties of interest in relation to their target antigen.

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Summary

In a first aspect, the present disclosure provides an animal comprising an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response.

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In some embodiments, the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in mounting a primary humoral immune response.

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In some embodiments, the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in

immunoglobulin isotype switching, BF cell maturation, and/or production of plasma and/or memory B cells.

5 In some embodiments, the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or both genes selected from CD40 and/or CD40L. In some embodiments, the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of CD40 and/or CD40L.

10 In some embodiments, the animal comprises an endogenous nucleotide sequence providing for recombinase-mediated disruption of expression of one or more genes involved in mounting a primary humoral immune response.

15 In some embodiments, the endogenous nucleotide sequence comprises target sequences for a recombinase flanking all or part of the nucleotide sequence of a gene involved in mounting a primary humoral immune response.

In some embodiments, the animal comprises an endogenous nucleotide sequence providing for inducible expression or activity of the recombinase.

20 In some embodiments, the endogenous nucleotide sequence providing for inducible expression or activity of the recombinase encodes a conditional system for controlling expression or activity of the recombinase.

25 In some embodiments, the target sequences for a recombinase are *loxP* sequences, and the recombinase is a Cre recombinase.

In some embodiments, the animal comprises an endogenous nucleotide sequence encoding one or more human immunoglobulin genes or gene segments.

30 In some embodiments, the animal is a mouse, a rat or a rabbit.

In some embodiments, the endogenous nucleotide sequence comprises target sequences for a recombinase flanking one or more exons of CD40. In some embodiments, the endogenous nucleotide sequence comprises target sequences for a recombinase flanking one or more exons of CD40L.

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In some embodiments, the animal comprises an endogenous nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:4.

40 In some embodiments, the animal comprises an endogenous nucleotide sequence encoding a conditional system for controlling expression and/or activity of a Cre recombinase.

In some embodiments, expression of the Cre recombinase is under the control of a promoter driving expression in B cell lineage cells.

- 5 In some embodiments, the animal comprises an endogenous nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:6.

10 The present disclosure also provides method for producing an antigen-binding molecule, comprising administering a peptide/polypeptide, or nucleic acid encoding a peptide/polypeptide, to an animal according to the present disclosure.

In some embodiments, the method comprises:

- 15 (i) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;
(ii) treating the animal to inhibit its ability to mount a primary immune response; and
(iii) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

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In some embodiments, the method comprises:

- (i) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;
(ii) administering a second peptide/polypeptide, or nucleic acid encoding the second
25 peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest; and
(iii) treating the animal to inhibit its ability to mount a primary immune response.

- 30 In some embodiments, treating the animal to inhibit its ability to mount a primary immune response comprises administering an agent for inducing expression or activity of the recombinase.

Also provided is a method for producing an antigen-binding molecule, wherein the method comprises:

- 35 (i) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;
(ii) treating the animal to inhibit its ability to mount a primary immune response; and
(iii) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

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In some embodiments, the method comprises treating the animal to inhibit its ability to mount a primary immune response before, during and/or after the administration of the second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide.

5 In some embodiments, treating the animal to inhibit its ability to mount a primary immune response comprises administering an agent that inhibits the expression of, or inhibits the activity of the product of, one or more genes involved in mounting a primary humoral immune response. In some embodiments, treating the animal to inhibit its ability to mount a primary immune response comprises administering an agent that reduces/blocks stimulation, differentiation, activation, maturation, and/or proliferation of naïve B
10 cells in the animal, reduces the number/proportion of naïve B cells in the animal, and/or inhibits immunoglobulin isotype switching and/or B cell maturation. In some embodiments, the agent inhibits the expression of, or inhibits the activity of the product of, one or both genes selected from CD40 and/or CD40L.

15 In some embodiments, the agent inhibits the activity of CD40 and/or CD40L. In some embodiments, the agent is selected from, or comprises, an antibody, antigen-binding molecule, polypeptide, decoy receptor, aptamer, sequestering agent or small molecule.

In some embodiments, the agent inhibits the expression of CD40 and/or CD40L. In some embodiments,
20 the agent is, or comprises, RNAi, siRNA, an antisense nucleic acid, an antisense oligonucleotide, or a gene editing system.

In some embodiments, the animal comprises an endogenous nucleotide sequence encoding one or more human immunoglobulin genes or gene segments. In some embodiments, the animal is a mouse, a rat or
25 a rabbit.

In some embodiments, the methods further comprise generating a hybridoma producing the antigen-binding molecule capable of binding to a protein/protein complex of interest.

30 In some embodiments, the methods further comprise isolating one or more antigen-binding molecules capable of binding to a protein comprising the amino acid sequence of interest.

In some embodiments, the methods further comprise formulating the antigen-binding molecule capable of binding to a protein comprising the amino acid sequence of interest to a pharmaceutical composition.
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In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence providing for inducible inhibition of a primary humoral immune response.

In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence
40 providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in mounting a primary humoral immune response.

In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in immunoglobulin isotype switching and/or B cell maturation, or one or more genes involved in stimulation, differentiation, activation and/or proliferation of naïve B cells..

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In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or both genes selected from CD40 and/or CD40L.

10 In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence providing for recombinase-mediated disruption of expression of one or more genes involved in mounting a primary humoral immune response.

The present disclosure also provides a nucleic acid, or a plurality of nucleic acids, comprising a
15 nucleotide sequence encoding all or part of the nucleotide sequence of a gene involved in mounting a primary humoral immune response, flanked by target sequences for a recombinase.

In some embodiments, the nucleic acid/plurality of nucleic acids further comprises a nucleotide sequence encoding a conditional system for controlling expression or activity of the recombinase.

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In some embodiments, the target sequences for a recombinase are *loxP* sequences, and the recombinase is a Cre recombinase.

In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence
25 comprising target sequences for a recombinase flanking one or more exons of CD40, and/or one or more exons of CD40L.

In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity
30 to SEQ ID NO:4.

In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence encoding a conditional system for controlling expression and/or activity of a Cre recombinase.

35 In some embodiments, expression of the Cre recombinase is under the control of a promoter driving expression in B cell lineage cells.

In some embodiments, the nucleic acid, or a plurality of nucleic acids, comprises a nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity
40 to SEQ ID NO:6.

The present disclosure also provides a vector, or a plurality of vectors, comprising the nucleic acid or plurality according to the present disclosure.

The present disclosure also provides a cell comprising the nucleic acid or plurality of nucleic acids or the vector or plurality of vectors according to the present disclosure. In some embodiments, the cell comprises an endogenous nucleotide sequence encoding one or more human immunoglobulin genes or gene segments.

In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is an embryonic stem cell. In some embodiments, the cell is a mouse cell, a rat cell or a rabbit cell.

In some embodiments, the mouse is a hyperimmune mouse or a mouse having a hyperimmune phenotype.

In some embodiments, the animal is a humanized mouse.

Description

The present disclosure is broadly concerned with the production of antibodies (particularly IgG antibodies, and preferably ultimately monoclonal IgG antibodies) possessing certain desirable functional properties, which are the product of binding to a region of a target protein/protein complex of interest.

This can be achieved by (i) immunizing an animal with a first peptide/polypeptide comprising an amino acid sequence of interest (*i.e.* (a) an amino acid sequence of a protein/protein complex of interest, or (b) an amino acid sequence which is similar to the amino acid sequence of (a)) to elicit a primary immune response directed against the amino acid sequence of interest, (ii) inhibiting the animal's ability to mount a primary immune response, and subsequently (iii) immunizing the animal with a second peptide/polypeptide comprising the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

In some embodiments, this can be achieved by (i) immunizing an animal comprising an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response with a first peptide/polypeptide comprising an amino acid sequence of interest (*i.e.* (a) an amino acid sequence of a protein/protein complex of interest, or (b) an amino acid sequence which is similar to the amino acid sequence of (a)) to elicit a primary immune response directed against the amino acid sequence of interest, (ii) inhibiting the animal's ability to mount a primary immune response, and subsequently (iii) immunizing the animal with a second peptide/polypeptide comprising the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

In some embodiments, this can be achieved by (i) immunizing an animal with a first peptide/polypeptide comprising an amino acid sequence of interest (*i.e.* (a) an amino acid sequence of a protein/protein complex of interest, or (b) an amino acid sequence which is similar to the amino acid sequence of (a)) to elicit a primary immune response directed against the amino acid sequence of interest, (ii) immunizing the

animal with a second peptide/polypeptide comprising the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest, and subsequently (iii) inhibiting the animal's ability to mount a primary immune response. In some cases, the animal may comprise an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response.

Inhibiting the ability of the animal to mount a primary immune response favors a secondary immune response to the amino acid sequence of interest on administration of the second peptide/polypeptide. Secondary immune responses are associated with the production of antibodies (in particular IgG antibodies) which bind to their target with high affinity.

In some embodiments, the second peptide/polypeptide is non-identical to the first peptide/polypeptide.

Inhibiting a primary immune response and/or promoting a secondary immune response

Aspects and embodiments of the present disclosure relate to the inhibition of a primary immune response (e.g. a primary humoral immune response) in an animal, e.g. using an agent. In some embodiments the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of a primary immune response.

The aim is to favor a secondary humoral immune response to the amino acid sequence of interest as presented by the second peptide/polypeptide over a primary humoral immune response to regions of the second peptide/polypeptide which are not present in the first peptide/polypeptide.

That is, it is intended to suppress a primary humoral immune response to regions of the second peptide/polypeptide other than the amino acid sequence of interest, and/or enhance a secondary humoral immune response to the amino acid sequence of interest as presented in the second peptide/polypeptide.

To be clear, it is not intended to inhibit a primary humoral immune response to the first peptide/polypeptide. In particular, it is not intended to prevent immunoglobulin isotype switching by, or to deplete, cells activated/stimulated to proliferate by administration of the first peptide/polypeptide, or cells derived from such cells (i.e. the progeny of cells activated/stimulated to proliferate by administration of the first peptide/polypeptide).

In some embodiments, inhibition of the ability to mount a primary humoral immune response does not inhibit the development of IgG-, IgA- and/or IgE-expressing cells from cells activated/stimulated to proliferate by administration of the first peptide/polypeptide. In some embodiments, the development of plasma B cells and/or memory B cells from cells activated/stimulated to proliferate by administration of the first peptide/polypeptide is not inhibited.

In some embodiments, inhibition of the ability to mount a primary humoral immune response does not deplete IgG-, IgA- and/or IgE-expressing cells (e.g. IgG-, IgA- and/or IgE- expressing cells produced by immunoglobulin isotype switching from cells activated/stimulated to proliferate by administration of the

first peptide/polypeptide). In some embodiments, plasma B cells and/or memory B cells (e.g. plasma B cells and/or memory B cells produced by immunoglobulin isotype switching from cells activated/stimulated to proliferate by administration of the first peptide/polypeptide) are not depleted.

5 Affinity maturation of memory B cells producing antigen-binding molecules capable of recognizing the amino acid sequence of interest is preferably favored over the generation of a primary humoral immune response to regions other than the amino acid sequence of interest following introduction of the second peptide/polypeptide.

10 In such embodiments, the agent may suppress the primary immune response to sequences in the second peptide/polypeptide which are not present in the first peptide/polypeptide.

Primary and secondary immune responses are components of the adaptive immune response. The adaptive immune response is described e.g. in *Janeway's Immunobiology* 9th Edn.; Murphy *et al.*, 2017

15 (Garland Science, Taylor & Francis), in particular at part IV.

The primary immune response refers to a response of the adaptive immune system of a subject following an initial exposure to an antigen.

20 Triggering of the primary immune response generally involves antigen uptake and processing and presentation on MHC Class II molecules by antigen presenting cells (APCs) such as dendritic cells. In the presence of appropriate costimulation, naïve T cells comprising a T cell receptor (TCR) specific for an MHC class II:peptide complex become activated and are stimulated to proliferate.

25 Antigen also binds to cognate B cell receptors (BCRs) expressed on the surface of naïve B cells. The bound antigen is internalized, processed and presented in the context of MHC class II:peptide complex on the surface of the B cell. Effector follicular T helper cells (T_{FH}) comprising a TCR specific for the MHC class II:peptide complex presented by the B cell are stimulated to produce cytokines such as IL-4 and IL-21, which induce B cell proliferation and differentiation into plasma B cells and memory B cells.

30 Antibodies initially produced by the primary immune response are predominantly IgM isotype antibodies, and bind to the target antigen with low affinity.

The secondary immune response refers to a response of the adaptive immune system of a subject
35 following exposure to an antigen to which the subject has already produced a primary immune response.

Upon subsequent exposure, antigen binds to the BCR of memory B cells (*i.e.* memory B cells generated by the primary immune response on initial exposure to the antigen), which internalise, process and present to, and thus activate, memory T_{FH} cells.

40 Upon antigen-binding, memory B cells in the germinal centers undergo V region somatic hypermutation, resulting in the production of closely related B cell clones having BCRs with different affinities for the

antigen. B cells with high affinity BCRs recognize antigen presented by follicular dendritic cells, and process and present antigen to T_{FH} cells, which promote the survival of the B cells through production of IL-21 and ligation of CD40. Activated B cells maturing in the germinal centers also undergo immunoglobulin class switching to produce IgG, IgA or IgE antibodies. B cells expressing high-affinity BCRs differentiate to mature plasma B cells, which produce large amounts of high-affinity antibody to the antigen.

As used herein, a “humoral immune response” refers to an immune response involving the production of antibodies from B cells.

A primary humoral immune response may be characterized by: activation and/or maturation of a naïve B cell (*i.e.* stimulation of a naïve B cell to proliferate, differentiate and/or undergo immunoglobulin class switching), production of IL-4 and/or IL-21 by a B cell, immunoglobulin class switching of a IgM- and/or IgD-expressing cell to an IgG-, IgE-, or IgA-expressing cell, differentiation of a naïve B cell to a plasma B cell, differentiation of a naïve B cell to a memory B cell, and/or production of antibodies (*e.g.* IgM antibodies) which bind to their target antigen with low affinity.

B cell development is described *e.g.* in Pieper *et al.*, J Allergy Clin Immunol (2013) 131(4):959-71, which is hereby incorporated by reference in its entirety. Characteristics of B cells are described in *e.g.* Carsetti *et al.*, Cytometry A. 2022;101(2):131-139, which is hereby incorporated by reference in its entirety.

As used herein, a “precursor cell to a naïve B cell” refers to a cell type upstream of a naïve B cell in course of B cell development. In some embodiments, a precursor cell to a naïve B cell may be selected from: a stem cell, a pro-B cell, an early pro-B cell, a late pro-B cell, a pre-B cell, a large pre-B cell, a small pre-B cell or an immature B cell.

A stem cell may be a hematopoietic stem cell, and may *e.g.* be characterized by expression (*e.g.* surface expression) of CD34, and/or lack of expression (*e.g.* surface expression) of CD10. An early pro-B cell may be characterized by expression (*e.g.* surface expression) of CD10, CD43, CD45 and/or MHC class II. A late pro-B cell may be characterized by expression (*e.g.* surface expression) of CD19, CD40, CD43, CD45 and/or MHC class II. A large pre-B cell may be characterized by expression (*e.g.* surface expression) of pre-BCR, CD19, CD40, CD43, CD45 and/or MHC class II. A small pre-B cell may be characterized by expression (*e.g.* surface expression) of pre-BCR, CD19, CD40, CD45 and/or MHC class II. An immature B cell may be characterized by expression (*e.g.* surface expression) of CD10, CD19, CD20, CD24, CD38, CD40, CD45, IgM and/or MHC class II, and/or lack of expression (*e.g.* surface expression) of CD27.

As used herein, a “naïve” B cell refers to a mature B cell which has not encountered the antigen for which the BCR of the B cell is specific. A naïve B cell may also be referred to as a mature naïve B cell, or a mature B cell. A naïve B cell may be characterized by expression of one or more of the following (*e.g.* at the cell surface): CD19, CD20, CD24, CD40, CD38, CD45, CD21, MHC Class II, IgM and IgD. A naïve B cell may be characterized by lack of expression (*e.g.* at the cell surface) of CD27.

As used herein, a “plasma” B cell refers to a B cell which expresses large amounts of soluble antibody. A plasma B cell may be characterized by expression of one or more of the following (e.g. at the cell surface): CD27, CD38, CD138, CD78, CD126, CXCR4 and BCMA. A plasma B cell may be characterized by lack of expression (e.g. at the cell surface): of CD20 and/or CD24.

As used herein, a “memory” B cell refers to a B cell formed in a germinal center following a primary immune response. A memory B cell may be characterized by expression of one or more of the following (e.g. at the cell surface): CD19, CD20, CD21, CD24, CD27, CD95, CD148, MHC Class II and TACI.

Reference to a given gene or protein in the present specification includes isoforms, fragments, variants or homologues of the gene/protein, from any species. It will be appreciated that in aspects and embodiments relating to a particular animal, the gene or protein is the appropriate homologue encoded by the genome of the animal. For example, where the animal is a mouse, the gene/protein may be the mouse homologue of the relevant gene/protein. In a further example of a transgenic mouse encoding the human homologue of the relevant gene/protein, the gene may be the human homologue of the relevant gene/protein.

A secondary humoral immune response may be characterized by: activation of a memory B cell (*i.e.* stimulation of a memory B cell to proliferate and/or differentiate), somatic hypermutation of a memory B cell, differentiation of a memory B cell to a plasma B cell, and/or production of antibodies (e.g. IgG antibodies) which bind to their target antigen with high affinity.

Some aspects and embodiments of the present disclosure are concerned with agents providing for inhibition of a primary humoral immune response in an animal. Some aspects and embodiments of the present disclosure are concerned with animals comprising endogenous nucleotide sequences providing for inducible inhibition of a primary humoral immune response in the animal. It will be appreciated that the effect of such inhibition can be to favor/promote a secondary humoral immune response in the animal.

Also provided by the present disclosure are agents, e.g. as described herein, for use in the methods disclosed herein, *i.e.* for inhibition of a primary humoral immune response in an animal. Also provided are the use of said agents, *i.e.* for inhibition of a primary humoral immune response in an animal.

Herein, “inhibition” may also be referred to as “antagonism”. Agents capable of inhibiting a response/expression/an activity may be referred to as “inhibitors” or “antagonists” of the relevant response/expression/activity.

In some embodiments, inhibition of a primary humoral immune response in accordance with the present disclosure comprises one or more of: inhibiting the expression of a gene involved in mounting a primary humoral immune response, inhibiting the activity of the product of a gene involved in mounting a primary humoral immune response, reducing the number/proportion of naïve B cells, reducing/blocking stimulation, differentiation, activation, maturation, and/or proliferation of naïve B cells e.g., by T cells,

inhibiting immunoglobulin isotype switching, inhibiting the expression of a gene involved in immunoglobulin isotype switching and/or inhibiting the activity of the product of a gene involved in immunoglobulin isotype switching.

5 **As used herein, “expression” may refer to gene or protein expression.** Gene expression encompasses transcription of DNA to RNA, and can be analyzed by various means known to those skilled in the art, for example by measuring levels of mRNA by quantitative real-time PCR (qRT-PCR), or by reporter-based methods. Similarly, protein expression can be measured by various methods well known in the art, *e.g.* by antibody-based methods, for example by western blot, immunohistochemistry, immunocytochemistry,
10 flow cytometry, ELISA, or reporter-based methods.

As used herein, a factor/activity/cell type which is “involved in” a given response/process (*e.g.* mounting an immune response, immunoglobulin isotype switching, *etc.*) refers to a factor/activity/cell type which is implicated in the relevant response/process. The factor/activity/cell type preferably contributes positively
15 to (*i.e.* promotes, potentiates) the relevant response/process, and may *e.g.* be required for the relevant response/process, meaning that the response/process does not occur in the absence of the factor/activity/cell type.

Aspects and embodiments of the present disclosure are concerned with inhibiting the expression (*i.e.* gene or protein expression) of one or more genes involved in mounting a primary humoral immune response, and/or inhibiting the activity of the product of one or more genes involved in mounting a primary humoral immune response.

In preferred embodiments, the one or more genes are not involved in, or required for, mounting a
25 secondary humoral immune response. In some embodiments, the one or more genes involved in mounting a primary humoral immune response are selected from CD40 and/or CD40L. In some embodiments, the one or more genes involved in mounting a primary humoral immune response is CD40.

Inhibition of gene or protein expression of a given gene may comprise *e.g.* inhibiting transcription of the
30 gene, inhibiting post-transcriptional processing (*e.g.* splicing) of RNA transcribed from the gene, reducing the stability of RNA transcribed from the gene, promoting degradation of RNA transcribed from the gene, inhibiting translation of RNA transcribed from the gene into protein, inhibiting post-translational processing of a polypeptide encoded by the gene, reducing the stability of a polypeptide encoded by the gene, or promoting degradation of a polypeptide encoded by the gene.

Gene expression can be analyzed by means well known to the skilled person. The level of RNA encoding a given gene can be determined *e.g.* by techniques such as RT-qPCR. Protein expression can also be determined by means well known to the skilled person. The level of a given protein/isoform thereof can be determined *e.g.* by antibody-based methods including western blot, immunohisto/cytochemistry, flow
40 cytometry, ELISA, *etc.*

Inhibition of gene or protein expression of a given gene may be to less than 1 times, *e.g.* one of ≤ 0.99 times, ≤ 0.95 times, ≤ 0.9 times, ≤ 0.85 times, ≤ 0.8 times, ≤ 0.75 times, ≤ 0.7 times, ≤ 0.65 times, ≤ 0.6 times, ≤ 0.55 times, ≤ 0.5 times, ≤ 0.45 times, ≤ 0.4 times, ≤ 0.35 times, ≤ 0.3 times, ≤ 0.25 times, ≤ 0.2 times, ≤ 0.15 times, ≤ 0.1 times, ≤ 0.05 times, or ≤ 0.01 times the level of expression observed in the uninhibited state. In

5 some embodiments, inhibition of gene or protein expression inhibits greater than 5%, *e.g.* one of $\geq 10\%$, $\geq 15\%$, $\geq 20\%$, $\geq 25\%$, $\geq 30\%$, $\geq 35\%$, $\geq 40\%$, $\geq 45\%$, $\geq 50\%$, $\geq 55\%$, $\geq 60\%$, $\geq 65\%$, $\geq 70\%$, $\geq 75\%$, $\geq 80\%$, $\geq 85\%$, $\geq 90\%$, $\geq 91\%$, $\geq 92\%$, $\geq 93\%$, $\geq 94\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$ or 100% of the expression observed in the uninhibited state.

10 Inhibition of gene or protein expression may be achieved *e.g.* by altering/disrupting the nucleotide sequence of the gene, or altering/disrupting nucleotide sequence required for expression of the gene (*e.g.* a regulatory sequence governing expression of the gene). In some embodiments, inhibiting gene or protein expression may comprise altering a nucleotide sequence, *e.g.* by substitution, deletion or insertion of one or more nucleotides. For example, in particular aspects and embodiments the present disclosure
15 contemplates inhibiting gene or protein expression by deletion of all or part of the nucleotide sequence of the relevant gene.

In some embodiments, altering/disrupting the nucleotide sequence, may comprise *e.g.* altering/removing a regulatory sequence (*e.g.* a promoter, an enhancer) for transcription of the gene, introducing a
20 premature stop codon in the sequence transcribed from the gene, altering the nucleotide sequence to encode a truncated and/or non-functional gene product, or altering the nucleotide sequence to encode a gene product which is misfolded and/or degraded.

Altering/disrupting the nucleotide sequence inhibit/prevent gene or protein expression from a gene may
25 be referred to as gene 'knockout'.

Nucleotides sequences may be disrupted *e.g.* by homologous recombination, or by target nucleic acid modification using site-specific nucleases (SSNs, also referred to herein as 'gene editing systems').

30 Modification by homologous recombination may involve the exchange of nucleic acid sequence through crossover events guided by homologous sequences, and is reviewed, for example, in Mortensen Curr Protoc Neurosci. (2007) Chapter 4:Unit 4.29 and Vasquez *et al.*, PNAS (2001) 98(15): 8403-8410 both of which are hereby incorporated by reference in their entirety. The homologous sequences flank all or part of the nucleotide sequence to be disrupted. Recombination may be catalysed by a recombinase. For
35 example, in particular aspects and embodiments the present disclosure contemplates disruption of a nucleotide sequence by homologous recombination between *loxP* sequences, catalysed by a *Cre* recombinase.

Disruption of a nucleotide sequence by homologous recombination can be achieved in an animal *e.g.* as
40 described hereinabove in relation to *Cre-LoxP*, *Flp-FRT* and *Dre-rox* systems.

Gene editing using SSNs is reviewed e.g. in Eid and Mahfouz, *Exp Mol Med.* (2016) 48(10): e265, which is hereby incorporated by reference in its entirety. Enzymes capable of creating site-specific double strand breaks (DSBs) can be engineered to introduce DSBs to target nucleic acid sequences of interest. DSBs may be repaired by either error-prone non-homologous end-joining (NHEJ), in which the two ends of the break are rejoined, often with insertion or deletion of nucleotides. Alternatively, DSBs may be repaired by highly homology-directed repair (HDR), in which a DNA template with ends homologous to the break site is supplied and introduced at the site of the DSB. SSNs capable of being engineered to generate target nucleic acid sequence-specific DSBs include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced palindromic repeats/CRISPR-associated-9 (CRISPR/Cas9) systems.

ZFN systems are reviewed e.g. in Umov *et al.*, *Nat Rev Genet.* (2010) 11(9):636-46, which is hereby incorporated by reference in its entirety. ZFNs comprise a programmable Zinc Finger DNA-binding domain and a DNA-cleaving domain (e.g. a *FokI* endonuclease domain). The DNA-binding domain may be identified by screening a Zinc Finger array capable of binding to the target nucleic acid sequence. TALEN systems are reviewed e.g. in Mahfouz *et al.*, *Plant Biotechnol J.* (2014) 12(8):1006-14, which is hereby incorporated by reference in its entirety. TALENs comprise a programmable DNA-binding TALE domain and a DNA-cleaving domain (e.g. a *FokI* endonuclease domain). TALEs comprise repeat domains consisting of repeats of 33-39 amino acids, which are identical except for two residues at positions 12 and 13 of each repeat which are repeat variable di-residues (RVDs). Each RVD determines binding of the repeat to a nucleotide in the target DNA sequence according to the following relationship: "HD" binds to C, "NI" binds to A, "NG" binds to T and "NN" or "NK" binds to G (Moscou and Bogdanove, *Science* (2009) 326(5959):1501.). CRISPR/Cas9 and related systems e.g. CRISPR/Cpf1, CRISPR/C2c1, CRISPR/C2c2 and CRISPR/C2c3 are reviewed e.g. in Nakade *et al.*, *Bioengineered* (2017) 8(3):265-273, which is hereby incorporated by reference in its entirety. These systems comprise an endonuclease (e.g. Cas9, Cpf1 *etc.*) and the single-guide RNA (sgRNA) molecule. The sgRNA can be engineered to target endonuclease activity to nucleic acid sequences of interest.

As used herein, a "product of" a gene may e.g. refer to a nucleic acid transcribed from the gene, or a peptide/polypeptide (including peptides/polypeptides modified with chemical moieties, e.g. carbohydrate and/or lipid moieties) produced by translation of a nucleic acid transcribed from the gene (and where appropriate, further post-translational processing).

Inhibition of the activity of the product of a given gene may comprise e.g. inhibiting gene or protein expression of the gene (e.g. as described hereinabove), or inhibiting one or more activities of the product of the gene. An activity of a product of a gene may e.g. be catalytic activity, binding (e.g. protein-protein interaction, such as ligand-receptor binding, multimerization, *etc.*), signalling, transport, storage, structural support *etc.*

Inhibition of an activity of a product of a given gene may be to less than 1 times, e.g. one of ≤ 0.99 times, ≤ 0.95 times, ≤ 0.9 times, ≤ 0.85 times, ≤ 0.8 times, ≤ 0.75 times, ≤ 0.7 times, ≤ 0.65 times, ≤ 0.6 times, ≤ 0.55 times, ≤ 0.5 times, ≤ 0.45 times, ≤ 0.4 times, ≤ 0.35 times, ≤ 0.3 times, ≤ 0.25 times, ≤ 0.2 times, ≤ 0.15 times,

≤0.1 times, ≤0.05 times, or ≤0.01 times the level of the activity observed in the uninhibited state. In some embodiments, inhibition an activity of a product of a given gene inhibits greater than 5%, e.g. one of ≥10%, ≥15%, ≥20%, ≥25%, ≥30%, ≥35%, ≥40%, ≥45%, ≥50%, ≥55%, ≥60%, ≥65%, ≥70%, ≥75%, ≥80%, ≥85%, ≥90%, ≥91%, ≥92%, ≥93%, ≥94%, ≥95%, ≥96%, ≥97%, ≥98%, ≥99% or 100% of the activity observed in the uninhibited state.

In some embodiments the agent is capable of decreasing the expression of a target gene or protein (*i.e.* a gene or protein involved in mounting a primary immune response), and/or is capable of decreasing activity of said target gene or protein. In some cases, the agent inhibits, degrades, silences, knocks down, reduces or otherwise decreases expression and/or activity of a gene or protein involved in mounting a primary immune response.

The agent may possess one or more of the following properties in relation to a target gene or protein (*i.e.* a gene or protein involved in mounting a primary immune response): acts to inhibit expression of the gene and/or protein; interferes with transcription of the gene; interferes with translation of mRNA encoding the protein; degrades mRNA encoding the protein; binds to the protein; sequesters the protein; competes for binding of the protein; and/or blocks activity of the protein.

In some embodiments the agent is capable of inhibiting the expression of, or inhibiting the activity of the product of, one or both genes selected from CD40 and/or CD40L.

Such agents may be used to treat an animal as described herein. In some embodiments, the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of a primary immune response, as described herein. In some embodiments the animal does not comprise an endogenous nucleotide sequence providing for inducible inhibition of a primary immune response.

In some embodiments, the agent is an antibody or antigen-binding molecule (both referred to herein as “antigen-binding molecule”) e.g. an anti-CD40 or anti-CD40L antibody. In some cases, the antigen-binding molecule is specific for a protein involved in mounting a primary immune response, for example CD40 or CD40L. In some cases, the antigen-binding molecule displays specific binding to a protein involved in mounting a primary immune response, for example CD40 or CD40L. In some cases, the antigen-binding molecule is an anti-CD40 or anti-CD40L antigen-binding molecule.

The antigen-binding molecule may be an antagonist antigen-binding molecule that inhibits or reduces a biological activity of a target protein involved in mounting a primary immune response, such as CD40 or CD40L.

The antigen-binding molecule may bind to a particular region of interest of a target protein involved in mounting a primary immune response, such as CD40 or CD40L. The antigen-binding region of an antigen-binding molecule may bind to a linear epitope of a target protein involved in mounting a primary immune response, such as CD40 or CD40L, consisting of a contiguous sequence of amino acids (*i.e.* an amino acid primary sequence). In some embodiments, the antigen-binding region molecule may bind to a

conformational epitope of a target protein involved in mounting a primary immune response, such as CD40 or CD40L, consisting of a discontinuous sequence of amino acids of the amino acid sequence.

The antigen-binding molecule may be a multispecific antigen-binding molecule. By “multispecific” it is meant that the antigen-binding molecule displays specific binding to more than one target. In some embodiments the antigen-binding molecule is a bispecific antigen-binding molecule. In some embodiments the antigen-binding molecule comprises at least two different antigen-binding domains (i.e. at least two antigen-binding domains, e.g. comprising non-identical VHs and VLs). Multispecific antigen-binding molecules may be provided in any suitable format, such as those formats described in described in Brinkmann and Kontermann MABs (2017) 9(2): 182-212, which is hereby incorporated by reference in its entirety.

In some embodiments the antigen-binding molecule binds to a target protein involved in mounting a primary immune response, such as CD40 or CD40L, and another target (e.g. an antigen other than the target protein), and so is at least bispecific. The term “bispecific” means that the antigen-binding molecule is able to bind specifically to at least two distinct antigenic determinants.

The ability of a given polypeptide to bind specifically to a given molecule or another given peptide/polypeptide can be determined by analysis according to methods known in the art, such as by ELISA, Surface Plasmon Resonance (SPR; see e.g. Hearty et al., *Methods Mol Biol* 2012, 907:411-442), Bio-Layer Interferometry (see e.g. Lad et al., *J Biomol Screen.* 2015, 20(4): 498-507), flow cytometry, or by a radiolabeled antigen-binding assay (RIA) enzyme-linked immunosorbent assay. Through such analysis binding to a given molecule can be measured and quantified. In some embodiments, the binding may be the response detected in a given assay. Binding affinity may be expressed in terms of dissociation constant (KD).

The region of a peptide/polypeptide to which an antibody binds can be determined by the skilled person using various methods well known in the art, including X-ray co-crystallography analysis of antibody-antigen complexes, peptide scanning, mutagenesis mapping, hydrogen-deuterium exchange analysis by mass spectrometry, phage display, competition ELISA and proteolysis-based ‘protection’ methods. Such methods are described, for example, in Gershoni et al., *BioDrugs*, 2007, 21(3):145-156, which is hereby incorporated by reference in its entirety.

In some embodiments the antigen-binding molecule inhibits the interaction between two binding partners, such as CD40 and CD40L. The ability of an antigen-binding molecule to inhibit interaction between two binding partners can be determined by analysis of the downstream functional consequences of such interaction in an appropriate assay e.g. by detecting the production of protein from a reaction using ELISA, Western blotting or electrophoresis methods.

A person skilled in the art will be able to produce suitable antigen binding molecules using e.g. techniques as described herein or those known in the art, see e.g. Chiu and Gilliland, *Curr Opin Struct Biol.* 2016, 38:163–173, Jakobovits A, *Curr Opin Biotechnol.* 1995 Oct;6(5):561-6, and Brüggemann M et al., *Arch*

Immunol Ther Exp (Warsz). 2015; 63(2): 101–108. One suitable technique is phage display technology, see e.g. Hammers and Stanley, *J Invest Dermatol*. 2014, 134(2): e17 and Bazan J et al., *Hum Vaccin Immunother*. 2012, 8(12): 1817–1828. Antigen-binding polypeptide chains may also be produced by techniques such as chemical synthesis (see e.g. Chandrudu et al., *Molecules* (2013), 18: 4373–4388), recombinant expression such as the techniques set out in Green and Sambrook, *Molecular Cloning: A Laboratory Manual* (4th Edition), Cold Spring Harbor Press, 2012, and in *Nat Methods*. (2008); 5(2): 135–146, or cell-free-protein synthesis (CFPS; see e.g., Zemella et al. *Chembiochem* (2015) 16(17): 2420–2431), all of which are hereby incorporated by reference in their entirety. The antigen-binding molecule may be monoclonal, i.e. a homogenous population of antibodies specifically targeting a single epitope on an antigen. Monoclonal antibodies to selected antigens may be prepared by known techniques, for example those disclosed in "Monoclonal Antibodies: A manual of techniques", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", J G R Hurrell (CRC Press, 1982). Chimaeric antibodies are discussed by Neuberger et al (1988, 8th International Biotechnology Symposium Part 2, 792–799). Suitable polyclonal antibodies can also be prepared using methods well known in the art.

The antigen-binding portion may be a part of an antibody (for example a Fab fragment) or a synthetic antibody fragment (for example a single chain Fv fragment [ScFv]). Antigen-binding fragments of antibodies, such as Fab and Fab2 fragments may also be used/provided as can genetically engineered antibodies and antibody fragments. The variable heavy (VH) and variable light (VL) domains of the antibody are involved in antigen recognition, a fact first recognized by early protease digestion experiments. Further confirmation was found by "humanization" of rodent antibodies. Variable domains of rodent origin may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parented antibody (Morrison et al (1984) *Proc. Natl. Acad. Sci. USA* 81, 6851–6855).

Antibodies and antigen-binding fragments according to the present disclosure comprise the complementarity-determining regions (CDRs) of an antibody which is capable of binding to the relevant target molecule, i.e. one or more proteins involved in mounting a primary humoral immune response as described herein.

The agent may be nucleic acid-based or comprise nucleic acid elements. The agent may promote silencing of gene expression via RNA-mediated interference (RNAi) or antisense degradation mechanisms, e.g. via RNase H.

In some embodiments the agent is, or comprises, an antisense nucleic acid. An "antisense nucleic acid" as referred to herein is a nucleic acid (e.g. DNA or RNA molecule) that is complementary to at least a portion of a specific target nucleic acid (e.g. an mRNA translatable into a protein, such as an FHR protein) and is capable of reducing transcription of the target nucleic acid (e.g. mRNA from DNA), reducing the translation of the target nucleic acid (e.g. mRNA) or altering transcript splicing (e.g. by a single stranded morpholino oligo). Antisense nucleic acids may be single stranded, e.g. gapmers, or may be double stranded e.g. siRNA. Antisense nucleic acids are capable of hybridizing to (e.g. selectively hybridizing to)

a target nucleic acid (e.g. target mRNA) via Watson-Crick base pairing. In some cases the antisense nucleic acids specifically bind to the target nucleic acid. In some cases, the antisense nucleic acid hybridizes to the target nucleic acid sequence (e.g. mRNA) under stringent hybridization conditions. In some cases, the antisense nucleic acid hybridizes to the target nucleic acid (e.g. mRNA) under moderately stringent hybridization conditions.

The nucleotide sequence of an antisense nucleic acid is sufficiently complementary to the target nucleic acid of interest such that it binds or hybridises to the target nucleic acid. Thus, if a skilled person knows the sequence of the target nucleic acid, it is easy and routine to design a suitable antisense nucleic acid that will hybridize to the target to achieve the desired effect.

The target RNA may be an mRNA that encodes for a protein involved in mounting a primary immune response, e.g. CD40 or CD40L.

In some cases the agent is capable of promoting RNA interference (RNAi). RNAi uses small double-stranded RNA molecules to cause degradation of target mRNA. Non-limiting examples of antisense nucleic acids for use as agents according to the present invention include siRNAs (including their derivatives or pre-cursors, such as nucleotide analogs), short hairpin RNAs (shRNA), micro RNAs (miRNA, including their long primary transcripts (pri-miRNAs) and partially processed 60-70 base pair hairpin transcripts (pre-miRNAs)), saRNAs (small activating RNAs) and small nucleolar RNAs (snoRNA) or certain of their derivatives or pre-cursors. Antisense nucleic acid molecules may stimulate RNA interference (RNAi).

siRNA nucleic acids are ~21-25 nucleotides in length and comprise a guide strand which hybridizes with the target mRNA, plus a complementary passenger strand (e.g., each complementary sequence of the double stranded siRNA is 21-25 nucleotides in length, and the double stranded siRNA is about 21-25 base pairs in length). They promote degradation of the target mRNA via RISC. Structure and function of siRNAs are well known in the art and are described in e.g. Kim and Rossi, *Biotechniques*. 2008 Apr; 44(5): 613–616. Suitable siRNA molecules for use in the methods of the present invention may be designed by schemes known in the art, see for example Elbashire et al., *Nature*, 2001 411:494-8; Amarzguioui et al., *Biochem. Biophys. Res. Commun.* 2004 316(4):1050-8; and Reynolds et al., *Nat. Biotech.* 2004, 22(3):326-30. Details for making siRNA molecules can be found in the websites of several commercial vendors such as Ambion, Dharmacon, GenScript, Invitrogen and OligoEngine. The sequence of any potential siRNA candidate generally can be checked for any possible matches to other nucleic acid sequences or polymorphisms of nucleic acid sequence using the BLAST alignment program (see the National Library of Medicine internet website). Typically, a number of siRNAs are generated and screened to obtain an effective drug candidate, see, U.S. Pat. No. 7,078,196. siRNAs can be expressed from a vector and/or produced chemically or synthetically. Synthetic RNAi can be obtained from commercial sources, for example, Invitrogen (Carlsbad, Calif.). RNAi vectors can also be obtained from commercial sources, for example, Invitrogen.

microRNAs (miRNAs) also regulate gene expression via RISC. They are initially expressed as long primary transcripts (pri-miRNAs), which are processed within the nucleus into 60-70 nucleotide hairpins (pre-miRNAs), which are further processed in the cytoplasm into small double stranded nucleic acids that interact with RISC and target mRNA. miRNAs comprise “seed sequences” that are essential for binding to target mRNA. “Seed sequences” usually comprise six nucleotides and are situated at positions 2-7 at the miRNA 5' end.

In some embodiments the agent comprises a double stranded nucleic acid molecule in which one strand is wholly or partially complementary to, or hybridizes with, an mRNA sequence encoding a protein involved in mounting a primary immune response, as described herein, e.g., CD40 or CD40L. In some embodiments the agent comprises a siRNA molecule comprising a guide strand complementary to, or that hybridizes with, a portion of an mRNA sequence that encodes all or part of a protein involved in mounting a primary immune response, as described herein, e.g., CD40 or CD40L. In some embodiments the agent comprises a miRNA molecule (pri-, pre- or mature miRNA) comprising a seed sequence capable of hybridizing to a portion of an mRNA sequence that encodes all or part of a protein involved in mounting a primary immune response, as described herein, e.g., CD40 or CD40L.

In some cases the agent is a single stranded antisense oligonucleotide (ASO). ASOs modify expression of a target RNA, either by altering splicing or by recruiting RNase H to degrade the target RNA. RNase H recognizes DNA:RNA hybrids formed when the ASO binds to the target RNA. ASOs tend to be 18-30 base pairs in length. Many ASOs are designed as chimeras, comprising a mix of bases with different chemistries, or as gapmers, comprising a central DNA portion surrounded by ‘wings’ of modified bases. ASOs are described in e.g. Scoles et al., Neurol Genet. 2019 Apr; 5(2): e323.

Antisense nucleic acids may comprise naturally occurring nucleotides or modifications such as e.g. phosphorothioate linkages, phosphorodiamidate linkages, methoxyethyl nucleotide modifications e.g. 2-MOE, ‘locked’ nucleic acids e.g. LNAs, peptide nucleic acids (PNAs), and/or 5'-methylcytosine modifications.

In some embodiments the agent comprises an antisense oligonucleotide that is capable of hybridizing to a portion of an mRNA sequence that encodes all or part of a protein involved in mounting a primary immune response, as described herein, e.g., CD40 or CD40L.

Antisense nucleic acids described herein may comprise or consist of nucleotide sequences having at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 81%, at least 82%, at least 83%, at least 84%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% complementarity to their target nucleic acid. Complementarity may be calculated over the whole length of the antisense nucleic acids and/or over all or part of the target nucleic acid to which the antisense nucleic acid binds.

The nucleic acid molecule may be an aptamer. The term "aptamer" as used herein refers to oligonucleotides (e.g. short oligonucleotides or deoxyribonucleotides), that bind (e.g. with high affinity and specificity) to proteins, peptides, and small molecules. Aptamers typically have defined secondary or tertiary structure owing to their propensity to form complementary base pairs and, thus, are often able to fold into diverse and intricate molecular structures. The three-dimensional structures are essential for aptamer binding affinity and specificity, and specific three-dimensional interactions drives the formation of aptamer-target complexes. Aptamers can be selected *in vitro* from very large libraries of randomized sequences by the process of systemic evolution of ligands by exponential enrichment (SELEX as described in Ellington AD, Szostak JW, *Nature* 1990, 346:818-822; Tuerk C, Gold L. *Science* 1990, 249:505-510) or by developing SOMAmers (slow off-rate modified aptamers) (Gold L et al. (2010) Aptamer-based multiplexed proteomic technology for biomarker discovery. PLoS ONE 5(12):e15004). SOMAmers are short, single stranded deoxyoligonucleotides with protein-like properties thanks to functional groups that mimic amino acid side chains. Applying the SELEX and the SOMAmer technology includes for instance adding functional groups that mimic amino acid side chains to expand the aptamer's chemical diversity. As a result, high affinity aptamers for a target may be enriched and identified.

Aptamers may be DNA or RNA molecules and may be single stranded or double stranded. The aptamer may comprise chemically modified nucleic acids, for example in which the sugar and/or phosphate and/or base is chemically modified. Such modifications may improve the stability of the aptamer or make the aptamer more resistant to degradation and may include modification at the 2' position of ribose.

Aptamers may be synthesized by methods which are well known to the skilled person. For example, aptamers may be chemically synthesized, e.g. on a solid support. Solid phase synthesis may use phosphoramidite chemistry. Briefly, a solid supported nucleotide is detritylated, then coupled with a suitably activated nucleoside phosphoramidite to form a phosphite triester linkage. Capping may then occur, followed by oxidation of the phosphite triester with an oxidant, typically iodine. The cycle may then be repeated to assemble the aptamer (e.g., see Sinha, N. D.; Biernat, J.; McManus, J.; Köster, H. *Nucleic Acids Res.* 1984, 12, 4539; and Beaucage, S. L.; Lyter, R. P. (1992). *Tetrahedron* 48 (12): 2223).

Aptamers may be peptides selected or engineered to bind specific target molecules. Peptide aptamers and methods for their generation and identification are reviewed in Reverdatto et al., *Curr Top Med Chem.* (2015) 15(12):1082-101, which is hereby incorporated by reference in its entirety. Peptide aptamers may optionally have a minimum length of one of 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acids. Peptide aptamers may optionally have a maximum length of one of 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 or 50 amino acids. Suitable peptide aptamers may optionally have a length of one of 2-30, 2-25, 2-20, 5-30, 5-25 or 5-20 amino acids.

Aptamers may have K_D 's in the nM or pM range, e.g. less than one of 500nM, 100nM, 50nM, 10nM, 1nM, 500pM, 100pM.

An aptamer or SOMAmer suitable for use as described herein may bind to a protein involved in mounting a primary immune response, as described herein, e.g., CD40 or CD40L. An aptamer or SOMAmer suitable for use as described herein may display specific binding for a protein involved in mounting a primary immune response, as described herein, e.g., CD40 or CD40L. The aptamer may inhibit the function of a protein involved in mounting a primary immune response, e.g., CD40 or CD40L, for example blocking its binding to its cognate binding partner or ligand.

An agent may be a sequestering agent, e.g., of protein involved in mounting a primary immune response as described herein. The agent may be a protein molecule.

The agent may be a small molecule. For example, the small molecule may bind to a protein involved in mounting a primary immune response as described herein and prevent/reduce its usual function and/or prevent/reduce its interaction with a cognate binding partner. The small molecule may prevent/reduce correct folding of the target protein.

An agent may be a decoy receptor. In some embodiments, a decoy receptor refers to a peptide or polypeptide capable of binding a protein involved in mounting a primary immune response as described herein. The receptor may be a receptor, including fragments and derivatives thereof, for a protein involved in mounting a primary immune response as described herein. A decoy receptor may be able to recognize and bind a specific ligand but may not be able to signal or activate a subsequent response. A decoy receptor may bind a protein involved in mounting a primary immune response as described herein to form a complex. A decoy receptor may act as an inhibitor of a protein involved in mounting a primary immune response as described herein by preventing/reducing the ability or availability of the proteins to bind to their receptor(s). A decoy receptor may act as an inhibitor of a protein involved in mounting a primary immune response by binding to a binding partner of said protein, e.g. in the region that would usually be bound by a binding partner, and preventing interaction between said protein and one or more binding partners. For example, the agent may be a molecule which binds to CD40L and may prevent/reduce its interaction with CD40.

A decoy receptor may be soluble (not membrane bound), or may be membrane bound e.g. expressed on a cell surface. Decoy receptors may be presented and/or administered on a surface of a nanocarrier, for example, a nanoparticle, liposome, bead, polymer, metal particle, dendrimer, nanotube or micro-sized silica rods, see e.g. Wilczewska AZ et al., *Pharmacol Rep.* 2012, 64(5):1020-1037.

Methods for detecting whether a decoy receptor competes for binding for a target protein may be for example SPR (see e.g. Hearty et al., *Methods Mol Biol* 2012, 907:411-442), competition ELISA assay or solid phase binding assays. Other suitable methods will be known in the art.

Agents that decrease the amount of a protein involved in mounting a primary immune response and/or decrease expression of a gene encoding a protein involved in mounting a primary immune response may fall into more than one of the categories above. For example, an antigen binding molecule or decoy receptor may also be a sequestering agent.

Any of the agents described herein may be optionally isolated and/or substantially purified.

Reducing the production of plasma and memory B cells

- 5 Aspects and embodiments of the present disclosure are concerned with reducing and/or blocking the stimulation of early-stage B cells (e.g. B cells which have not encountered the antigens for which the respective BCRs of the B cells are specific, or B cells which have encountered said antigens but which have not yet differentiated or proliferated into plasma or memory B cells).
- 10 The purpose is to inhibit/block the co-stimulation of B cells by activated T cells, and thus block B cell proliferation, differentiation and activation into antibody-secreting cells (e.g. plasma B cells or memory B cells). This acts to inhibit the initiation of adaptive humoral immunity via the failure to form functional germinal centers, and reduces/prevents the ability of the animal to mount a primary humoral immune response to an antigen following subsequent challenge.
- 15 It is not intended to inhibit the stimulation, activation, differentiation and/or proliferation of B cells that have been stimulated to proliferate by administration of the first peptide/polypeptide, or cells derived from such cells (*i.e.* the progeny of cells activated/stimulated to proliferate by administration of the first peptide/polypeptide).
- 20 In some embodiments, treatment of the animal to inhibit/block the stimulation, activation, differentiation and/or proliferation of B cells is performed after a period of time sufficient for B cells (or cells derived from such cells) to have been stimulated/activated/differentiated/proliferated by administration of the first peptide/polypeptide. In some embodiments, such treatment of the animal is performed after a period of
- 25 time sufficient for the cells stimulated/activated/differentiated/proliferated by administration of the first peptide/polypeptide (or cells derived from such cells) to have differentiated into plasma B cells and/or memory B cells.
- In some embodiments, treatment of the animal to inducibly inhibit/block the stimulation, activation,
- 30 differentiation and/or proliferation of B cells is performed after a period of time sufficient for B cells (or cells derived from such cells) to have been stimulated/activated/differentiated/proliferated by administration of the first peptide/polypeptide. In some embodiments, such inducible treatment of the animal is performed after a period of time sufficient for the cells that have been
- stimulated/activated/differentiated/proliferated by administration of the first peptide/polypeptide (or cells
- 35 derived from such cells) to have differentiated into plasma B cells and/or memory B cells.
- In some embodiments, inhibiting/blocking/reducing the stimulation, activation, differentiation and/or proliferation of B cells does not comprise inhibiting/blocking/reducing IgG-, IgE-, or IgA-expressing cells. In some embodiments, inhibiting/blocking/reducing the stimulation, activation, differentiation and/or
- 40 proliferation of B cells does not comprise inhibiting/blocking/reducing plasma B cells and/or memory B cells.

In some embodiments, inhibiting/blocking/reducing the stimulation, activation, differentiation and/or proliferation of B cells is achieved by inhibition of the expression of one or more factors expressed by such cells. For example, inhibition of the stimulation, activation, differentiation and/or proliferation of B cells might be achieved by inhibition of the expression of CD40 and/or CD40L, which are involved in directing all phases of the B cell response to antigens (see e.g. Janeway CA Jr et al., Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001. B-cell activation by armed helper T cells, which is hereby incorporated by reference in its entirety).

Aspects and embodiments of the present disclosure are concerned with inhibiting the production (i.e. differentiation, proliferation) of plasma B cells or memory B cells in response to challenge with an antigen.

The production of said B cells is inhibited to reduce/prevent the ability of the animal to mount a primary humoral immune response to an antigen following subsequent challenge.

It is not intended to deplete/remove cells activated/stimulated to proliferate by administration of the first peptide/polypeptide, or cells derived from such cells (*i.e.* the progeny of cells activated/stimulated to proliferate by administration of the first peptide/polypeptide).

In some embodiments, treatment of the animal to inhibit the production of plasma or memory B cells is performed after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have undergone immunoglobulin isotype switching (*i.e.* to IgG-, IgE-, or IgA-expressing cells). In some embodiments, treatment of the animal to inhibit the production of plasma or memory B cells is performed after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have differentiated into plasma B cells and/or memory B cells.

In some embodiments, treatment of the animal to inducibly inhibit the production of plasma or memory B cells is performed after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have undergone immunoglobulin isotype switching (*i.e.* to IgG-, IgE-, or IgA-expressing cells). In some embodiments, treatment of the animal to inducibly inhibit the production of plasma or memory B cells is performed after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have differentiated into plasma B cells and/or memory B cells.

In some embodiments, inhibiting the production of plasma or memory B cells does not comprise reducing the number/proportion of IgG-, IgE-, or IgA-expressing cells. In some embodiments, inhibiting the production of plasma or memory B cells does not comprise reducing the number/proportion of plasma B cells and/or memory B cells produced by administration of the first peptide/polypeptide.

Inhibiting immunoglobulin isotype switching

Aspects and embodiments of the present disclosure are concerned with inhibiting immunoglobulin isotype switching. The general purpose is to remove the ability of the animal to mount an IgG response to regions of the second peptide/polypeptide which are dissimilar to regions of the first peptide/polypeptide following subsequent immunization.

Some aspects and embodiments of the present disclosure comprise inhibiting the expression (gene or protein expression) of a gene involved in immunoglobulin isotype switching and/or inhibiting the activity of the product of a gene involved in immunoglobulin isotype switching.

Immunoglobulin class switching is also referred to as “isotype switching” and “class switch recombination”, and is reviewed *e.g.* in Stavnezer and Schrader, J Immunol. (2014) 193(11): 5370–5378, which is hereby incorporated by reference in its entirety.

Mature naïve B cells express both IgM and IgD. Activation by binding of antigen causes the cells to proliferate, and if they encounter the appropriate factors (*e.g.* IL-4, CD40L) they are triggered via signalling through cytokine receptors and CD40 to undergo class switch recombination to switch from expressing IgM and IgD to expression of IgG, IgE, or IgA. During class switching, the constant region of the immunoglobulin heavy chain changes but the variable regions, and therefore antigenic specificity, stay the same.

Immunoglobulin class switching involves replacement of the μ and δ heavy chain constant (CH) regions of the expressed Ig with γ , ϵ or α CH regions, and occurs by deletional recombination between two different switch (S) regions. Class switch recombination (CSR) is instigated by activation-induced cytidine deaminase (AICDA), which converts cytosines in S regions to uracils. The uracils are subsequently removed by two DNA repair pathways, resulting in mutations, single-strand DNA breaks, and the double-strand breaks required for CSR.

Aspects and embodiments of the present disclosure concern inhibition of immunoglobulin class switching of IgM- and/or IgD-expressing B cells to IgG-, IgE-, and/or IgA-expressing cells. In particular embodiments, the present disclosure concerns inhibition of immunoglobulin class switching of IgM- and/or IgD-expressing B cells to IgG-expressing B cells.

It is not intended to inhibit immunoglobulin isotype switching by cells activated/stimulated to proliferate by administration of the first peptide/polypeptide, or cells derived from such cells (*i.e.* the progeny of cells activated/stimulated to proliferate by administration of the first peptide/polypeptide).

In some embodiments, treatment of the animal to inhibit, *e.g.* inducibly inhibit, immunoglobulin isotype switching is performed after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have undergone immunoglobulin isotype switching (*i.e.* to IgG-, IgE-, or IgA-expressing cells). In some embodiments, treatment of the animal to inhibit, *e.g.* inducibly inhibit, immunoglobulin isotype switching is performed

after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have differentiated into plasma B cells and/or memory B cells.

- 5 In some embodiments, inhibiting immunoglobulin isotype switching does not comprise inhibiting immunoglobulin isotype switching by cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells).

10 CD40/CD40L signalling plays an important role in immunoglobulin class switching. CD40 is expressed on B cells, while CD40L is expressed on activated T cells. CD40 knockout mice have been shown not to mount an IgG response in response to T-cell-dependent antigens (Kawabe *et al.*, Immunity (1994) 1: 167-178), and B cells from CD40 knockout mice do not undergo isotype switching in response to *in vitro* stimulation with sCD40L and IL-4 (Castigli *et al.*, PNAS (1994) 91(25): 12135-12139). Moreover, CD40L knockout mice fail to produce an antigen-specific IgG1 response following immunization with a thymus-
15 dependent antigens (Xu *et al.*, Immunity (1994) 1:423-431). Thus inhibiting the expression of, or activity of the product of, CD40 and/or CD40L will impair immunoglobulin class switching.

In some embodiments, inhibition of immunoglobulin class switching comprises inhibition of gene or protein expression of a factor by a T cell (*e.g.* a cytokine, cell surface protein, *etc.*) which is involved in
20 immunoglobulin class switching. In some embodiments, inhibition of immunoglobulin class switching comprises inhibition of the activity of a factor expressed by a T cell (*e.g.* a cytokine, cell surface protein, *etc.*) which is involved in immunoglobulin class switching.

In some embodiments, inhibition of immunoglobulin class switching comprises inhibition of the expression
25 or activity of one or more factors involved in immunoglobulin class switching. In some embodiments, inhibition of immunoglobulin class switching comprises inhibition of the expression of, or activity of the product of, one or both of CD40 and/or, CD40L.

It will be appreciated that inhibition of immunoglobulin class switching can also be achieved *e.g.* by
30 reducing the number/proportion of naïve B cells, inhibiting/blocking stimulation, differentiation, activation, maturation, and/or proliferation of naïve B cells, or inhibiting T cell mediated help, *e.g.* as described hereinabove. That is, inhibition of immunoglobulin class switching can be achieved by depletion/removal of the population of cells which would otherwise undergo immunoglobulin class switching.

35 Aspects and embodiments of the present disclosure comprise treating an animal to inhibit a primary immune response (*e.g.* a primary humoral immune response), and/or promote a secondary immune response (*e.g.* a secondary humoral immune response).

Aspects and embodiments of the present disclosure comprise treating an animal comprising an
40 endogenous nucleotide sequence providing for inducible inhibition of a primary immune response so as to inhibit a primary immune response (*e.g.* a primary humoral immune response), and/or promote a secondary immune response (*e.g.* a secondary humoral immune response).

In some embodiments, the methods of the disclosure comprise administering an agent, e.g. as disclosed hereinabove, which induces inhibition of a primary immune response, and/or which promotes a secondary immune response, in the animal. In some embodiments, the agent induces inhibition of a primary immune response in the animal.

In some embodiments, the agent is effective to inhibit a primary immune response, and/or promote a secondary immune response, in the animal. In some embodiments the agent inhibits the expression of a gene involved in mounting a primary humoral immune response, inhibits the activity of the product of a gene involved in mounting a primary humoral immune response, reduces the number/proportion of naïve B cells, reduces/inhibits the stimulation, differentiation, activation, maturation, and/or proliferation of naïve B cells e.g. via co-stimulation by T cells, inhibits immunoglobulin isotype switching, inhibits the expression of a gene involved in immunoglobulin isotype switching and/or inhibits the activity of the product of a gene involved in immunoglobulin isotype switching.

In some embodiments, the agent induces expression or activity of one or more factors resulting in inhibition of a primary immune response, and/or promotion of a secondary immune response.

Aspects and embodiments of the present disclosure relate to the use of such an agent in methods of producing an antigen-binding molecule, e.g. according to methods of producing an antigen-binding molecule as described herein.

In some embodiments, the agent is capable of inhibiting the expression (gene or protein expression) of a gene involved in mounting a primary humoral immune response. In some embodiments, the agent is an agent capable of inhibiting the expression (gene or protein expression) of a gene involved in immunoglobulin isotype switching. In some embodiments, the agent is an agent capable of inhibiting the expression (gene or protein expression) of a gene involved in stimulation of naïve B cells to proliferate, differentiate and/or activate into antibody-secreting cells.

In some embodiments, the agent is capable of altering/disrupting the nucleotide sequence of a target gene (*i.e.* a gene involved in mounting a primary immune response), or altering/disrupting nucleotide sequence required for expression of the target gene (*e.g.* a regulatory sequence governing expression of the target gene, transcription factor).

In some embodiments, the agent is capable of inducing alteration/disruption of the nucleotide sequence of a target gene (*i.e.* a gene involved in mounting a primary immune response) by homologous recombination. In some embodiments, the agent is capable of increasing the expression or activity of a recombinase capable of altering/disrupting the nucleotide sequence of a target gene (*i.e.* a gene involved in mounting a primary immune response).

In some embodiments the animal comprises an endogenous nucleotide sequence that encodes a tamoxifen/4-hydroxytamoxifen-controlled system for controlling activity of the recombinase.

In aspects and embodiments employing such systems, an agent capable of inhibiting a primary immune response, and/or promoting a secondary immune response may be tamoxifen/4-hydroxytamoxifen.

Similarly, methods of the present disclosure concerning such systems may comprise administering

5 tamoxifen/4-hydroxytamoxifen to the animal to inhibit the ability of the animal to mount a primary immune response.

In some embodiments, an agent capable of inhibiting a primary immune response, and/or promoting a secondary immune response may be administered to the animal on multiple occasions. In some

10 embodiments, the agent is administered in a quantity, and/or with a periodicity, selected to achieve a desired level of inhibition of the primary immune response, or a desired level of a secondary immune response.

For example, an animal comprising an endogenous nucleotide sequence providing for recombinase-

15 mediated disruption of expression of one or more genes involved in mounting a primary humoral immune response and an endogenous nucleotide sequence providing for inducible expression or activity of the recombinase, may be administered an agent for inducing expression or activity of the recombinase on multiple occasions, in order to maintain a sufficient level of expression/activity of the recombinase to provide for sustained disruption of expression of the one or more genes involved in mounting a primary

20 humoral immune response. By way of illustration, Example 12 herein describes multiple administrations of tamoxifen to *Cd40^{flox/flox}; Cd79a^{+/-}CreERT2* mice in order to maintain knockout of Cd40 expression by B cells.

In some embodiments, in the context of a method according to the present disclosure, an agent capable of inhibiting a primary immune response, and/or promoting a secondary immune response, is

25 administered to the animal more than one time, e.g. one of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 19 or 20 or more times. Individual administrations of the agent may be separated by a predetermined time interval, which may be from 2 to 14 days, e.g. 3 to 12 days, 5 to 10 days, or 5 to 9 days. In some embodiments, following initial administration of the agent to the animal, subsequent administrations of the agent may be given every 2 days, 3 days (plus or minus 1 day), 4 days (plus or

30 minus 1 or 2 days), 5 days (plus or minus 1 or 2 days), 6 days (plus or minus 1 or 2 days), 7 days (plus or minus 1, 2 or 3 days), 8 days (plus or minus 1, 2 or 3 days), 9 days (plus or minus 1, 2 or 3 days), 10 days (plus or minus 1, 2 or 3 days), 11 days (plus or minus 1, 2 or 3 days), 12 days (plus or minus 1, 2, 3 or 4 days), 13 days (plus or minus 1, 2, 3 or 4 days) or 14 days (plus or minus 1, 2, 3 or 4 days).

35 Nucleotide sequences

Aspects and embodiments of the present disclosure relate to animals comprising an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response.

As used herein, a nucleotide sequence "providing for" inducible inhibition of a primary humoral immune

40 response may encode one or more factors involved in (e.g. which are required for, or which allow for) inducible inhibition of a primary humoral immune response.

In some embodiments, the nucleotide sequence provides for site-specific recombinase-mediated inhibition of expression of one or more (*e.g.* one of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more) genes involved in mounting a primary humoral immune response.

- 5 In some embodiments, the nucleotide sequence provides for site-specific recombinase-mediated inhibition of expression of one or both of CD40 and/or.

Site-specific recombinase (SSR) systems for inhibition of gene expression are well known in the art. Such SSR systems include *e.g.* Cre-*LoxP*, Flp-*FRT* and Dre-*rox* systems, and are described *e.g.* in Branda and
10 Dymecki, *Developmental Cell* (2004) 6(1): 7-28 and Kim *et al.*, *Lab Anim Res.* (2018) 34(4): 147–159, both of which are hereby incorporated by reference in their entirety. Variants of such SSR systems and other SSR systems are also well known in the art and can similarly be employed to the ends of inhibiting expression of a target gene of interest.

- 15 Targeted disruption of a nucleotide sequence can be achieved by providing target sequences for a recombinase either side of (*i.e.* **upstream/5' of and downstream/3' of**) all or part of the nucleotide sequence of the gene, or a nucleotide sequence required for expression of the gene. In the presence of the corresponding recombinase, the homologous recombination occurs between the target sequences, disrupting of the nucleotide sequence of the gene or the nucleotide sequence required for expression of
20 the gene.

In the Cre-*loxP* system, a Cre recombinase binds to inverted repeats of *loxP* target sequences and promotes recombination and excision of the nucleotide sequence between the *loxP* target sequences. As
used herein, "a Cre recombinase" refers to any peptide/polypeptide having the catalytic activity of Cre
25 recombinase. A Cre recombinase may comprise the amino acid sequence of UniProtKB Q71TG5-1, v1, or an amino acid sequence having at least 60%, preferably one of 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of UniProtKB Q71TG5-1, v1. Cre recombinases include *e.g.* fusion proteins of Cre recombinase, including *e.g.* CreERT and CreERT2 described hereinbelow.

30 Whilst the above example describes the use of SSR systems for targeted excision of a nucleotide sequence, SSR systems can also be employed for targeted inversion, insertion and translocation of nucleotide sequences.

- 35 SSR-mediated gene knockout, in particular knockout using the Cre-*loxP* system, is described *e.g.* in Kim *et al.*, *Lab Anim Res.* (2018) 34(4): 147–159, which is incorporated by reference herein.

It will be appreciated that alteration/disruption using a SSR system in accordance with the present disclosure inhibits/prevents gene or protein expression of the gene. In some embodiments,
40 alteration/disruption using a SSR system inhibits/prevents the production of a product encoded by the unaltered nucleotide sequence of the gene.

In some embodiments, alteration/disruption using a SSR system reduces/prevents transcription of the gene, introduces a premature stop codon in the sequence transcribed from the gene, alters the nucleotide sequence to encode a truncated and/or non-functional gene product, or alters the nucleotide sequence to encode a gene product which is misfolded and/or degraded.

5

In some embodiments, the nucleotide sequence of the present disclosure encodes factors providing for site-specific recombinase-mediated disruption of expression of one or more genes involved in mounting a primary humoral immune response.

10 In some embodiments, the nucleotide sequence comprises target sequences for a recombinase flanking all or part of the nucleotide sequence of a gene involved in mounting a primary humoral immune response. In some embodiments, the nucleotide sequence comprises target sequences for a recombinase flanking all or part of a nucleotide sequence required for expression of the gene.

15 In some embodiments, the target sequences for a recombinase are *loxP* sequences, and the recombinase is a Cre recombinase. In some embodiments, the target sequences for a recombinase are *FRT* sequences, and the recombinase is Flp recombinase. In some embodiments, the target sequences for a recombinase are *rox* sequences, and the recombinase is Dre recombinase.

20 In some embodiments, the nucleotide sequence flanked by target sequences for a recombinase is a nucleotide sequence required for expression of a gene product from the gene. In some embodiments, the nucleotide sequence flanked by target sequences for a recombinase encodes all or part of one or more exons of the gene. In some embodiments, the nucleotide sequence flanked by target sequences for a recombinase encodes all or part of a regulatory sequence controlling expression of the gene, *e.g.* a
25 promoter or an enhancer.

It will be appreciated that target sequences for a recombinase “flanking” a given nucleotide sequence are provided either side of the given nucleotide sequence. That is, they are provided 5' of and 3' to the given nucleotide sequence, in the context of the endogenous nucleotide sequence of the present disclosure. By
30 way of illustration, an endogenous nucleotide sequence of the present disclosure may comprise the following arrangement of nucleotide sequences:

5'-[target sequence for a recombinase]-[all or part of the nucleotide sequence of a target gene/nucleotide sequence required for expression of the target gene]-[target sequence for a recombinase]-3'

35

In some embodiments, a target sequence for a recombinase may be within about 5, 10, 50, 100, 250, 500 or 1000 bases of the first and/or last base of the nucleotide sequence of the target gene/nucleotide sequence required for expression of the target gene. That is, in some embodiments the final base of target sequence for a recombinase may be provided within about 5, 10, 50, 100, 250, 500 or 1000 bases
40 of the first base of the nucleotide sequence of the target gene/nucleotide sequence required for expression of the target gene, and/or the first base of target sequence for a recombinase may be

provided within about 5, 10, 50, 100, 250, 500 or 1000 bases of the final base of the nucleotide sequence of the target gene/nucleotide sequence required for expression of the target gene.

In some aspects and embodiments, the endogenous nucleotide sequence of the present disclosure

5 further comprises a nucleotide sequence encoding the recombinase, *i.e.* the recombinase corresponding to the relevant target sequences for a recombinase. By way of illustration, in embodiments where the target sequences for a recombinase are *loxP* sequences, the endogenous nucleotide sequence may further comprise a nucleotide sequence encoding a Cre recombinase.

10 The endogenous nucleotide sequence may further comprise nucleotide sequence encoding regulatory nucleotide sequences (*e.g.* promoter and/or enhancer) for expression of the recombinase. The regulatory nucleotide sequences may be operably linked to the nucleotide sequence encoding the recombinase. In some embodiments the endogenous nucleotide sequence encodes an expression cassette for the recombinase.

15 In aspects and embodiments of the present disclosure, the endogenous nucleotide sequence provides for inducible inhibition of a primary humoral immune response. Inhibition of a primary humoral immune response may be inducible, *e.g.* in response to a given chemical or physical treatment.

20 In embodiments employing SSR systems, SSR-mediated inhibition of gene expression may be inducible by increasing the level or activity of the relevant recombinase, *e.g.* by administering the recombinase and/or increasing expression of the recombinase.

In embodiments employing an endogenous nucleotide sequence comprising target sequences for a
25 recombinase flanking all or part of the nucleotide sequence of a gene involved in mounting a primary humoral immune response, or flanking all or part of a nucleotide sequence required for expression of the gene, inhibition of expression of the relevant gene may be induced by administering the recombinase to the animal, administering nucleic acid (*e.g.* vector) encoding the recombinase to the animal, and/or treating the animal to increase the expression or activity of the recombinase.

30 It will be appreciated that increasing the level of the recombinase and/or activity of the recombinase in cells comprising the endogenous nucleotide sequence comprising target sequences for a recombinase flanking the target nucleotide sequence of interest potentiates recombination, and thus disruption of the target gene and consequent inhibition of its expression.

35 In some embodiments, upregulation of expression/activity of the recombinase is chemically inducible. Chemically-inducible SSR-mediated gene knockout is described *e.g.* in Kim *et al.*, Lab Anim Res. (2018) 34(4): 147–159 (incorporated by reference hereinabove).

40 In some embodiments, the endogenous nucleotide sequence encodes a conditional system for controlling expression or activity of the recombinase.

In aspects and embodiments employing such conditional systems, an agent capable of inhibiting a primary immune response, and/or promoting a secondary immune response may be an agent inducing expression or activity of the recombinase.

- 5 In some embodiments, the endogenous nucleotide sequence comprises a nucleotide sequence encoding a conditional expression system for controlling expression of the recombinase.

“Conditional expression” may also be referred to herein as “inducible expression”, and refers to gene/protein expression contingent on certain conditions, e.g. the presence of a particular agent.

- 10 Conditional expression systems are well known in the art and are reviewed e.g. in Ryding *et al.* Journal of Endocrinology (2001) 171, 1-14, which is hereby incorporated by reference in its entirety.

Conditional expression systems include systems which employ tetracycline-controlled transcriptional activation, such as Tet-On and Tet-Off systems.

- 15 The Tet-On system employs nucleic acid encoding a reverse tetracycline transactivator (rtTA) protein, which is a fusion of the tetracycline repressor (TetR) protein mutated at four amino acid positions to reverse the response to tetracycline/doxycycline, and the activation domain of VP16. In the absence of tetracycline (or a derivative thereof such as doxycycline) rtTA does not bind to TetO operator sequences
20 and the polypeptide is not expressed. In the presence of tetracycline/doxycycline, rtTA binds to TetO sequences in the TRE and activates transcription of the nucleic acid downstream of the promoter. Tet-On systems are described in Das *et al.*, Curr Gene Ther. (2016)16(3):156-67 (hereby incorporated by reference in its entirety), and include systems using optimized rtTA variants such as the Tet-On Advanced system (which uses the rtTA variant protein rtTA2^s-M2) and Tet-On 3G system.

- 25 The Tet-On Advanced system is also described in Urlinger *et al.* Proc. Natl. Acad. Sci. U.S.A. (2000) 97(14):7963–8 (hereby incorporated by reference in entirety), and Tet-On 3G is described in Zhou *et al.*, Gene Ther. 13(19):1382-1390 (hereby incorporated by reference in entirety).

- 30 The Tet-Off system employs nucleic acid encoding a tetracycline transactivator (tTA) protein, which is a fusion of the tetracycline repressor (TetR) protein and the activation domain HSV protein VP16. In the absence of tetracycline (or a derivative thereof such as doxycycline) tTA binds to TetO operator sequences, which form a tetracycline-response element (TRE), located just upstream of a minimal promoter (e.g. CMV promoter). Binding of tTA to the TetO sequences in the TRE activates transcription of
35 the nucleic acid downstream of the promoter. In the presence of tetracycline/doxycycline tTA is unable to bind TetO sequences in the TRE, and transcription of the nucleic acid downstream of the promoter is repressed. The Tet-Off system is described in Bujard *et al.* Proc. Natl. Acad. Sci. U.S.A. (1992) 89(12):5547–51 (hereby incorporated by reference in entirety).

- 40 Other tetracycline-controlled systems include the T-REx conditional expression system described in Yao *et al.*, Human Gene Therapy (1998) 9(13): 1939-1950 (hereby incorporated by reference in entirety). In the T-REx system, TetR is expressed under the control of a CMV promoter, and in the absence of

tetracycline/doxycycline TetR binds to two Tet operator 2 (TetO2) sequences upstream of the region of interest and repression of transcription of the region of interest. When tetracycline/doxycycline is added to the system it binds to TetR and causes its release from the TetO2 sequences, thereby releasing the region of interest from transcriptional repression.

5

In some embodiments the endogenous nucleotide sequence of the present disclosure comprises nucleotide sequence(s) encoding elements of a system for providing conditional expression of the recombinase. In some embodiments the endogenous nucleotide sequence encodes a tetracycline/doxycycline-controlled transcriptional activation system for controlling expression of the recombinase.

10

In aspects and embodiments employing such systems, an agent capable of inhibiting a primary immune response, and/or promoting a secondary immune response may be tetracycline/doxycycline. Similarly, methods of the present disclosure concerning such systems may comprise administering tetracycline/doxycycline to the animal to inhibit the ability of the animal to mount a primary immune response.

15

In some embodiments the endogenous nucleotide sequence encodes a conditional system for controlling activity of the recombinase.

20

In some embodiments, the recombinase encoded by the endogenous nucleotide sequence comprises a moiety providing for inducible regulation of recombinase activity. Regulation of recombinase activity may be achieved *e.g.* by influencing subcellular localization of the recombinase. That is, regulation of recombinase activity may be achieved by controlling access to target sequences for the recombinase.

25

Inducible recombinase activity systems are described *e.g.* in Kim *et al.*, Lab Anim Res. (2018) 34(4): 147–159 (incorporated by reference hereinabove). The tamoxifen-inducible Cre system employs a fusion protein comprising Cre recombinase fused to the estrogen receptor containing a mutated ligand binding domain (ER-LBD), known as CreER recombinase. CreER is normally localized to the cytoplasm of cells expressing the fusion protein, in a form that binds to HSP90. However, binding of synthetic steroids such as tamoxifen or 4-hydroxytamoxifen disrupts interaction between HSP90 and CreER, and CreERT (*i.e.* CreER-tamoxifen) translocates to the nucleus where it binds *loxP* target sequences and exerts recombinase activity. CreERT2 is a variant of CreER which is ~10 times more sensitive to 4-OHT *in vivo*.

30

In some embodiments, nuclear translocation of the recombinase is inducible. In some embodiments, the recombinase comprises a moiety comprising or consisting of the estrogen receptor having a mutated ligand binding domain (ER-LBD). In some embodiments, the recombinase is a Cre recombinase. In some embodiments, the Cre recombinase is CreERT, CreERT2 or a variant thereof. In preferred embodiments, the Cre recombinase is CreERT2.

40

In some embodiments the endogenous nucleotide sequence encodes a tamoxifen/4-hydroxytamoxifen-controlled system for controlling activity of the recombinase.

In aspects and embodiments employing such systems, an agent capable of inhibiting a primary immune response, and/or promoting a secondary immune response may be tamoxifen/4-hydroxytamoxifen.

Similarly, methods of the present disclosure concerning such systems may comprise administering

5 tamoxifen/4-hydroxytamoxifen to the animal to inhibit the ability of the animal to mount a primary immune response.

Expression of the recombinase may be under the control of regulatory sequences driving expression in cells of hematopoietic origin, *e.g.* regulatory sequences driving expression in B cell lineage cells.

10 Accordingly, in some embodiments the endogenous nucleotide sequence encodes a recombinase under the control of regulatory sequence(s) (*e.g.* a promoter) driving expression in cells of hematopoietic origin. In some embodiments the endogenous nucleotide sequence encodes a recombinase under the control of regulatory sequence(s) (*e.g.* a promoter) driving expression in B cell lineage cells.

15 In some embodiments, expression of the recombinase may be under the control of cell type- or tissue-specific regulatory sequence(s). For example, expression of the recombinase may be under the control of cell type- or tissue-specific promoter or enhancer. In this way, expression of the recombinase, and thus SSR-mediated gene knockout, may be restricted to target cells or tissues of interest. Accordingly, in some embodiments the endogenous nucleotide sequence encodes a recombinase under the control of cell
20 type- or tissue-specific regulatory sequence(s), *e.g.* a cell type- or tissue-specific promoter.

In some embodiments the endogenous nucleotide sequence encodes a recombinase under the control of hematopoietic cell or tissue-specific regulatory sequence(s), *e.g.* a hematopoietic cell or tissue-specific promoter. In some embodiments the endogenous nucleotide sequence encodes a recombinase under the
25 control of B cell lineage-specific regulatory sequence(s), *e.g.* a B cell lineage-specific promoter.

In some embodiments the endogenous nucleotide sequence encodes a recombinase (*e.g.* a Cre recombinase, *e.g.* CreERT2) under the control of the CD79A promoter.

30 In some embodiments, the endogenous nucleotide sequence encodes target sequences for a recombinase (*e.g.* *loxP* sequences) flanking one or more exons of one or more genes involved in mounting a primary humoral immune response. In some embodiments, one or more genes involved in mounting a primary humoral immune response are selected from: CD40 and/or CD40L.

35 In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase removes the flanked exons of the relevant gene(s). In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase removes the entire coding sequence of the relevant gene(s). In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase removes the promoter for transcription of the relevant
40 gene(s). In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase removes/disrupts one or more splice donor and/or acceptor sites of encoded by the relevant gene(s). In some embodiments, recombinase-mediated excision of the region

flanked by target sequences for a recombinase removes the translation initiation codon for translation of RNA encoded by the relevant gene(s). In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase introduces a frameshift in the nucleotide sequence of the relevant gene(s). In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase has the result that the locus encodes a truncated and/or non-functional form of the protein(s) encoded by the relevant gene(s). In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase results in non-sense-mediated degradation of the RNA transcribed from the locus.

In some embodiments, the endogenous nucleotide sequence encodes target sequences for a recombinase (e.g. *loxP* sequences) flanking one or more exons of CD40. In some embodiments, the endogenous nucleotide sequence encodes target sequences for a recombinase flanking one or more of exons 2, 3, 4 and 5 of CD40. In some embodiments, the endogenous nucleotide sequence encodes target sequences for a recombinase flanking exons 2 to 5 of CD40. In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase introduces a frameshift in the nucleotide sequence encoding CD40. In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase has the result that the CD40 locus encodes a truncated and/or non-functional form of CD40. In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase results in non-sense-mediated degradation of the RNA transcribed from the CD40 locus.

In some embodiments, the endogenous nucleotide sequence encodes target sequences flanking the region of CD40 shown in SEQ ID NO:3. In some embodiments, the endogenous nucleotide sequence provides for excision of the region of CD40 shown in SEQ ID NO:3.

In some embodiments, the endogenous nucleotide sequence comprises, or consists of a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:4, e.g. one of $\geq 60\%$, $\geq 61\%$, $\geq 62\%$, $\geq 63\%$, $\geq 64\%$, $\geq 65\%$, $\geq 66\%$, $\geq 67\%$, $\geq 68\%$, $\geq 69\%$, $\geq 70\%$, $\geq 71\%$, $\geq 72\%$, $\geq 73\%$, $\geq 74\%$, $\geq 75\%$, $\geq 76\%$, $\geq 77\%$, $\geq 78\%$, $\geq 79\%$, $\geq 80\%$, $\geq 81\%$, $\geq 82\%$, $\geq 83\%$, $\geq 84\%$, $\geq 85\%$, $\geq 86\%$, $\geq 87\%$, $\geq 88\%$, $\geq 89\%$, $\geq 90\%$, $\geq 91\%$, $\geq 92\%$, $\geq 93\%$, $\geq 94\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$ or 100% nucleotide sequence identity to SEQ ID NO:4. In some embodiments, the endogenous nucleotide sequence comprises, or consists of, the nucleotide sequence of SEQ ID NO:4.

In some embodiments, following recombinase-mediated excision the CD40 locus comprises a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:5, e.g. one of $\geq 60\%$, $\geq 61\%$, $\geq 62\%$, $\geq 63\%$, $\geq 64\%$, $\geq 65\%$, $\geq 66\%$, $\geq 67\%$, $\geq 68\%$, $\geq 69\%$, $\geq 70\%$, $\geq 71\%$, $\geq 72\%$, $\geq 73\%$, $\geq 74\%$, $\geq 75\%$, $\geq 76\%$, $\geq 77\%$, $\geq 78\%$, $\geq 79\%$, $\geq 80\%$, $\geq 81\%$, $\geq 82\%$, $\geq 83\%$, $\geq 84\%$, $\geq 85\%$, $\geq 86\%$, $\geq 87\%$, $\geq 88\%$, $\geq 89\%$, $\geq 90\%$, $\geq 91\%$, $\geq 92\%$, $\geq 93\%$, $\geq 94\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$ or 100% nucleotide sequence identity to SEQ ID NO:5. In some embodiments, following recombinase-mediated excision the CD40 locus comprises the nucleotide sequence of SEQ ID NO:5.

In some embodiments, the endogenous nucleotide sequence encodes a recombinase, *e.g.* a Cre recombinase. In preferred embodiments, the Cre recombinase is CreERT2.

In some embodiments, the endogenous nucleotide sequence encodes a recombinase (*e.g.* a Cre recombinase, *e.g.* CreERT2) under the control of a conditional system for controlling expression and/or activity of the recombinase. In some embodiments, the endogenous nucleotide sequence encodes a tamoxifen/4-hydroxytamoxifen-controlled system for controlling activity of the recombinase.

In some embodiments, expression of the recombinase is under the control of a regulatory sequence (*e.g.* a promoter) driving expression in cells of hematopoietic origin. In some embodiments, expression of the recombinase is under the control of a regulatory sequence (*e.g.* a promoter) driving expression in B cell lineage cells. In some embodiments, expression of the recombinase is under the control of the CD79A promoter.

In some embodiments, the endogenous nucleotide sequence comprises a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:6, *e.g.* one of $\geq 60\%$, $\geq 61\%$, $\geq 62\%$, $\geq 63\%$, $\geq 64\%$, $\geq 65\%$, $\geq 66\%$, $\geq 67\%$, $\geq 68\%$, $\geq 69\%$, $\geq 70\%$, $\geq 71\%$, $\geq 72\%$, $\geq 73\%$, $\geq 74\%$, $\geq 75\%$, $\geq 76\%$, $\geq 77\%$, $\geq 78\%$, $\geq 79\%$, $\geq 80\%$, $\geq 81\%$, $\geq 82\%$, $\geq 83\%$, $\geq 84\%$, $\geq 85\%$, $\geq 86\%$, $\geq 87\%$, $\geq 88\%$, $\geq 89\%$, $\geq 90\%$, $\geq 91\%$, $\geq 92\%$, $\geq 93\%$, $\geq 94\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$ or 100% nucleotide sequence identity to SEQ ID NO:6. In some embodiments, endogenous nucleotide sequence comprises the nucleotide sequence of SEQ ID NO:6.

Animals

The present disclosure relates to animals for the production of antigen-binding molecules, in which a primary immune response can be inhibited, *e.g.* using a method described herein.

Aspects and embodiments of the present disclosure relate to animals comprising an endogenous nucleotide sequence providing for inducible inhibition of a primary immune response. It will be appreciated that endogenous nucleotide sequence provides for inducible inhibition of a primary humoral immune response in the animal.

In aspects and embodiments of the present disclosure, an animal may comprise an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response according to an embodiment described herein.

An animal according to the present disclosure may be an individual/subject of any species of animal. In preferred embodiments, the animal is a non-human animal.

The animal is preferably an individual/subject of a species commonly used for the production of antibodies by immunization. For example, in some embodiments the animal may be a mouse, rat, hamster, llama, guinea pig, rabbit, goat, chicken, primate (*e.g.* non-human primate, *e.g.* a monkey), sheep, donkey, cow, cat, dog, pig or horse. In some embodiments the animal is a mammal (*e.g.* a non-human mammal).

In some embodiments the animal is an individual/subject of a species of the order *Rodentia* (e.g. an individual/subject of a species of the genus *Mus*, *Rattus* or *Cavia*) or *Lagomorpha* (e.g. an individual/subject of a species of the family *Leporidae*). In some embodiments, the animal is a mouse, a rat or a rabbit.

5 In some embodiments, the animal is a mouse (that is, in some embodiments the animal is an individual/subject of a species of the genus *Mus*; e.g. an individual/subject of the species *Mus musculus*).

10 In some embodiments, the animal is a rat (e.g. an individual/subject of the genus *Rattus*; e.g. an individual/subject of the species *Rattus norvegicus* or *Rattus rattus*).

In some embodiments, the animal is a rabbit (e.g. an individual/subject of a species of the genus *Oryctolagus*; e.g. an individual/subject of the species *Oryctolagus cuniculus*).

15 An animal according to the present disclosure may have a genome comprising a nucleotide sequence providing for inducible inhibition of a primary humoral immune response. The nucleotide sequence providing for inducible inhibition of a primary humoral immune response is preferably comprised in genomic DNA of the animal. That is, the nucleotide sequence may be integrated into or form part of the genomic DNA of cells of the animal.

20 An animal according to the present disclosure may have a genome comprising, or may comprise genomic DNA comprising, a nucleotide sequence providing for inducible inhibition of a primary humoral immune response may be said to comprise an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response.

25 An animal according to the present disclosure may comprise more than one (e.g. one of 2, 3, 4, 5, 6, 7, 8, 9 or 10 or more) endogenous nucleotide sequences providing for inducible inhibition of a primary humoral immune response.

30 An animal according to the present disclosure may comprise plural (e.g. one of 2, 3, 4, 5, 6, 7, 8, 9 or 10 or more) endogenous nucleotide sequences, each endogenous nucleotide sequence conforming to an embodiment of an endogenous nucleotide sequence as described herein. In such embodiments, the plural endogenous nucleotide sequences may provide for inducible inhibition of the expression or activity of different genes/products thereof. By way of illustration, an animal according to the present disclosure
35 may comprise an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or activity of a product of, CD40.

In some embodiments, the animal comprises endogenous nucleotide sequence(s) providing for inducible inhibition of the expression of, or activity of a product of, one or more genes involved in mounting a
40 primary humoral immune response. In some embodiments, the animal comprises endogenous nucleotide sequence(s) providing for inducible inhibition of the expression of, or activity of a product of, one or both of CD40 and/or CD40L.

In some embodiments, the animal comprises endogenous nucleotide sequence(s) providing for inducible knockout of one or more genes involved in mounting a primary humoral immune response. In some embodiments, the animal comprises endogenous nucleotide sequence(s) providing for inducible knockout of one or both of CD40 and/or CD40L.

As used herein, “inducible knockout” refers to gene knockout which is inducible *e.g.* in response to a given chemical or physical treatment. Inducible knockout may also be referred to as “conditional knockout”. Inducible gene knockout technology is described *e.g.* in Kim *et al.*, Lab Anim Res. (2018) 34(4): 147–159. Knockout may be inducible by treatment resulting in an increase in the level of expression or activity of a factor mediating gene knockout. For example, knockout may be mediated by a site-specific recombinase (SSR) system, and may be inducible by treatment resulting in an increase in the level of expression or activity of the relevant recombinase, which may effect gene knockout through binding to target sequences for the recombinase flanking all or part of the nucleotide sequence of a target gene. Knockout may be restricted to cell type(s) or tissues(s) of interest, *e.g.* by placing expression of the relevant recombinase under the control of regulatory sequence(s) (*e.g.* a promoter or enhancer) governing expression in the cell type(s) or tissues(s) of interest.

In some embodiments, an animal according to the present disclosure comprises an endogenous nucleotide sequence encoding target sequences for a recombinase (*e.g.* *loxP* sequences) flanking one or more exons of one or more genes involved in mounting a primary humoral immune response. In some embodiments, one or more genes involved in mounting a primary humoral immune response are CD40 and/or CD40L.

In some embodiments, the animal comprises an endogenous nucleotide sequence encoding target sequences for a recombinase (*e.g.* *loxP* sequences) flanking one or more exons of CD40. In some embodiments, the animal comprises an endogenous nucleotide sequence encoding target sequences for a recombinase flanking one or more of exons 2, 3, 4 and 5 of CD40. In some embodiments, the animal comprises an endogenous nucleotide sequence encoding target sequences for a recombinase flanking exons 2 to 5 of CD40. In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase introduces a frameshift in the nucleotide sequence encoding CD40. In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase has the result that the CD40 locus encodes a truncated and/or non-functional form of CD40. In some embodiments, recombinase-mediated excision of the region flanked by target sequences for a recombinase results in non-sense-mediated degradation of the RNA transcribed from the CD40 locus.

In some embodiments, the animal comprises an endogenous nucleotide sequence encoding target sequences flanking the region of CD40 shown in SEQ ID NO:3. In some embodiments, the animal comprises an endogenous nucleotide sequence providing for excision of the region of CD40 shown in SEQ ID NO:3.

In some embodiments, the animal comprises an endogenous nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:4, e.g. one of $\geq 60\%$, $\geq 61\%$, $\geq 62\%$, $\geq 63\%$, $\geq 64\%$, $\geq 65\%$, $\geq 66\%$, $\geq 67\%$, $\geq 68\%$, $\geq 69\%$, $\geq 70\%$, $\geq 71\%$, $\geq 72\%$, $\geq 73\%$, $\geq 74\%$, $\geq 75\%$, $\geq 76\%$, $\geq 77\%$, $\geq 78\%$, $\geq 79\%$, $\geq 80\%$, $\geq 81\%$, $\geq 82\%$, $\geq 83\%$, $\geq 84\%$, $\geq 85\%$, $\geq 86\%$, $\geq 87\%$, $\geq 88\%$, $\geq 89\%$, $\geq 90\%$, $\geq 91\%$, $\geq 92\%$, $\geq 93\%$, $\geq 94\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$ or 100% nucleotide sequence identity to SEQ ID NO:4. In some embodiments, the animal comprises an endogenous nucleotide sequence comprising, or consisting of, the nucleotide sequence of SEQ ID NO:4.

In some embodiments, following recombinase-mediated excision the animal comprises a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:5, e.g. one of $\geq 60\%$, $\geq 61\%$, $\geq 62\%$, $\geq 63\%$, $\geq 64\%$, $\geq 65\%$, $\geq 66\%$, $\geq 67\%$, $\geq 68\%$, $\geq 69\%$, $\geq 70\%$, $\geq 71\%$, $\geq 72\%$, $\geq 73\%$, $\geq 74\%$, $\geq 75\%$, $\geq 76\%$, $\geq 77\%$, $\geq 78\%$, $\geq 79\%$, $\geq 80\%$, $\geq 81\%$, $\geq 82\%$, $\geq 83\%$, $\geq 84\%$, $\geq 85\%$, $\geq 86\%$, $\geq 87\%$, $\geq 88\%$, $\geq 89\%$, $\geq 90\%$, $\geq 91\%$, $\geq 92\%$, $\geq 93\%$, $\geq 94\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$ or 100% nucleotide sequence identity to SEQ ID NO:5. In some embodiments, following recombinase-mediated excision the animal comprises the nucleotide sequence of SEQ ID NO:5.

In some embodiments, an animal according to the present disclosure may comprise more than one endogenous nucleotide sequence providing for inducible inhibition of a primary immune response according to the present disclosure. In some embodiments, the animal may comprise one of 1, 2, 3, 4, 5, 6, 7, 9 or 10 endogenous nucleotide sequences according to the present disclosure. In such embodiments, the plural endogenous nucleotide sequences may each independently conform to any embodiment of an endogenous nucleotide sequence described herein. In some embodiments wherein the animal comprises plural endogenous nucleotide sequences according to the present disclosure, the individual endogenous nucleotide sequences may provide for inducible inhibition of non-identical genes involved in mounting a primary humoral immune response (e.g. CD40 and/or CD40L).

In some embodiments, an animal according to the present disclosure comprises an endogenous nucleotide sequence encoding a recombinase, e.g. a Cre recombinase. In preferred embodiments, the Cre recombinase CreERT2.

In some embodiments, an animal according to the present disclosure comprises an endogenous nucleotide sequence encoding a recombinase (e.g. a Cre recombinase, e.g. CreERT2) under the control of a conditional system for controlling expression and/or activity of the recombinase. In some embodiments, the animal disclosure comprises an endogenous nucleotide sequence encoding a tamoxifen/4-hydroxytamoxifen-controlled system for controlling activity of the recombinase.

In some embodiments, expression of the recombinase in the animal is under the control of a regulatory sequence (e.g. a promoter) driving expression in cells of hematopoietic origin. In some embodiments, expression of the recombinase in the animal is under the control of a regulatory sequence (e.g. a promoter) driving expression in B cell lineage cells. In some embodiments, expression of the recombinase in the animal is under the control of the CD79A promoter.

In some embodiments, the animal comprises an endogenous nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:6, e.g. one of $\geq 60\%$, $\geq 61\%$, $\geq 62\%$, $\geq 63\%$, $\geq 64\%$, $\geq 65\%$, $\geq 66\%$, $\geq 67\%$, $\geq 68\%$, $\geq 69\%$, $\geq 70\%$, $\geq 71\%$, $\geq 72\%$, $\geq 73\%$, $\geq 74\%$, $\geq 75\%$, $\geq 76\%$, $\geq 77\%$, $\geq 78\%$, $\geq 79\%$, $\geq 80\%$, $\geq 81\%$, $\geq 82\%$, $\geq 83\%$, $\geq 84\%$, $\geq 85\%$, $\geq 86\%$, $\geq 87\%$, $\geq 88\%$, $\geq 89\%$, $\geq 90\%$, $\geq 91\%$, $\geq 92\%$, $\geq 93\%$, $\geq 94\%$, $\geq 95\%$, $\geq 96\%$, $\geq 97\%$, $\geq 98\%$, $\geq 99\%$ or 100% nucleotide sequence identity to SEQ ID NO:6. In some embodiments, the animal comprises an endogenous nucleotide sequence comprising, or consisting of, the nucleotide sequence of SEQ ID NO:6.

An animal comprising an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response may comprise such an endogenous nucleotide sequence as a consequence of having been genetically engineered to comprise such an endogenous nucleotide sequence. Accordingly, in some embodiments, the animal is a genetically-engineered animal comprising an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response. A genetically engineered animal may also be referred to as a transgenic animal.

Methods for genetically engineering animals to comprise a nucleotide sequence of interest are well known to the skilled person, and are described e.g. in Huijbers, *Methods Mol Biol* (2017) 1642:1-19, Sumiyama *et al.*, *PLoS One* (2018) 13(9):e0203056 and Asfaw *et al.*, *Cogent Food & Agriculture* (2019) 5(1):1686802, both of which are hereby incorporated by reference in their entirety. Such methods include e.g. pronuclear microinjection, which is described e.g. in Pu *et al.*, *Methods Mol Biol* (2019) 1874:17-41 (hereby incorporated by reference in its entirety), and e.g. SSN system-mediated genetic modification of germ cells, fertilized eggs or embryos, which is described e.g. in Lee *et al.*, *Drug Discovery Today: Disease Models* (2016) 20: 13-20 (hereby incorporated by reference in its entirety).

Methods for producing genetically engineering animals include e.g. methods comprising transfecting embryonic stem cells with nucleic acid sequence(s) for genomic integration via homologous recombination, selecting cells having integrated the nucleic acid sequence(s) into their genomic DNA, introducing the genetically-modified embryonic stem cell into a blastocyst, and intrauterine implantation of the blastocyst comprising the genetically-modified embryonic stem cell, for gestation.

Nucleotide sequences of interest can be prepared using recombinant DNA techniques, and vectored into cells/embryos by viral transduction, or introduced by microinjection, electroporation, *etc.* Cells/embryos/animals comprising the nucleotide sequence of interest can be identified by suitable screening, e.g. by southern blot, PCR, *etc.*

A major obstacle to the use of monoclonal antibodies produced from immunized animals in humans is their xenogenic origin. The host mounts an immune response to the non-host antibody, resulting in elimination, and potentially also undesirable side effects. Various approaches have been undertaken to reduce or eliminate their immunogenicity, such as the production of chimeric antibodies comprising human Fc regions, and humanization in wherein the variable domains of the antibody are engineered for similarity to human antibody sequences.

More recently, transgenic techniques have been employed, in which the animal's endogenous immunoglobulin gene loci are replaced with their human homologues. Monoclonal antibodies produced from such mice using traditional hybridoma techniques are fully human. Human Ig transgenic mouse strains such as Xenomouse (Abgenix; Green *et al.*, Nat. Genet. (1994) 7:13–21., Green, J Immunol Methods (1999) 231(1-2):11–23), UltiMAb (Medarex; Lonberg and Huszar, Int Rev Immunol (1995) 13:65–93, Lonberg, Nat Biotechnol (2005) 23:1117–1125), and Velocimmune (Regeneron; Murphy, PNAS (2014) 111(14): 5153-5158) have produced several human monoclonal antibodies that have been approved with acceptable safety and efficacy profile. Using such antibody discovery platforms a significant hurdle was overcome, by reducing the immunogenicity of the antibodies produced while retaining the practicality of mouse immunization. The mice are engineered to retain a robust B cell response, and repeat immunization with human antigens results in a robust secondary immune response and the capacity to develop a diverse repertoire of mAbs.

In aspects and embodiments of the present disclosure, the animal comprises an endogenous nucleotide sequence encoding one or more human immunoglobulin genes or gene segments. In some embodiments, the animal comprises genomic DNA encoding one or more human immunoglobulin genes or gene segments.

In some embodiments, the genome of the animal encodes human immunoglobulin VH region and/or VL region sequences. In some embodiments, the genome of the animal encodes human immunoglobulin Fc region sequence. In some embodiments, the genome of the animal encodes human immunoglobulin VH region, VL region and/or Fc region sequences.

Animals comprising endogenous human immunoglobulin genes or gene segments are useful for producing antibodies comprising fully human Fv (*i.e.* VH and VL regions). Transgenic mice encoding human immunoglobulin genes/gene segments are described *e.g.* in Lu *et al.*, J Biomed Sci. 2020; 27: 1 and Brüggemann *et al.*, Arch Immunol Ther Exp (Warsz). (2015) 63(2):101–108, and include Xenomouse (Abgenix; Green *et al.*, Nat. Genet. (1994) 7:13–21, Green, J Immunol Methods (1999) 231(1-2):11–23), UltiMAb (Medarex; Lonberg and Huszar, Int Rev Immunol (1995) 13:65–93, Lonberg, Nat Biotechnol (2005) 23:1117–1125), TransChromo™ Mouse (Ishida *et al.*, Cloning Stem Cells. (2002) 4:91–102), and Velocimmune® (Regeneron; Murphy, PNAS (2014) 111(14): 5153-5158).

In some embodiments, the animal of the present disclosure comprises an endogenous nucleotide sequence encoding human immunoglobulin V, D and/or J genes, or segments thereof.

In some embodiments, the animal of the present disclosure comprises an endogenous nucleotide sequence encoding the human immunoglobulin genes or gene segments encoded by the genome of an animal described in Lu *et al.*, J Biomed Sci. 2020; 27: 1, Brüggemann *et al.*, Arch Immunol Ther Exp (Warsz). (2015) 63(2):101–108, Green *et al.*, Nat. Genet. (1994) 7:13–21, Green, J Immunol Methods (1999) 231(1-2):11–23, Lonberg and Huszar, Int Rev Immunol (1995) 13:65–93, Lonberg, Nat Biotechnol (2005) 23:1117–1125, Ishida *et al.*, Cloning Stem Cells. (2002) 4:91–102 or Murphy, PNAS (2014)

111(14): 5153-5158, US 7,135,287 B1, US 7,105,348 B2, or US 2006/059575 A1, all of which are hereby incorporated by reference in their entirety.

In some embodiments, antibodies produced by the immune system of the animal of the present disclosure comprise fully human VH region and/or VL region sequences. In some embodiments, antibodies produced by the immune system of the animal of the present disclosure comprise a fully human Fc region sequence. In some embodiments, antibodies produced by the immune system of the animal of the present disclosure comprise fully human VH region, VL region and/or Fc region sequences. In some embodiments, antibodies produced by the immune system of the animal of the present disclosure comprise a fully human amino acid sequence.

In some embodiments, an animal according to the present disclosure has been immunized with a first peptide/polypeptide comprising an amino acid sequence of interest (*i.e.* (a) an amino acid sequence of a protein/protein complex of interest, or (b) an amino acid sequence which is similar to the amino acid sequence of (a)) to elicit a primary immune response directed against the amino acid sequence of interest.

In some embodiments, the animal according to the present disclosure is an individual/subject of a particular strain of mouse. In some embodiments, the animal is a C57BL/6 mouse, a BALB/c mouse, a A/J mouse, a CD1 mouse, a ICR mouse, a 129S2/SvPas mouse, or a FVB/N mouse. The mouse strains recited in the preceding sentence are described *e.g.* in The Jackson Laboratory Handbook on Genetically Standardized Mice, 6th Edition, October 2009 (Jackson Laboratory, Ed. Kevin Flurkey and Joanne M. Curren).

In some embodiments, the animal is a genetically-engineered mouse (*i.e.* a transgenic mouse).

In some embodiments, the animal is a genetically-engineered mouse in which the endogenous immunoglobulin gene loci are replaced with their human homologues. In some embodiments, the animal is a Xenomouse mouse, UltiMAb mouse, TransChromo mouse or Velocimmune mouse. In some embodiments, the animal is a mouse described in Lu *et al.*, J Biomed Sci. 2020; 27: 1, Brüggemann *et al.*, Arch Immunol Ther Exp (Warsz). (2015) 63(2):101–108, Green *et al.*, Nat. Genet. (1994) 7:13–21, Green, J Immunol Methods (1999) 231(1-2):11–23, Lonberg and Huszar, Int Rev Immunol (1995) 13:65–93, Lonberg, Nat Biotechnol (2005) 23:1117–1125, Ishida *et al.*, Cloning Stem Cells. (2002) 4:91–102, Murphy, PNAS (2014) 111(14): 5153-5158, US 7,135,287 B1, US 7,105,348 B2 or US 2006/059575 A1.

In some embodiments, the animal is a genetically-engineered mouse having increased longevity (*i.e.* as compared to equivalent mice lacking such genetic modification). Mice comprising genetic modification resulting in an increased lifespan are described *e.g.* in Ladiges *et al.*, Aging Cell. (2009) 8(4):346-52 (which is hereby incorporated by reference in its entirety); see in particular Table 1 thereof. In some embodiments, the animal is a mouse described in Table 1 of Ladiges *et al.*, Aging Cell. (2009) 8(4):346-52. In some embodiments, the animal is an Ames Dwarf mouse, an αMUPA Tg mouse, a p66shc^{-/-} mouse, a GHR/BP^{-/-} mouse, a Ghrhr^{lit/lit} mouse, a Snell Dwarf mouse, a Igf1r^{+/-} mouse, a FIRKO mouse, a

Klotho Tg mouse, a Mit CAT Tg mouse, a MT Tg heart mouse, a UCP2 Tg brain mouse, a PappA^{-/-} mouse, a AC5^{-/-} mouse, a *Surf1*^{-/-} mouse, a PEPCKTg muscle mouse, a *Irs1*^{-/-} mouse, a *Irs2*^{+/-} mouse, a *Irs2*^{+/-} brain mouse, or a IGF-1 Tg heart mouse.

5 In some embodiments, the animal is a mouse having low incidence of spontaneous tumors, e.g. a genetically-engineered mouse having low incidence of spontaneous tumors (*i.e.* as compared to equivalent mice lacking such genetic modification). In some embodiments, the animal is a mouse described in Rithidech *et al.*, Blood Cells Mol Dis. (1999) 25(1):38-45.

10 In some embodiments, the animal is a mouse having defective immune cell tolerance, e.g. a genetically-engineered mouse having defective immune cell tolerance. In some embodiments, the animal is a mouse described in Khattri *et al.*, J Immunol. (2001) 167(11):6312-6320.

In some embodiments, the animal is a mouse having chronic immune activation, e.g. a genetically-engineered mouse having chronic immune activation. In some embodiments, the animal is a mouse described in Subramanian *et al.*, Proc Natl Acad Sci U S A. (2006) 103(26):9970-9975.

In some embodiments, the animal is a mouse having a dysregulated autoimmune or hyperimmune phenotype, e.g. a genetically-engineered mouse. In some embodiments, the animal is a mouse comprising genetic variation that spontaneously gives rise to a dysregulated autoimmune or hyperimmune phenotype. In some embodiments, the animal is a mouse in which a dysregulated autoimmune or hyperimmune phenotype is/has been induced by treatment with a chemical or peptide/polypeptide. In some embodiments, the mouse is a NOD mouse, a NZB/W F1 mouse, a MRL mouse, a BXSB mouse, a *lpr* mouse, a *gld* mouse, a motheaten mouse, a Scurfy mouse, a Baff Tg mouse, a *Bcl2* Tg mouse, a *Bim*^{-/-} mouse, a *C1qa*^{-/-} mouse, a *C4*^{-/-} mouse, a *Cd19* Tg mouse, a *Cd19*^{Cre}-*Traf3*^{fl/fl} mouse, a *Cd22*^{-/-} mouse, a *Cd40l* Tg mouse, a *Cd45E613R* KI mouse, a *Ctla4*^{-/-} mouse, a *Dnase1*^{-/-} mouse, a *Foxp3*^{-/-} mouse, a *G2a*^{-/-} mouse, a *aGadd45a*^{-/-} mouse, a *aGadd45a*^{-/-}*p21cip1/waf*^{-/-} mouse, a *Gadd45b*^{-/-}*Gadd45g*^{-/-} mouse, a *Il4* Tg mouse, a *LatY136F* KI mouse, a *Lck*^{Cre}-*Pten*^{fl/fl} mouse, a *Lyn*^{-/-} mouse, a *Man2a1*^{-/-} mouse, a *Mark2*^{-/-} mouse, a *Mfge8*^{-/-} mouse, a *Ox40*^{Cre}-*Pten*^{fl/fl} mouse, a *p65P13K* Tg mouse, a *Pd1*^{-/-} mouse, a *Pkba* Tg mouse, a *Prkcd*^{-/-} mouse, a *Pten*^{+/-} mouse, a *Taci*^{-/-} mouse, or a *Tyro3*^{-/-}*Axl*^{-/-}*Mertk*^{-/-} mouse.

In some embodiments, the animal is a mouse having autoimmune encephalitis, e.g. a genetically-engineered mouse having autoimmune encephalomyelitis. In some embodiments, the animal is a mouse having autoimmune encephalitis in an SJL background, as described e.g. in Rajan *et al.*, J Immunol (1996) 157 (2): 941–949. In some embodiments, the animal is a mouse having collagen-induced arthritis in a DBA/1 background, as described e.g. in Courtenay *et al.*, Nature (1980) 283(5748):666-8. In some embodiments, the animal is a mouse having Imiquimod-induced psoriasis in a BALB/c or C57BL/6 background, as described e.g. in Van der Fits *et al.*, J Immunol (2009) 182(9):5836-45.

40 In some embodiments, the animal is a hyperimmune mouse, e.g. a genetically-engineered hyperimmune mouse. Genetically-engineered hyperimmune mice produce a more robust immune response to antigenic

material than equivalent mice lacking such genetic modification. Genetically-engineered hyperimmune mice include DiversimAb mice (Abveris) and DivergimAb mice (Abveris). In some embodiments, the animal is a DiversimAb mouse or a DivergimAb mouse.

- 5 The inventors have advantageously discovered that the use of a hyperimmune mouse for generating the animal(s) of the invention is useful for generating antibodies with a high affinity and titer.

Accordingly, in some embodiments, the animal is a **hyperimmune mouse**. “Hyperimmune mouse” (which may also be referred to as an “autoimmune mouse”) as referred to herein may refer to a mouse with a hyperimmune, dysregulated autoimmune, or highly immuno-reactive background or phenotype and/or a mouse of a hyperimmune, dysregulated autoimmune, or highly immuno-reactive strain.

In some embodiments, the hyperimmune mouse is a genetically-engineered mouse.

- 15 In some embodiments, the hyperimmune mouse may have one or more of the following properties:
- An elevated immune response (or a strong or overactive adaptive response) as compared to a mouse without a hyperimmune, dysregulated autoimmune or highly immune-reactive background or phenotype, *e.g.* as characterised by an enlarged pool of naïve B cells, enhanced activation of the primary or secondary immune response following immunization with an antigen, or increased activation of B cells following immunization with an antigen.
 - An altered tolerance to antigens (self or foreign) as compared to a mouse without a hyperimmune, dysregulated autoimmune or highly immuno-reactive background or phenotype, *e.g.* as characterised by an increased retention of self-reactive B cells, unchecked stimulation and proliferation of self-reactive B cells, or loss of negative selection against somatic hypermutation-generated self-reactivity.
 - Normal, or close to normal, development of B and T cells.
 - A lifespan compatible with the length of antibody discovery campaigns.
 - Strong T-dependent antibody responses.
 - Expanded germinal centers and B cell population
 - Capable of production of class-switched auto-antibodies.
 - Capable of producing high sera antibody titers when immunized with self-epitopes *e.g.* epitopes or antigens with high homology (>80%) to self.
 - Capable of producing high sera antibody titers when immunized with poorly immunogenic antigens *i.e.* antigens that do not trigger a strong immune response in a mouse without a hyperimmune, dysregulated autoimmune or highly immuno-reactive background or phenotype .

Accordingly, in some embodiments, the animal (*e.g.* the hyperimmune mouse) may be capable of producing strong antibody titers when immunized with self-epitopes or poorly immunogenic antigens.

- 40 Generation of a hyperimmune mouse can be achieved using one or more of the following:

- Mutations that affect B cell activation, proliferation and survival e.g. altered BCR and co-receptor signalling or loss of Fas-FasL dependent apoptosis (such as mice described in Miyamoto et al. Nature (2002) 416, 865-869 and Groom et al. J Clin Invest. (2002) 109(1):59-68.
- Alterations to antigen presentation e.g. using negative regulation of cytokine signalling for APC recruitment and migration (such as CCL2) or using mutations in antigen processing genes (such as TAP1 or LMP2).
- Mutations that affect T_{reg} and T_{FH} activation and function e.g. mutations that alter TCR signalling or result in loss of FOXP3-mediated differentiation (e.g. as described in Zahorsky-Reeves and Wilkinson. European Journal of Immunology (2001) 31(1) 196-204) .
- spontaneously acquired genetic mutations that result in an autoimmune phenotype e.g. the NZB/W F1 mouse.

In some embodiments, the hyperimmune mouse is a NOD mouse, a NZB/W F1 mouse, a MRL mouse, a BXSB mouse, a *lpr* mouse, a *gld* mouse, a motheaten mouse, a Scurfy mouse, a Baff Tg mouse, a *Bcl2* Tg mouse, a *Bim*^{-/-} mouse, a *C1qa*^{-/-} mouse, a *C4*^{-/-} mouse, a *Cd19* Tg mouse, a *Cd19*^{Cre}-*Traf3*^{fl/fl} mouse, a *Cd22*^{-/-} mouse, a *Cd40l* Tg mouse, a *Cd45E613R* KI mouse, a *Ctla4*^{-/-} mouse, a *Dnase1*^{-/-} mouse, a *Foxp3*^{-/-} mouse, a *G2a*^{-/-} mouse, a *aGadd45a*^{-/-} mouse, a *aGadd45a*^{-/-}*p21cip1/waf*^{-/-} mouse, a *Gadd45b*^{-/-} *Gadd45g*^{-/-} mouse, a *Il4* Tg mouse, a *LatY136F* KI mouse, a *Lck*^{Cre}-*Pten*^{fl/fl} mouse, a *Lyn*^{-/-} mouse, a *Man2a1*^{-/-} mouse, a *Mark2*^{-/-} mouse, a *Mfge8*^{-/-} mouse, a *Ox40*^{Cre}-*Pten*^{fl/fl} mouse, a *p65P13K* Tg mouse, a *Pd1*^{-/-} mouse, a *Pkba* Tg mouse, a *Prkcd*^{-/-} mouse, a *Pten*^{+/-} mouse, a *Taci*^{-/-} mouse, or a *Tyro3*^{-/-}*Axl*^{-/-} *Mertk*^{-/-} mouse.

In some embodiments, the hyperimmune mouse is a NZB/W F1 mouse. The NZB/W F1 mouse (also referred to herein as the "NZBWF1" or "NZBWF1/J" mouse) is the New Zealand Black (NZB) x New Zealand White (NZW) F1 mouse as described e.g. in Dubois *et al.* JAMA (1966) 195(4):285-289 and in Bagavant *et al.*, Autoimmun Rev. (2020) 19(2): 102686.

In some embodiments, the hyperimmune mouse comprises endogenous nucleotide sequence(s) providing for inducible knockout of CD40. In some embodiments, the mouse is a NZBWF1/J mouse. In some embodiments, the mouse is a hyperimmune *CD40*^{flx/flx}; *Cd79a*^{+/-CreERT2} mouse. In some embodiments, the mouse is a NZBWF1/J *CD40*^{flx/flx}; *Cd79a*^{+/-CreERT2} mouse.

In some embodiments, the animal is a humanized mouse. The humanized mouse may have been engrafted with cells or tissue from a human. The humanized mouse may also be a transgenic mice.

In some embodiments, the humanized mouse is as described in, e.g., Chen and Murawsky. Front Immunol (2018) Volume 9 and Ma, B., Osborn, M. (2021). Transgenic Animals for the Generation of Human Antibodies. In: Rüker, F., Wozniak-Knopp, G. (eds) Introduction to Antibody Engineering. Learning Materials in Biosciences. Springer, Cham. https://doi.org/10.1007/978-3-030-54630-4_5.

In some embodiments, the animal is a humanized hyperimmune mouse.

Producing antigen-binding molecules

Aspects of the present disclosure concern methods for eliciting the production of an antigen-binding molecules capable of binding to a protein of interest.

- 5 Aspects of the present disclosure are concerned with the generation of antigen-binding molecules, *e.g.* for subsequent purification. Aspects of the present disclosure are concerned with the generation of populations of cells producing antigen-binding molecules.

10 As referred to herein, and in the context of the various aspects and embodiments of the present disclosure, an “antigen-binding molecule” refers to a molecule capable of binding to a target antigen, and may *e.g.* be an antibody/immunoglobulin. In some embodiments an antibody/immunoglobulin according to the present disclosure is an IgG (*e.g.* IgG1, IgG2, IgG3, IgG4), IgA (*e.g.* IgA1, IgA2), IgD, IgE or IgM. In preferred embodiments, the antibody/immunoglobulin is an IgG.

15 Antigen-binding molecules/cells producing antigen-binding molecules may be generated for downstream use, *e.g.* in therapeutic, research, imaging and/or diagnostic applications. The methods of the present disclosure may be employed to produce antigen-binding molecules having particular properties of interest relevant to therapeutic, research, imaging and/or diagnostic applications.

20 Briefly, the methods comprise introducing material into an animal which is recognized by the immune system of the animal to be foreign (*i.e.* non-host), resulting in the selective production by the animal of antibodies which are capable of binding to the material. The material may comprise, or may be processed to, an antigen. The ability of the immune system to produce antibodies capable of binding specifically to antigens can be used to generate antibodies for detecting molecules of interest in various research,
25 diagnostic, imaging, therapeutic and prophylactic applications.

Methods for producing antibodies are well known in the art, and are described, for example, in Antibodies: A Laboratory Manual, Second Edition, 2014; Edward A. Greenfield, Cold Spring Harbor Laboratory Press, which is hereby incorporated by reference in its entirety. In particular, Chapter 6 provides detailed
30 description of methods for immunizing animals for the production of antibodies.

In particular, aspects of the methods of the present disclosure are concerned with producing monoclonal antibodies. Such methods may comprise isolating antigen-binding molecule-producing cells from subjects. Such methods may comprise generating monoclonal hybridomas from cells isolated from
35 subjects, wherein the hybridomas produce antibodies of a single type (*i.e.* of a single specificity).

Methods of antibody production involves introducing antigen into an animal to elicit the production of antibodies which can then be recovered from the animal.

40 Aspects and embodiments of the methods of the present disclosure comprise:

(i) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;

(ii) treating the animal to inhibit its ability to mount a primary immune response; and
(iii) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

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In some embodiments, treatment of the animal to inhibit its ability to mount a primary immune response is performed after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have undergone immunoglobulin isotype switching (*i.e.* to IgG-, IgE-, or IgA-expressing cells). In some embodiments, treatment of the animal to inhibit its ability to mount a primary immune response is performed after a period of time sufficient for the cells activated/stimulated to proliferate by administration of the first peptide/polypeptide (or cells derived from such cells) to have differentiated into plasma B cells and/or memory B cells.

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In some embodiments a method for producing an antigen-binding molecule comprises one or more of:

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preparing/formulating a peptide/polypeptide/nucleic acid/cell to be introduced into an animal;
introducing a peptide/polypeptide/nucleic acid/cell into an animal;
detecting and/or monitoring production of antigen-binding molecules by the animal;
detecting and/or monitoring production of cells expressing/comprising antigen-binding molecules by the animal;

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collecting antigen-binding molecules produced by the animal;
isolating/purifying antigen-binding molecules produced by the animal;
collecting cells producing antigen-binding molecules from the animal;
isolating/purifying cells producing antigen-binding molecules from the animal;
generating a hybridoma producing antigen-binding molecules;

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culturing cells producing antigen-binding molecules; and
isolating/purifying antigen-binding molecules produced by cells in culture.

In some embodiments, methods comprise isolating antigen-binding molecule-producing cells from the animal. In some embodiments, antigen-binding molecule-producing cells are harvested from the blood of the animal (*e.g.* from PBMCs obtained from the blood of the animal), or from an organ of the animal (*e.g.* the spleen). In some embodiments the isolated antigen-binding molecule-producing cells are cultured *in vitro*. In some embodiments the methods comprise culturing cells isolated from the subject *in vitro*.

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In some embodiments, methods comprise isolating antigen-binding molecules capable of binding to the protein of interest. In some embodiments, the antigen-binding molecules are isolated from the animal. The antigen-binding molecules may be recovered from *e.g.* the blood, plasma, serum, or ascites of the animal.

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In some embodiments, antigen-binding molecules may be isolated from cells obtained from the animal. In some embodiments, the cells are B cells. In some embodiments, the antigen-binding molecules may be isolated from cell culture supernatant from B cells cultured *in vitro*.

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In some embodiments, antigen-binding molecules are obtained from a hybridoma producing antigen-binding molecules capable of binding to the protein of interest. In some embodiments, the methods comprise isolating antigen-binding molecules capable of binding to the protein of interest from a culture of a hybridoma produced according to the present disclosure. In some embodiments, the antigen-binding molecules are obtained from cell culture supernatant of a culture of a hybridoma. In some embodiments, the antigen-binding molecules are obtained from the blood, plasma, serum, or ascites of an animal immunized with a hybridoma.

Methods for isolating (*i.e.* purifying) antigen-binding molecules from an antigen-binding molecule containing sample (*e.g.* cell, cell extract, cell culture medium, blood, plasma, serum, ascites) are well known to the skilled person, and are described in detail in *Antigen-binding molecules: A Laboratory Manual*, Second Edition, 2014; Edward A. Greenfield, Cold Spring Harbor Laboratory Press (incorporated by reference herein above), in particular at Chapter 10. The methods include, for example, ion exchange chromatography, protein A or protein G based purification, gel electrophoresis, dialysis, and affinity purification based on target binding.

An isolated or purified antigen-binding molecule as used herein refers to a composition comprising an antibody, of which at least 80%, 90%, 95%, 99% or 100% of the composition (by weight, or by weight of the protein component of the composition) is the antigen-binding molecule component of the composition.

In some embodiments, the methods of the present disclosure employ single B cell cloning. Single B cell cloning for the production of monoclonal antibodies is described *e.g.* in Carbonetti *et al.*, *J Immunol Methods* (2017) 448: 66–73 and Lei *et al.* *Front Microbiol* (2019) 10:672, both of which are hereby incorporated by reference in their entirety. Such methods generally comprise culturing B cells obtained from an animal as single clones *in vitro*, *e.g.* in the presence of factors promoting proliferation of the B cells and/or production of antibodies from the B cells. B cells may be obtained from the blood of the animal (*e.g.* from populations of PBMCs derived from the blood of the animal), or from an organ of the animal (*e.g.* the spleen). B cells may be sorted into single cell cultures by FACS, or another cell sorting technique. B cells expressing antibodies having properties of interest may be sequenced in order to determine the amino acid sequence of the antibody and/or the nucleic acid sequence encoding the antibody.

Methods for hybridoma formation are well known to the skilled person, and are described, for example, in *Antibodies: A Laboratory Manual*, Second Edition, 2014; Edward A. Greenfield, Cold Spring Harbor Laboratory Press (incorporated by reference herein above); in particular, at Chapter 7. Briefly, an animal is immunized in accordance with the present disclosure, stimulating an adaptive immune response, and B lymphocytes are isolated from the animal and fused with a suitable myeloma cell line to produce a hybridoma.

Antigen-binding molecule production by a subject (*e.g.* antigen-binding molecule titer) may be determined as described herein, prior to hybridoma production. If the titer is too low, one or more booster steps may be performed as described herein, and antigen-binding molecule production monitored (*e.g.* by repeated

blood sampling), until a sufficiently high titer is achieved. In some embodiments, the methods comprise selecting a subject for hybridoma production on the basis of antigen-binding molecule production or titer. Antigen-binding molecule production or titer may be determined in e.g. a blood, plasma, serum or ascites sample obtained from the animal.

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In some embodiments, subjects and/or cells may be selected in accordance with methods for producing an antigen-binding molecule based on detection of production an antigen-binding molecule capable of binding to the protein of interest.

- 10 In some embodiments, collecting cells producing antigen-binding molecules comprises harvesting the spleen and/or lymph nodes of a subject. In some embodiments producing a hybridoma comprises fusing a cell (e.g. a B cell) capable of producing an antigen-binding molecule obtained from a subject with a myeloma cell. In some embodiments the fusing a cell (e.g. a B cell) capable of producing an antigen-binding molecule obtained from a subject with a myeloma cell comprises co-centrifugation in polyethylene glycol (PEG). In some embodiments producing a hybridoma comprises selected by culture of cells in selective media such as media containing hypoxanthine-aminopterin-thymidine (HAT).
- 15

- Hybridoma colonies may be tested for production of antibody capable of binding to the protein of interest, and/or other peptides and polypeptides, as described herein, e.g. by immunoprecipitation, immunoblotting, or in vitro binding assays (e.g. flow cytometry, ELISA, etc.). In some embodiments, antibody production or antibody titer may be determined in cell culture supernatant from hybridoma cultured *in vitro*.
- 20

- The present disclosure also provides an antigen-binding molecule capable of binding to a protein of interest, wherein the antigen-binding molecule is obtained by, or obtainable by, a method for producing an antigen-binding molecule as described herein.
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- In some embodiments, the methods further comprise formulating antigen-binding molecules to a composition, e.g. a pharmaceutical composition. In some embodiments the methods comprise mixing an antigen-binding molecule with a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. Pharmaceutical compositions may be formulated in fluid (including gel) or solid (e.g. tablet) form. Fluid formulations may be formulated for administration by injection or via catheter to a selected region of the human or animal body. The present disclosure also provides a pharmaceutical composition formed by a method according to the present disclosure.
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Antigen-binding molecules produced by the methods of the present disclosure may be produced on a large scale using methods known to the skilled person.

- Hybridomas can be propagated either in *in vitro* culture using standard methods of cell culture, or *in vivo*, e.g. as ascites in a host animal. In some embodiments, the methods of the present disclosure comprise propagating the hybridoma by *in vitro* cell culture. In some embodiments, the methods comprise propagating the hybridoma *in vivo* by injecting a host animal with the hybridoma.
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In some embodiments, antigen-binding molecules can be made using recombinant DNA techniques known to the skilled person. For example, a polynucleotide encoding an antibody can be derived from a B cell or hybridoma cell producing an antibody, *e.g.*, by reverse transcription PCR (RT-PCR) using oligonucleotide primers that specifically amplify the genes encoding the heavy and light chains of the antibody, and the sequence of the polynucleotide can be determined. Isolated polynucleotides encoding the heavy and light chains can be cloned into suitable expression vectors which produce the monoclonal antibodies when transfected into host cells such as *E. coli*, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin proteins.

Amino acid sequence of interest

An “amino acid sequence of interest” as used herein refers to (i) an amino acid sequence of a protein/protein complex of interest, or (ii) an amino acid sequence which is similar to the amino acid sequence of the protein/protein complex of interest (*i.e.* an amino acid sequence which is similar to the amino acid sequence of (i)).

As used herein, an amino acid sequence which is “similar to” a reference amino acid sequence is an amino acid sequence having some shared character with the reference amino acid sequence. In some embodiments an amino acid sequence which is “similar to” a reference amino acid sequence is an amino acid sequence comprised in a protein which is an isoform, variant or homolog of the protein comprising the reference amino acid sequence. In some embodiments, an amino acid sequence which is “similar to” a reference amino acid sequence (*e.g.* an amino acid sequence of a protein of interest) comprises one of at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the reference amino acid sequence. In some embodiments, an amino acid sequence which is “similar to” a reference amino acid sequence does not differ by more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids to the reference amino acid sequence.

Conversely, an amino acid sequence which is “dissimilar to” a reference amino acid sequence is an amino acid sequence having comprises less than 100%, *e.g.* one of less than 90%, 80%, 70%, 60%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or less than 5% sequence identity to the reference amino acid sequence. In some embodiments, an amino acid sequence which is “dissimilar to” a reference amino acid sequence differs by more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids to the reference amino acid sequence.

Comparison of a given amino acid sequence (*i.e.* a query sequence) to a reference amino acid sequence can be performed by aligning the sequences and comparing the amino acids at corresponding positions. In some embodiments, the sequence comparison is performed over a region of the query sequence and reference sequences which is at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 15 to 20, 20 to 25, or 25 to 30, 30 to 60, 40 to 80, or 80 to 100 amino acids in length.

As used herein, a “protein” includes peptides and polypeptides. In some embodiments a protein of interest may be a peptide of interest, or a polypeptide of interest. A “peptide” is a chain of two or more

amino acid monomers linked by peptide bonds. A peptide typically has a length in the region of about 2 to 50 amino acids. A "polypeptide" is a polymer chain of two or more peptides. Polypeptides typically have a length greater than about 50 amino acids.

- 5 Reference to peptides and polypeptides herein also encompasses complexes comprising such peptides/polypeptides, which may be homo- or hetero-multimeric complexes (e.g. formed by non-covalent interactions) comprising two or more (e.g. 2, 3, 4, 5, 6, 7, 8 or more) peptides/polypeptides.

- 10 A protein of interest may be any protein. A protein of interest may for example be a protein of diagnostic, prognostic, imaging or therapeutic relevance. In some embodiments a protein of interest may be a candidate therapeutic target for an antigen-binding molecule. **"A protein of interest" as used herein means "one or more protein(s) of interest".** That is, an antibody capable of binding to a protein may be to more than one protein of interest. **Reference to a "protein of interest" herein also includes a protein complex of interest,** which may be a homo- or hetero-multimeric complex (e.g. formed by non-covalent interactions)
15 comprising two or more (e.g. 2, 3, 4, 5, 6, 7, 8 or more) polypeptides.

- In some embodiments, the protein of interest is a protein whose expression/activity, or whose upregulated expression/activity, is positively associated with a disease or disorder (e.g. a cancer, an infectious disease or an autoimmune disease). In some embodiments, the protein of interest is expressed by a
20 pathogen/infectious agent, cell, or a cell of a tissue, which it is desirable to destroy or remove. In some embodiments, the protein of interest is expressed by a pathogen/infectious agent, cell, or a cell of a tissue to which it is desirable to direct a humoral immune response. In some embodiments the protein of interest is associated with a cancer, an infectious disease, or an autoimmune disease. Pathogens include prokaryotic (bacteria), eukaryotic (e.g. protozoan, helminth, fungus) and viral pathogens. In some
25 embodiments, the protein of interest is expressed by a cancer cell, an infectious agent, a cell infected with an infectious agent or an autoimmune effector cell (i.e. an effector of an autoimmune pathology). In some embodiments the protein of interest is a disease/disorder-associated (e.g. cancer-associated and/or autoimmune disease) variant of a protein.

- 30 In some embodiments the antigen-binding molecules generated in accordance with the methods of the present disclosure are capable of recognizing proteins related to the protein of interest.

- A "protein related to the protein of interest" refers to a protein having some shared character with a reference protein of interest. For example, the methods of the present disclosure can be used to elicit
35 antigen-binding molecules capable of recognizing variants of a protein of a pathogen/infectious agent. That is, the methods can be employed to elicit broadly-neutralizing antigen-binding molecules. In some embodiments a protein related to the protein of interest comprises the amino acid sequence of interest. In some embodiments a protein related to the protein of interest comprises the amino acid sequence of the protein of interest.

- 40 Related proteins include isoforms, fragments, variants or homologs (e.g. paralogs, orthologs) of the protein of interest, including proteins which are members of the same protein family. Isoforms, fragments,

variants or homologs of a given protein of interest may optionally be characterized as having an amino acid sequence having at least 60%, preferably one of 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% amino acid sequence identity to the amino acid sequence of the reference protein.

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As used herein, “sequence identity” refers to the percent of nucleotides/amino acid residues in a subject sequence that are identical to nucleotides/amino acid residues in a reference sequence, after aligning the sequences and, if necessary, introducing gaps, to achieve the maximum percent sequence identity between the sequences. Pairwise and multiple sequence alignment for the purposes of determining percent sequence identity between two or more amino acid or nucleic acid sequences can be achieved in various ways known to a person of skill in the art, for instance, using publicly available computer software such as ClustalOmega (Söding, J. 2005, Bioinformatics 21, 951-960), T-coffee (Notredame *et al.* 2000, J. Mol. Biol. (2000) 302, 205-217), Kalign (Lassmann and Sonnhammer 2005, BMC Bioinformatics, 6(298)) and MAFFT (Kato and Standley 2013, Molecular Biology and Evolution, 30(4) 772–780 software. When using such software, the default parameters, *e.g.* for gap penalty and extension penalty, are preferably used.

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In some embodiments, an amino acid sequence of interest may be *e.g.* a consensus or majority sequence for more than one related protein in a corresponding region. In some embodiments, an amino acid sequence of interest may be a consensus sequence for the corresponding region to the amino acid sequence of a protein of interest for two or more isoforms, homologs or variants of the protein of interest.

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In some embodiments, the amino acid sequence of interest is an antigenic amino acid sequence. The term “antigenic” as used herein refers to the ability to stimulate an immune response, in particular an adaptive immune response (*e.g.* a B cell- and/or T cell-mediated immune response). In some embodiments the amino acid sequence of interest is capable of stimulating a B cell-mediated immune response. In some embodiments the amino acid sequence of interest is a sequence of amino acids which forms, or is predicted to form, a B cell epitope. In some embodiments the amino acid sequence of interest is capable of stimulating the production of antigen-binding molecules.

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In some embodiments, the amino acid sequence of interest is a consecutive sequence of amino acids of the protein of interest, or a similar sequence. In some embodiments, for example in embodiments where the amino acid sequence of interest folds to form a discontinuous epitope, the amino acid sequence of interest is a discontinuous sequence of amino acids of the protein of interest, or a similar sequence.

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In some embodiments the amino acid sequence of interest is a continuous sequence of amino acids providing a linear epitope. In some embodiments the amino acid sequence of interest is a continuous sequence of amino acids which folds to provide a discontinuous epitope. In some embodiments the amino acid sequence of interest is a discontinuous sequence of amino acids which together form a discontinuous epitope.

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In some embodiments the amino acid sequence of interest is a continuous sequence of amino acids of a protein of interest, or a similar sequence. In some embodiments the amino acid sequence of interest is a discontinuous sequence of amino acids of a protein of interest, or a similar sequence. In some
 5 embodiments the amino acid sequence of interest is a discontinuous sequence of amino acids of a protein complex of interest, or a similar sequence. In some embodiments the amino acid sequence of interest is a discontinuous sequence of amino acids formed of sequences of amino acids from two or more polypeptides of a protein complex of interest, or a similar sequence.

In some embodiments the amino acid sequence of interest has a length of one of 5 to 100, 5 to 50, 5 to
 10 40, 5 to 35, 5 to 30, 5 to 25, 5 to 20, 5 to 19, 5 to 18, 5 to 17, 5 to 16, 5 to 15, 5 to 14, 5 to 13, 5 to 12, 5 to 11, or 5 to 10 amino acids. In some embodiments the amino acid sequence of interest has a length of one of 10 to 100, 10 to 50, 10 to 40, 10 to 35, 10 to 30, 10 to 25, 10 to 20, 10 to 19, 10 to 18, 10 to 17, 10 to 16, 10 to 15, 10 to 14, 10 to 13, or 10 to 12 amino acids. In some embodiments, the amino acid sequence of interest has a length of one of 15 to 100, 15 to 50, 15 to 40, 15 to 35, 15 to 30, 15 to 25, 15 to 20, 15 to
 15 19, 15 to 18, or 15 to 17 amino acids. In some embodiments, the amino acid sequence of interest has a length of one of 20 to 100, 20 to 50, 20 to 40, 20 to 35, 20 to 30, or 20 to 25 amino acids. In some embodiments, the amino acid sequence of interest has a length of 5 to 30 amino acids.

Where the amino acid sequence of interest provides, or is predicted to provide, a discontinuous epitope,
 20 the amino acid sequence of interest may refer either to the continuous sequence of amino acids which folds to provide the discontinuous epitope, or the discontinuous sequence of amino acids which forms the discontinuous epitope.

Where the amino acid sequence of interest provides a discontinuous epitope, the overall length of the
 25 discontinuous epitope may be about 5 to 30 amino acids. Discontinuous epitopes may comprise *e.g.* 2, 3, 4, 5, 6 or 7 non-continuous sequences of amino acids. Such non-continuous sequences may each comprise *e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids, but preferably less than *e.g.* 30, 25, 20 or 15 amino acids.

The skilled person is able to identify antigenic sequences of a protein of interest by methods known in the
 30 art. For some proteins, sequences of amino acids which are known or predicted to be antigenic are provided in databases such as BciPep, AntiJen, AntigenDB, SPTR, FIMM, HPTAA, IEDB, Epitome, MHCBN, MHCPEP, MPID-T2, Protegen. Antigenic amino acid sequences of proteins can also be identified by literature searches, for example using the internet.

Sequences of amino acids which are predicted to form B cell epitopes, and that are therefore likely to be
 effective in raising antibodies, can be predicted from a query sequence by a variety of methods. The
 skilled person is able to predict whether a sequence of amino acids is antigenic by comparison to known
 or predicted antigenic sequences (*e.g.* for other proteins), and/or based on properties of the amino acid
 40 sequence. See, for example, El-Manzalawy and Honavar, Immunome Res, 2010, 6(Suppl 2): S2, which is hereby incorporated by reference in its entirety. Such methods take into account, for example, the hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of

sequences of amino acids. Whether a peptide/polypeptide comprises a T cell epitope can be determined e.g. using prediction methods as described in Desai *et al.*, 2014 Methods Mol Biol 1184:333-364.

Some of the methods take into account three-dimensional structure, and can be used to predict

5 conformational epitopes. Software which can be used to identify/predict antigenic sequences of amino acids includes EMBOSS: antigenic, BepiPred, IEDB Analysis Resource, SVMTriP, and SCRATCH, ElliPro, COBEPro, BEPro, PEPITO and DiscoTope.

Antigenic sequences of a protein of interest can also be identified by experimentally determining whether
10 an amino acid or sequence of amino acids is antigenic. For example, the skilled person is able to determine whether a given amino acid sequence is antigenic, for example by immunizing a subject (e.g. a mammal) with a peptide of the amino acid sequence and determining whether an adaptive immune response is elicited. One or more of the described approaches may be employed separately or in combination in the methods of the present disclosure to identify antigenic sequences of amino acids of a
15 protein of interest.

When a sequence of amino acids is assessed for similarity to a reference amino acid sequence of a protein of interest which provides, or is predicted to provide, a discontinuous epitope, the sequence of amino acids may be aligned to the continuous sequence of amino acids which folds to form the
20 discontinuous epitope, or the discontinuous sequence of amino acids which together form the discontinuous epitope.

First peptide/polypeptide

Methods of the present disclosure comprise administering a first peptide/polypeptide, or nucleic acid
25 encoding the first peptide/polypeptide, to an animal, wherein first peptide/polypeptide comprises an amino acid sequence of interest. In some embodiments the first peptide/polypeptide consists of, or consists essentially of, the amino acid sequence of interest.

It will be appreciated that the first peptide/polypeptide encompasses peptides/polypeptides comprising or
30 consisting of: (i) an amino acid sequence of a protein of interest, or (ii) an amino acid sequence which is similar to the amino acid sequence of the protein of interest (*i.e.* an amino acid sequence which is similar to the amino acid sequence of (i)).

In some embodiments the first peptide/polypeptide comprises the amino acid sequence of interest, and
35 additionally comprises further amino acids. In some embodiments the first peptide/polypeptide comprises the amino acid sequence of interest and one of 1-5, 1-10, 1-15, 1-20, 1-25, 1-30, 1-40 or 1-50 additional amino acids, at one or both ends (*i.e.* the N- or C-terminus) of the amino acid sequence of interest.

In some embodiments the additional amino acids correspond to the amino acids provided at those
40 positions relative to the amino acid sequence of interest, in the context of the protein from which the amino acid sequence of interest is derived. By way of illustration, where an amino acid sequence of interest corresponds to amino acid positions 20 to 30 of the amino acid sequence of a protein of interest,

and wherein the first peptide/polypeptide comprises the amino acid sequence of interest and an additional 5 amino acids at the N-terminus of the amino acid sequence of interest, those additional 5 amino acids may correspond to positions 15 to 19 of the amino acid sequence of the protein of interest.

- 5 The first peptide/polypeptide may comprise more than one peptide/polypeptide chain. For example, the first peptide/polypeptide may be a complex of peptides/polypeptides, e.g. comprising 2, 3, 4, 5 or 6 or more peptides/polypeptides. The peptides/polypeptides may be conjugated to one other, or administered as a composition (e.g. mixture) of two or more peptides/polypeptides that are not conjugated.
- 10 In some embodiments the first peptide/polypeptide is provided as a conjugate with a carrier protein. As used herein, a “carrier protein” refers to a protein which can be used to elicit an immune response to the peptide/polypeptide to which it is conjugated (*i.e.* which it acts as a “carrier” for). Due to their size and complexity, carrier proteins induce an immune response, including to the conjugated peptide/polypeptide. Many proteins can be used as carriers and are chosen based on immunogenicity, solubility, and
- 15 availability of useful functional groups through which conjugation with a peptide/polypeptide of interest can be achieved. Carrier proteins are well known in the art of immunology, and are described e.g. in “Thermo Scientific Pierce Antibody Production and Purification Technical Handbook”, Version 2, (2010); Thermo Scientific, USA (1601975 09/10), which is hereby incorporated by reference in its entirety.
- 20 In some embodiments the carrier protein is selected from keyhole limpet hemocyanin (KLH), *Concholepas concholepas* hemocyanin (CCH; also known as Blue Carrier Protein), bovine serum albumin (BSA), cationized BSA (cBSA), hepatitis B core antigen (HBc), thyroglobulin and ovalbumin (OVA). In some embodiments the carrier protein is selected from KLH, BSA, HBc and OVA.
- 25 Peptide/polypeptides may be conjugated to carrier proteins via means well known to the skilled person., including e.g. amine-sulfhydryl crosslinking (e.g. using succinimidyl 6-((beta-maleimidopropionamido) hexanoate) SMPH), EDC conjugation (carboxyl and amino crosslinking), maleimide conjugation (sulfhydryl crosslinking), glutaraldehyde conjugation (amine-amine crosslinking) (see “Thermo Scientific Pierce Antibody Production and Purification Technical Handbook”, Version 2, (2010); Thermo Scientific,
- 30 USA (1601975 09/10)).

A “first peptide/polypeptide” as used herein, may refer to at least one peptide/polypeptide (*i.e.* one or more peptides/polypeptides). Similarly, a “nucleic acid encoding a first peptide/polypeptide” may refer to a nucleic acid encoding at least one peptide/polypeptide, or at least one nucleic acid encoding one or more

35 peptides/polypeptides. For example, a “first peptide/polypeptide” may refer to 2, 3, 4, 5, 6, 7, 8, 9, 10 or more peptides and/or polypeptides, e.g., in a mixture or conjugated to one another. A “nucleic acid encoding a first peptide/polypeptide” may refer to a nucleic acid encoding 2, 3, 4, 5, 6, 7, 8, 9, 10 or more peptides and/or polypeptides, or 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleic acids encoding 2, 3, 4, 5, 6, 7, 8, 9, 10 or more peptides and/or polypeptides. A “first peptide/polypeptide” or “nucleic acid encoding a first

40 peptide/polypeptide” may be a mixture of at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 peptides/polypeptides or nucleic acids encoding peptides/polypeptides.

The two or more peptides/polypeptides/nucleic acids may be administered simultaneously and/or sequentially, as described herein.

Second peptide/polypeptide

5 Methods of the present disclosure comprise administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to an animal, wherein second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

10 In some embodiments, the second peptide/polypeptide is identical to the first peptide/polypeptide. In some embodiments, the second peptide/polypeptide is non-identical to the first peptide/polypeptide.

It will be appreciated that a peptide/polypeptide which is “non-identical” to a reference peptide/polypeptide comprises an amino acid sequence having less than 100% sequence identity to the amino acid sequence of the reference peptide/polypeptide. In some embodiments, the second peptide/polypeptide has an amino acid sequence having less than 100%, e.g. one of less than 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91% or 90% sequence identity to the amino acid sequence of the first peptide/polypeptide. In some embodiments, the second peptide/polypeptide has an amino acid sequence having at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the amino acid sequence of the first peptide/polypeptide.

It will be appreciated that the second peptide/polypeptide encompasses peptides/polypeptides comprising: (a) the amino acid sequence of interest which is comprised in the first peptide/polypeptide, or (b) an amino acid sequence which is similar to the amino acid sequence of interest which is comprised in the first peptide/polypeptide.

It will in turn be appreciated that (as explained hereinabove) the amino acid sequence of interest comprised in the first peptide/polypeptide can be (i) an amino acid sequence of a protein of interest, or (ii) an amino acid sequence which is similar to the amino acid sequence of the protein of interest (i.e. an amino acid sequence which is similar to the amino acid sequence of (i)).

In some embodiments the second peptide/polypeptide comprises the amino acid sequence of interest, and additionally comprises further amino acids. In some embodiments second peptide/polypeptide comprises at least 5, 10, 15, 20, 40, 50, 80, 100, 200, or 300 additional amino acids at one or both ends (i.e. the N- or C-terminus) of the amino acid sequence of interest. In some embodiments second peptide/polypeptide comprises one of at least 1-5, 1-10, 1-15, 1-20, 1-40, 1-50, 1-80, or 1-100, 1-200 or 1-300 additional amino acids at one or both ends (i.e. the N- or C-terminus) of the amino acid sequence of interest.

40 The second peptide/polypeptide may comprise more than one peptide/polypeptide chain. For example, the second peptide/polypeptide may be a complex of peptides/polypeptides, e.g. comprising 2, 3, 4, 5 or 6 or more peptides/polypeptides. The peptides/polypeptides may be conjugated to one other, or

administered as a composition (e.g. mixture) of two or more peptides/polypeptides that are not conjugated.

Peptides/polypeptides and nucleic acids described herein may be administered in the form of cells comprising/expressing the peptide/polypeptide/nucleic acid, or synthetic agents comprising the peptide/polypeptide/nucleic acid.

In some embodiments, the second peptide/polypeptide may further comprise 1 or more amino acids at either or both ends of the second peptide/polypeptide. In some embodiments, the second peptide/polypeptide may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acids, or one of 1-20, 1-15, 1-10, 1-8, 1-6, 1-5, 1-4, or 1-3 amino acids at one end, or both ends.

In some embodiments, the second peptide/polypeptide is capable of eliciting the production of antigen-binding molecules capable of binding to the protein of interest and an isoform, variant or homolog of the protein of interest.

A "second peptide/polypeptide" as used herein, may refer to at least one peptide/polypeptide (i.e. one or more peptides/polypeptides). Similarly, a "nucleic acid encoding a second peptide/polypeptide" may refer to a nucleic acid encoding at least one peptide/polypeptide, or at least one nucleic acid encoding one or more peptides/polypeptides. For example, a "second peptide/polypeptide" may refer to 2, 3, 4, 5, 6, 7, 8, 9, 10 or more peptides and/or polypeptides, e.g., in a mixture or conjugated to one another. A "nucleic acid encoding a second peptide/polypeptide" may refer to a nucleic acid encoding 2, 3, 4, 5, 6, 7, 8, 9, 10 or more peptides and/or polypeptides, or 2, 3, 4, 5, 6, 7, 8, 9, 10 or more nucleic acids encoding 2, 3, 4, 5, 6, 7, 8, 9, 10 or more peptides and/or polypeptides. A "second peptide/polypeptide" or "nucleic acid encoding a second peptide/polypeptide" may be a mixture of at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 peptides/polypeptides or nucleic acids encoding peptides/polypeptides.

The two or more peptides/polypeptides/nucleic acids may be administered simultaneously and/or sequentially, as described herein.

Nucleic acid encoding peptides/polypeptides

As described herein, the peptides/polypeptides of the present disclosure may be expressed from nucleic acid encoding the peptides/polypeptides. The nucleic acid may be, or may be comprised in, a vector. In some embodiments, the nucleic acid may be DNA encoding a peptide/polypeptide described herein.

Nucleic acid/vector may be administered to a subject in order to express a peptide/polypeptide as described herein. The nucleic acid/vector may be present in a cell and the cell may be administered to the animal. The nucleic acid/vector may be incorporated into the genome of a cell, and the cell may be administered to the animal. The nucleic acid/vector may provide for recombinant expression of a peptide/polypeptide as described herein.

A “vector” as used herein is a nucleic acid molecule used as a vehicle to transfer exogenous nucleic acid into a cell. The vector may be a vector for expression of the nucleic acid in the cell. Such vectors may include a promoter sequence operably linked to the nucleotide sequence encoding the sequence to be expressed. A vector may also include a termination codon and expression enhancers. Any suitable
5 vectors, promoters, enhancers and termination codons known in the art may be used to express a peptide or polypeptide from a vector according to the present disclosure. The term “operably linked” may include the situation where a selected nucleic acid sequence and regulatory nucleic acid sequence (e.g. promoter and/or enhancer) are covalently linked in such a way as to place the expression of nucleic acid sequence under the influence or control of the regulatory sequence (thereby forming an expression
10 cassette). Thus a regulatory sequence is operably linked to the selected nucleic acid sequence if the regulatory sequence is capable of effecting transcription of the nucleic acid sequence. The resulting transcript(s) may then be translated into a desired peptide(s)/polypeptide(s). Suitable vectors include plasmids, binary vectors, DNA vectors, mRNA vectors, viral vectors (e.g. gammaretroviral vectors (e.g. murine Leukemia virus (MLV)-derived vectors), lentiviral vectors, adenovirus vectors, adeno-associated
15 virus vectors, vaccinia virus vectors and herpesvirus vectors), transposon-based vectors, and artificial chromosomes (e.g. yeast artificial chromosomes).

In some embodiments, the vector may be a eukaryotic vector, e.g. a vector comprising the elements necessary for expression of protein from the vector in a eukaryotic cell. In some embodiments, the vector
20 may be a mammalian vector, e.g. comprising a cytomegalovirus (CMV) or SV40 promoter to drive protein expression.

Methods for immunizing a subject with nucleic acid encoding a peptide/polypeptide of interest to elicit an antibody response is described, for example, in Aurisicchio *et al.* (2012) J Cell Physiol 227: 3381-3388,
25 which is hereby incorporated by reference in its entirety.

In some embodiments of the methods of the present disclosure, nucleic acid encoding a peptide/polypeptide as described herein is administered to an animal. In such embodiments, the peptide or polypeptide is expressed in the animal following immunization.
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Administrations

Aspects of the present disclosure involve administration of peptides/polypeptides, or nucleic acids encoding peptides/polypeptides, to an animal. Aspects of the present disclosure also include administering agents for inducing inhibition of the ability of an animal to mount primary immune response
35 and/or promotion of the ability of an animal to mount a secondary immune response to an animal.

A step of “administering” a peptide/polypeptide/nucleic acid to an animal comprises introducing the peptide/polypeptide/nucleic acid into an animal one or more times. In some embodiments, “administering” a peptide/polypeptide/nucleic acid into an animal comprises one of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 separate
40 introductions of the peptide/polypeptide/nucleic acid into an animal.

By way of illustration, in some embodiments the method comprises introducing a first peptide/polypeptide (or nucleic acid encoding the first peptide/polypeptide) as defined herein into an animal on 2 occasions, and introducing a second peptide/polypeptide (or nucleic acid encoding the second peptide/polypeptide) as defined herein into the animal on 2 occasions.

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In some embodiments the method comprises introducing a first peptide/polypeptide (or nucleic acid encoding the first peptide/polypeptide) as defined herein into an animal on one of 3, 4, 5 or 6 occasions, and introducing a second peptide/polypeptide (or nucleic acid encoding the second peptide/polypeptide) as defined herein into the animal on one of 1, 2, 3 or 4 occasions.

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In some embodiments the method comprises introducing a first peptide/polypeptide (or nucleic acid encoding the first peptide/polypeptide) as defined herein into an animal on 2 occasions, and introducing a second peptide/polypeptide (or nucleic acid encoding the second peptide/polypeptide) as defined herein into the animal on 1 occasion.

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Similarly, a step of “administering” an agent resulting in inhibition of a primary immune response and/or promoting a secondary immune response comprises introducing the agent into the animal one or more times. In some embodiments, “administering” the agent comprises one of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 separate introductions of the agent into the animal.

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In some embodiments the method comprises introducing an agent as defined herein into the animal on one of 1, 2, 3 or 4 occasions.

In some embodiments the time interval between individual introductions of an administration step comprising plural introductions is one of at least 24 hours, 36 hours, 48 hours, 72 hours, 4 days, 5 days, 7 days, 10 days or 12 days. In some embodiments, the time interval between individual introductions of an administration step comprising plural introductions is about 5-30 days, 7-20 days, *e.g.* about 10-16 days. In some embodiments, the time interval between individual introductions of an administration step comprising plural introductions is about 2-30 days, 5-20 days, *e.g.* about 6-8 days.

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Introductions in accordance with an administration step described herein may be of identical materials, or non-identical materials, provided the materials satisfy the requirements of the material to be administered in accordance with the administration step.

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By way of illustration, administration of a first peptide/polypeptide as defined herein (or nucleic acid encoding the same) may comprise simultaneous or sequence introduction of the same peptide/polypeptide (or nucleic acid encoding the same), or of two or more (*e.g.* one of 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) non-identical peptides/polypeptides each independently meeting the requirements of a first peptide/polypeptide described herein (or nucleic acid encoding the same). Likewise, administration of a second peptide/polypeptide as defined herein (or nucleic acid encoding the same) may comprise simultaneous or sequence introduction of the same peptide/polypeptide (or nucleic acid encoding the same), or of two or more (*e.g.* one of 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) non-identical peptides/polypeptides

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each independently meeting the requirements of a second peptide/polypeptide described herein (or nucleic acid encoding the same).

Individual introductions of an administration step described herein may be sequential. That is, in some embodiments a peptide/polypeptide/nucleic acid is introduced into a subject, and after a given time interval a separate introduction is performed.

In some embodiments, a peptide/polypeptide may be administered in the form of an agent comprising or expressing the peptide/polypeptide. In some embodiments, a peptide/polypeptide may be administered in the form of an agent comprising nucleic acid encoding peptide/polypeptide. In some embodiments, a nucleic acid may be administered in the form of an agent comprising the nucleic acid.

The agent may be any agent capable of delivering the peptide/polypeptide/nucleic acid. In some embodiments the agent is a cell. In some embodiments the agent is a virus, or a virus-like particle.

In some particular embodiments, a peptide/polypeptide may be administered in the form of a cell comprising or expressing the peptide/polypeptide. In some embodiments, a peptide/polypeptide may be administered in the form of a cell comprising nucleic acid encoding peptide/polypeptide. In some embodiments, a nucleic acid may be administered in the form of a cell comprising the nucleic acid.

A cell expressing a peptide/polypeptide may endogenously express the peptide/polypeptide. That is, the peptide/polypeptide may be encoded by, and/or expressed from, nucleic acid of the cell prior to any introduction of nucleic acid encoding the peptide/polypeptide into the cell. A cell expressing a peptide/polypeptide may exogenously express the peptide/polypeptide. That is, the peptide/polypeptide may be encoded by, and/or expressed from, nucleic acid which has been introduced into the cell. In some embodiments the cell expressing a peptide/polypeptide may be or have been modified to express or overexpress the peptide/polypeptide.

In some embodiments, the peptide/polypeptide/nucleic acid/cell/agent administered is isolated or purified. An isolated or purified peptide/polypeptide/nucleic acid/cell/agent as used herein refers to a composition comprising the peptide/polypeptide/nucleic acid/cell/agent of which at least 80%, 90%, 95%, 99% or 100% of the composition (by weight) is the peptide/polypeptide/nucleic acid/cell/agent component of the composition.

In some embodiments, the peptide/polypeptide/nucleic acid is administered in a cell or protein-containing extract.

A peptide/polypeptide/nucleic acid/cell/agent can be introduced into a subject by any suitable means, such as those described, for example, in *Antibodies: A Laboratory Manual*, Second Edition, 2014; Edward A. Greenfield, Cold Spring Harbor Laboratory Press (incorporated by reference herein above), in particular at Chapter 6.

Materials to be introduced into an animal can be formulated as appropriate to the material, route of introduction, the animal and the desired response.

For example, peptides, polypeptides (optionally conjugated to a carrier protein such as KLH, BSA or OVA, *etc.*), cells and agents may be diluted in sterile saline, and may combined with an adjuvant (*e.g.* Complete or Incomplete Freund's Adjuvant, an aluminium salt (*e.g.* aluminium sulfate (alum), aluminium phosphate, aluminium hydroxide), CpG or an adjuvant described in Lee and Nguyen, Immune Netw. (2015) 15(2):51-57, which is hereby incorporated by reference in its entirety) to form a stable emulsion. Nucleic acids (optionally provided as liposome-nucleic acid complexes), may be injected in aqueous solution in saline, introduced by pneumatic (jet) injection in aqueous solution, or coated on gold beads and introduced by gene gun.

Where materials are introduced into an animal by injection, any suitable site for injection may be used. For example, injections may be intraperitoneal, intravascular (*e.g.* intravenous or intraarterial), intradermal, subcutaneous, intramuscular, intraosseous, intrathecal, epidural, intracardiac, intraarticular, intracavernous, and intravitreal. Preferred routes for injection in accordance with the methods of the present disclosure are intraperitoneal and intravascular (*e.g.* intravenous or intraarterial).

Appropriate amounts of peptide/polypeptide/nucleic acid/agent or number of cells for an individual introduction can be readily determined by the skilled person, *e.g.* by reference to Antibodies: A Laboratory Manual, Second Edition, 2014; Edward A. Greenfield, Cold Spring Harbor Laboratory Press (incorporated by reference herein above). Appropriate volumes and concentrations of formulations for introductions can also readily be determined by the skilled person.

In some embodiments, peptides/polypeptides/nucleic acids/cells may be formulated differently for different introductions, and/or different administration steps. For example, in some embodiments, different carrier proteins or adjuvants may be used in different introductions and/or different administrations steps. In some embodiments, one or more adjuvants may be used in one or more introductions and/or administration steps, and a different adjuvant or no adjuvant may be used in one or more other introductions and/or administration steps according to the methods of the present disclosure.

By way of example, in an embodiment of a method according to the present disclosure a step of administering a first peptide/polypeptide to a subject may comprise introducing the first peptide/polypeptide formulated with *e.g.* **Complete Freund's Adjuvant and CpG into a subject, and a step of administering a second peptide/polypeptide to the subject may comprise introducing the second peptide/polypeptide formulated with *e.g.* Incomplete Freund's Adjuvant and CpG.**

A peptide/polypeptide/nucleic acid/cell/agent according to the present disclosure may be introduced into the animal in an amount appropriate to bring about the desired response.

For example, introducing a peptide/polypeptide as defined herein into an animal may comprise introducing one of 5 µg, 10 µg, 20 µg, 25 µg, 30 µg, 35 µg, 40 µg, 45 µg, 50 µg, 60 µg, 70 µg, 80 µg, 100

µg, 150 µg, 200 µg, 250 µg, 300 µg, 400 µg or 500 µg of the peptide/polypeptide (e.g., in total, or per administration). In some embodiments introducing a peptide/polypeptide as defined herein into an animal may comprise introducing one of 5-500 µg, 10-200 µg, 20-80 µg or ~50 µg of the peptide/polypeptide into an animal (e.g., in total, or per administration).

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For example, introducing an agent as defined herein into an animal may comprise introducing one of 5 µg, 10 µg, 20 µg, 25 µg, 30 µg, 35 µg, 40 µg, 45 µg, 50 µg, 60 µg, 70 µg, 80 µg, 100 µg, 150 µg, 200 µg, 250 µg, 300 µg, 400 µg or 500 µg of the agent (e.g., in total, or per administration). In some embodiments introducing an agent as defined herein into an animal may comprise introducing one of 5-500 µg, 10-300

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µg, 50-200 µg or ~100 µg of the agent into an animal (e.g., in total, or per administration).

Introducing an agent as defined herein into an animal may comprise introducing one of 1 mg/kg, 2 mg/kg, 5 mg/kg, 10 mg/kg, 12 mg/kg, 15 mg/kg, 20 mg/kg or 25 mg/kg of the agent (e.g., in total, or per administration). In some embodiments introducing an agent as defined herein into an animal may

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comprise introducing one of 1-25 mg/kg, 2-20 mg/kg, 5-15 mg/kg or ~10 mg/kg of the agent into an animal (e.g., in total, or per administration).

Administration steps of the methods of the present disclosure may be sequential. That is, in some embodiments an administration step as described herein is performed, and after a given time interval a separate administration step is performed.

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The time interval between administration steps performed sequentially is preferably a time interval appropriate to the desired response in the animal. By way of example, in an embodiment of the method of the present disclosure following administration of a first peptide/polypeptide as described herein (or

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nucleic acid encoding the same), a period of time sufficient for the animal to produce one or more antigen-binding molecules to the first peptide/polypeptide may be allowed to pass before administration of a second peptide/polypeptide as described herein (or nucleic acid encoding the same).

In some embodiments the time interval between administration steps performed sequentially is one of at least 24 hours, 36 hours, 48 hours, 72 hours, 4 days, 5 days, 7 days, 10 days, 14 days, 18 days, 21 days, or 28 days. In some embodiments, administration steps performed sequentially are separated by a period of about 3 to 21 days, e.g. about 5 to 18 days, 7 to 16 days, or 12-16 days. In some embodiments, administration steps performed sequentially are separated by a period of about 14 days. In some

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embodiments, administration steps performed sequentially are separated by a period of about 23 days. In some embodiments, administration steps performed sequentially are separated by a period of about 30 days.

In embodiments where administration steps comprise plural, separate introductions of material into an animal, time intervals provided herein may be between the final introduction of one administration step, and the first introduction of the subsequent administration step.

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In some embodiments, administration steps of the methods of the present disclosure may be performed simultaneously. That is, in some embodiments an administration step as described herein is performed at the same time as or immediately before/after another administration step as described herein.

5 In particular embodiments, the methods of the present disclosure involve simultaneous administration of an agent capable of inhibiting a primary immune response and/or promoting a secondary immune response to the animal with a second peptide/polypeptide as described herein (or nucleic acid encoding the same).

10 The time interval between administration steps performed simultaneously is preferably less than one of 72 hours, 48 hours, 36 hours, 24 hours, 12 hours, or 6 hours.

In some embodiments, where administration steps performed simultaneously, the agents of the separate administration steps are formulated together as a single preparation for administration to the animal. In
15 some embodiments, the agents of the separate administration steps are formulated as separate preparations for administration to the animal.

Administration steps performed sequentially or simultaneously herein may be introduced into the animal by the same route, or by different routes.

20 In some embodiments administration of an agent for inhibiting a primary immune response and/or promoting a secondary immune response in the animal is performed before, at the same time as (e.g., simultaneously with), and/or after administration with the second peptide/polypeptide. In some embodiments administration of an agent for inhibiting a primary immune response and/or promoting a
25 secondary immune response in the animal is performed before and/or after administration with the second peptide/polypeptide, e.g., one or more administrations up to 5 days before administration of the second peptide/polypeptide and/or one or more administrations up to 10 days after administration of the second peptide/polypeptide.

30 In some embodiments an agent for inhibiting a primary immune response and/or promoting a secondary immune response in the animal is administered 3 and/or 1 day(s) before administration of the second peptide/polypeptide, and 1, 3 and/or 6 days after administration of the second peptide/polypeptide.

In some embodiments an agent for inhibiting a primary immune response and/or promoting a secondary
35 immune response in the animal is administered 1, 3 and/or 6 days after administration of the second peptide/polypeptide.

In aspects of the present disclosure that comprise administration of an agent capable of inhibiting a primary immune response and/or promoting a secondary immune response to the animal, it will be appreciated that administration of the agent is such that the primary immune response to the second
40 peptide/polypeptide is inhibited, and/or such that the secondary immune response is promoted.

Likewise, it will be appreciated that administration of the first peptide/polypeptide as described herein (or nucleic acid encoding the same), administration of an agent for inducible inhibition of a primary immune response and/or promoting a secondary immune response to the animal and administration of the second peptide/polypeptide as described herein (or nucleic acid encoding the same) is such that the primary immune response to the first peptide/polypeptide is not substantially inhibited, but the primary immune response to the second peptide/polypeptide is inhibited, and/or the secondary immune response is promoted.

In some embodiments, the methods comprise one or more further administration steps as described herein.

For the purposes of the following statements, the administration steps are as follows:

(a) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal described herein, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;

(b) administering to the animal an agent for inhibiting, e.g., inducibly inhibiting, a primary immune response and/or promoting a secondary immune response in the animal; and

(c) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest, and wherein the second peptide/polypeptide is non-identical to (e.g. larger than) the first peptide/polypeptide.

In some embodiments the methods of the present disclosure comprise one of more of the following combinations of the administration steps identified above:

(1) (a) + (c)

(2) (a) + (b) + (c)

(3) (a) + (c) + (a)

(4) (a) + (b) + (c) + (a)

(5) (a) + (b) + (c) + (b) + (a)

(6) (a) + (c) + (b)

(7) (a) + (b) + (c) + (b)

In some embodiments, the administration steps are as follows:

(a) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal described herein, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;

(b) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest, and wherein the second peptide/polypeptide is non-identical to (e.g. larger than) the first peptide/polypeptide; and

(c) administering to the animal an agent for inhibiting, e.g., inducibly inhibiting, a primary immune response and/or promoting a secondary immune response in the animal.

In some embodiments, the administration steps are as follows:

- 5 (a) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal described herein, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;
- (b) administering to the animal an agent for inhibiting, e.g., inducibly inhibiting, a primary immune response and/or promoting a secondary immune response in the animal;
- 10 (c) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest, and wherein the second peptide/polypeptide is non-identical to (e.g. larger than) the first peptide/polypeptide; and
- 15 (d) administering to the animal an agent for inhibiting, e.g., inducibly inhibiting, a primary immune response and/or promoting a secondary immune response in the animal.

In some embodiments the methods further comprise a booster step. A “booster step” may comprise introduction of a peptide/polypeptide/nucleic acid as described herein into an animal in the absence of a carrier or adjuvant.

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Booster steps are well known to person skilled in the art of immunology. Booster steps may be included in methods for producing antigen-binding molecules, for example to increase titer, for example prior to isolating antigen-binding molecules, or prior to harvesting cells for hybridoma generation.

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Booster steps may be included in methods for generating immunity to proteins/pathogens, for example to elicit an anamnestic response.

In some embodiments, a booster step is given prior to isolation of antigen-binding molecules. In some embodiments, a booster step is given prior to harvesting of B lymphocytes for hybridoma production. In some embodiments, a booster step is performed at least about 12 hours to 5 days, about 1-4 days, or about 2-3 days prior to harvesting B lymphocytes for hybridoma production.

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In some embodiments, a booster step comprises administration to the animal of a peptide/polypeptide/nucleic acid which has already been administered to the animal, for example in a preceding administration step according to (a) or (c) above.

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In some embodiments, the methods comprise performing more than one (e.g. one of 2, 3, 4, 5 or 6) booster steps. Plural booster steps may be separated by one of at least 12 hours, 24 hours, 36 hours, 48 hours or 72 hours. In some embodiments, booster steps may be separated by a period of about 12-48, e.g. ~24 hours.

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A booster step may comprise introduction of the of peptide/polypeptide/nucleic acid by injection. In some embodiments, particularly embodiments relating to methods for producing antigen-binding molecules, a booster step may comprise injecting the peptide/polypeptide/nucleic acid into the abdomen of the animal.

5 Evaluation of antigen-binding molecule production

The methods of the present disclosure may further comprise monitoring/evaluating the immune response of an animal subjected to one or more administration steps as described herein. In some embodiments, the methods comprise detecting the presence of an immune response capable of recognizing a peptide/polypeptide/amino acid sequence of interest.

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In some embodiments, the methods comprise detecting the presence of an antigen-binding molecule capable of binding to a peptide/polypeptide/amino acid sequence of interest. In some embodiments, the methods comprise detecting the presence of an immune cell/population of immune cells capable of producing an antigen-binding molecule capable of binding to a peptide/polypeptide/amino acid sequence of interest.

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In some embodiments, the methods comprise detecting the presence of an antigen-binding molecule possessing one or more functional properties of interest, or an immune cell/population of immune cells capable of producing such an antigen-binding molecule. A functional property of interest may *e.g.* be the ability to antagonize or agonize a function of the protein/protein complex of interest (*e.g.* catalytic activity, binding (*e.g.* protein-protein interaction, such as ligand-receptor binding or multimerization), signalling, transport, storage, structural support *etc.*). Antigen-binding molecules can be analyzed for such functional properties *e.g.* using appropriate assays of such function(s) of the protein/protein complex of interest. Immune responses can be analyzed by methods well known to the skilled person. For example, at an appropriate period of time following administration in accordance with the methods described herein, a sample (*e.g.* a blood sample) may be obtained from the subject and analyzed for antigen-binding molecule production and/or the presence of cells displaying a property of interest (*e.g.* production of antigen-binding molecule capable of recognizing antigen of interest).

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Assays for the detection and quantification of antigen-binding molecule production are well known to the skilled person, and are described, for example, in *Antibodies: A Laboratory Manual*, Second Edition, 2014; Edward A. Greenfield, Cold Spring Harbor Laboratory Press, 2014 (incorporated by reference hereinabove), in particular at Chapter 15. For example, following one or more administration steps according to the present disclosure, a blood, plasma, serum or ascites sample may be collected from the subject, and analyzed *e.g.* by ELISA or flow cytometry. **"Plasma"** as used herein refers to the liquid component of blood, lacking the cellular component, and may be the fluid portion of the blood obtained after removal of the blood cells. **"Serum"** as used herein refers to plasma lacking clotting factors (*e.g.* fibrinogens), and may be the fluid portion of the blood obtained after removal of the fibrin clot and blood cells. **"Ascites"** as used herein refers to fluid obtained from the peritoneal cavity.

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Immunoassays may be used to detect antigen-binding molecule production. Immunoassays may be used to determine whether a sample obtained from a subject contains antigen-binding molecule capable of binding to a given peptide or polypeptide.

- 5 In some embodiments the methods comprise quantifying antigen-binding molecule in a sample, *e.g.* by determining antibody titer. Antibody titer is a measurement of the amount of antibody a subject has produced that is capable of recognizing (*i.e.* binding to) a given antigen. Antibody titer is expressed as the inverse of the highest dilution of the test sample (*e.g.* a serum sample) which gives a positive result for the detection of the antigen in *e.g.* an immunoassay.

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As used herein, "binding" by an antigen-binding molecule refers to specific interaction between an antigen-binding molecule and its cognate antigen. "Specific interaction" is interaction between antibody and antigen which is not non-specific. Antigen-binding molecule:antigen binding is mediated by non-covalent interactions such as Van der Waals forces, electrostatic interactions, hydrogen bonding, and
15 hydrophobic interactions. In particular, the interaction is between the antigen-binding site of an antigen-binding molecule and its cognate epitope in an antigen. An epitope is a part of an antigen which is contacted by an antigen-binding molecule. In particular, the epitope is the part of the antigen to which the antigen-binding molecule binds. An epitope is provided by an antigenic sequence of amino acids. An epitope may be linear, consisting of a contiguous sequence of amino acids (*i.e.* an amino acid primary
20 sequence). Alternatively, an epitope may be conformational, consisting of a discontinuous sequence of amino acids of an amino acid sequence of any antigen. The amino acids of the discontinuous sequence of amino acids may be located in different regions of a peptide/polypeptide, and may be positioned in close proximity when the antigen is folded, *e.g.* into its native structure.

- 25 The ability of a given antigen-binding molecule to bind to a given protein can be analyzed using techniques which are well known to the skilled person, which include ELISA, immunoblot (*e.g.* western blot), immunoprecipitation, Surface Plasmon Resonance (SPR; see *e.g.* Hearty *et al.*, *Methods Mol Biol* (2012) 907:411-442) or Bio-Layer Interferometry (see *e.g.* Lad *et al.*, (2015) *J Biomol Screen* 20(4): 498-507) and flow cytometry, amongst others. Through such analysis interaction between a protein/domain
30 and an antigen-binding molecule can be measured and quantified. Such methods may involve expressing the protein/domain, contacting the expressed protein/domain with an antigen-binding molecule and detecting formation of a non-covalent complex of the protein/domain and the antigen-binding molecule.

The particular region of a given binding partner to which an antigen-binding molecule binds can
35 furthermore be analyzed using methods well known in the art, including X-ray co-crystallography analysis of antibody-antigen complexes, hydrogen-deuterium exchange analysis by mass spectrometry, cryo-electron microscopy, peptide scanning and mutagenesis mapping. Such methods are described, for example, in Gershoni *et al.*, *BioDrugs*, 2007, 21(3):145-156, and Abbott *et al.*, *Immunology* (2014) 142: 526-535, both of which are hereby incorporated by reference in their entirety.

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Further articles of the disclosure

The present disclosure also provides a nucleic acid, or a plurality of nucleic acids, comprising a nucleotide sequence as described herein.

- 5 In some embodiments, the nucleic acid/plurality is purified or isolated, e.g. from other nucleic acid, or naturally-occurring biological material. In some embodiments the nucleic acid/plurality comprises or consists of DNA and/or RNA.

- 10 It will be appreciated that in some embodiments, a plurality of nucleic acids according to the present disclosure may comprise one or more (e.g. one of 1, 2, 3, 4, 5, 6, 7, 9 or 10) nucleotide sequences according to the present disclosure. In such embodiments, plural nucleotide sequences may independently conform to any embodiment of a nucleotide sequence described herein.

- 15 The present disclosure also provides a vector or a plurality of vectors comprising the nucleic acid/plurality of nucleic acids according to the present disclosure.

- The nucleic acid/plurality may be contained in a vector or a plurality of vectors. A “vector” as referred to herein is a nucleic acid molecule used as a vehicle to transfer exogenous nucleic acid into a cell. A vector may be a vector for expression of the nucleic acid in the cell (i.e. an expression vector.). Vectors may include a promoter sequence operably linked to the nucleotide sequence encoding the sequence to be expressed. A vector may also include a termination codon and expression enhancers. Any suitable vectors, promoters, enhancers and termination codons known in the art may be used.

- 25 Suitable vectors include plasmids, binary vectors, DNA vectors, mRNA vectors, viral vectors (e.g. gammaretroviral vectors (e.g. murine Leukemia virus (MLV)-derived vectors), lentiviral vectors, adenovirus vectors, adeno-associated virus vectors, vaccinia virus vectors and herpesvirus vectors), transposon-based vectors, and artificial chromosomes (e.g. yeast artificial chromosomes).

- 30 In some embodiments, a vector may be a eukaryotic vector, e.g. a vector comprising the elements necessary for expression in a eukaryotic cell. In some embodiments, a vector may be a mammalian vector, e.g. comprising a cytomegalovirus (CMV) or SV40 promoter.

- 35 The present disclosure also provides a cell comprising or expressing a nucleic acid/plurality or vector/plurality. The cell may be a eukaryotic cell, e.g. an animal cell. The cell may be a non-human animal cell, e.g. a mouse, rat, hamster, llama, guinea pig, rabbit, goat, chicken, primate (e.g. non-human primate, e.g. a monkey), sheep, donkey, cow, cat, dog, pig or horse cell. In some embodiments the cell is a mammalian cell (e.g. a non-human mammalian cell). In some embodiments the cell is from an animal of a species of the order *Rodentia* (e.g. a species of the genus *Mus*, *Rattus* or *Cavia*) or *Lagomorpha* (e.g. a species of the family *Leporidae*). In some embodiments the cell is a mouse cell.

- 40 In some embodiments, the cell is a multipotent cell, e.g. a pluripotent cell. In some embodiments, the cell is a stem cell. In some embodiments, the cell is an embryonic stem cell.

Also provided is an embryo comprising a cell according to the present disclosure. Also provided is a blastocyst comprising a cell according to the present disclosure.

- 5 Such nucleic acids, vectors, cells, embryos and blastocysts are useful in the production of animals according to the present disclosure. Accordingly, the present disclosure also provides an animal produced by intrauterine implantation of a blastocyst comprising a cell according the present disclosure.

Preparation of peptides/polypeptides

- 10 Peptides and polypeptides for use in methods described herein may be prepared according to methods known to the skilled person.

Polypeptides may be prepared by chemical synthesis, *e.g.* liquid or solid phase synthesis. For example, peptides/polypeptides can be synthesized using the methods described in, for example, Chandrudu *et al.*,
15 Molecules (2013), 18: 4373-4388, which is hereby incorporated by reference in its entirety. Alternatively, peptides/polypeptides may be produced by recombinant expression. Molecular biology techniques suitable for recombinant production of peptides/polypeptides are well known in the art, such as those set out in Green and Sambrook, Molecular Cloning: A Laboratory Manual (4th Edition), Cold Spring Harbor Press, 2012, and in Nat Methods. (2008); 5(2): 135-146 both of which are hereby incorporated by
20 reference in their entirety.

For recombinant production according to the present disclosure, any cell suitable for the expression of peptides/polypeptides may be used. The cell may be a prokaryote or eukaryote. In some embodiments the cell is a prokaryotic cell, such as a cell of archaea or bacteria. In some embodiments the bacteria may
25 be Gram-negative bacteria such as bacteria of the family *Enterobacteriaceae*, for example *Escherichia coli*. In some embodiments, the cell is a eukaryotic cell such as a yeast cell, a plant cell, insect cell or a mammalian cell, *e.g.* CHO, HEK (*e.g.* HEK293), HeLa or COS cells. In some embodiments, the cell is a CHO cell that transiently or stably expresses the polypeptides.

30 In some cases the cell is not a prokaryotic cell because some prokaryotic cells do not allow for the same folding or post-translational modifications as eukaryotic cells. In addition, very high expression levels are possible in eukaryotes and proteins can be easier to purify from eukaryotes using appropriate tags. Specific plasmids may also be utilized which enhance secretion of the peptide/polypeptide into the media.

35 In some embodiments polypeptides may be prepared by cell-free-protein synthesis (CFPS), *e.g.* according using a system described in Zemella *et al.* Chembiochem (2015) 16(17): 2420-2431, which is hereby incorporated by reference in its entirety.

Production may involve culture or fermentation of a eukaryotic cell modified to express the
40 peptides/polypeptides of interest. The culture or fermentation may be performed in a bioreactor provided with an appropriate supply of nutrients, air/oxygen and/or growth factors. Secreted proteins can be collected by partitioning culture media/fermentation broth from the cells, extracting the protein content,

and separating individual proteins to isolate secreted peptides/polypeptides. Culture, fermentation and separation techniques are well known to those of skill in the art, and are described, for example, in Green and Sambrook, *Molecular Cloning: A Laboratory Manual* (4th Edition; incorporated by reference herein above).

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Bioreactors include one or more vessels in which cells may be cultured. Culture in the bioreactor may occur continuously, with a continuous flow of reactants into, and a continuous flow of cultured cells from, the reactor. Alternatively, the culture may occur in batches. The bioreactor monitors and controls environmental conditions such as pH, oxygen, flow rates into and out of, and agitation within the vessel such that optimum conditions are provided for the cells being cultured.

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Following culturing the cells that express the peptides/polypeptides, the peptides/polypeptides of interest may be isolated. Any suitable method for separating proteins from cells known in the art may be used. In order to isolate the polypeptide, it may be necessary to separate the cells from nutrient medium. If the peptides/polypeptides are secreted from the cells, the cells may be separated by centrifugation from the culture media that contains the secreted peptides/polypeptides of interest. If the peptides/polypeptides of interest collect within the cell, protein isolation may comprise centrifugation to separate cells from cell culture medium, treatment of the cell pellet with a lysis buffer, and cell disruption e.g. by sonification, rapid freeze-thaw or osmotic lysis.

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It may then be desirable to isolate the peptides/polypeptides of interest from the supernatant or culture medium, which may contain other protein and non-protein components. A common approach to separating protein components from a supernatant or culture medium is by precipitation. Proteins of different solubilities are precipitated at different concentrations of precipitating agent such as ammonium sulfate. For example, at low concentrations of precipitating agent, water soluble proteins are extracted. Thus, by adding different increasing concentrations of precipitating agent, proteins of different solubilities may be distinguished. Dialysis may be subsequently used to remove ammonium sulfate from the separated proteins.

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Other methods for distinguishing different proteins are known in the art, for example ion exchange chromatography and size chromatography. These may be used as an alternative to precipitation, or may be performed subsequently to precipitation.

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Once the peptides/polypeptides of interest have been isolated from culture it may be desired or necessary to concentrate the peptides/polypeptides. A number of methods for concentrating proteins are known in the art, such as ultrafiltration or lyophilization.

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Sequence Identity

Pairwise and multiple sequence alignment for the purposes of determining percent identity between two or more amino acid or nucleic acid sequences can be achieved in various ways known to a person of skill in the art, for instance, using publicly available computer software such as ClustalOmega (Söding, J. 2005, *Bioinformatics* 21, 951-960), T-coffee (Notredame *et al.* 2000, *J. Mol. Biol.* (2000) 302, 205-217),

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Kalign (Lassmann and Sonnhammer 2005, BMC Bioinformatics, 6(298)) and MAFFT (Katoh and Standley 2013, Molecular Biology and Evolution, 30(4) 772–780) software. When using such software, the default parameters, *e.g.* for gap penalty and extension penalty, are preferably used.

5 Sequences

[illegible]

[illegible]

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3	Region of <i>Cd40</i> removed following Cre recombination in the <i>Cd40</i> CKO mouse	TTGCCCTCTGTGGGGTGAATCTTATCTCTTTGGTGGCAGGGAGCTGGGGACAGAAACCGGGAG AAGGGCTGAGGCCAGCTTGAGCCAGCAGTCTCGGGACTCTGGAGGAAGAACTGGAGTTCTCC CTACCTGCTGCGTCTTTGGGAGCACTGAAGAGTCTGTGCATCTGTTCCGATTAGAGGGTTCT GCGTTCTTGCTTTGGTAGATGGCAGTAAGACGATGTGACAACAGAGTAAAAAAAAAATAGACC TCACACTCTGGGGGCTCACTTTTCTGCTTTGGATTTCCACATCAGCTACAGCCTGCGTCTTGGC TAACTTTCAACATGCCGGTGAAGATCCCTTCCAGCTGTCCAATCTGTTTTAGGTCCATCTAG GGCAGTGTGTTACGTGCAGTGACAACAGTAACTCCACGATGGCCAGTCTGTGATTTGTGCC AGCCAGGTGAGATGCTAGCCCTCTGCCCGTACCAAGACCCCTTTCCCTCTTGGATTGCTGGT GGATGCAGACCCCATATATAGACTGTGAACCTAAAGTCTAAATGACCCATTTCTCCCTCTTCCCT GATGCCAGAATACCCCAAGCTGTCCCGTCTCTTCCATCTTCTTACTCGTGAGGGTCTGAGAT ATCCATTCCCAAACCTCAACCTCTCACCTCCAGTCTGGCTCCCTGGGTTGTGACACAGTCTG TGTCACAGGATTGGCCCAACCTGCCTCATATCCTCCTGTTTTTACGGAAGCCGACTGACAAGCC ACTGCACAGCTCTTGAGAAGACCCAATGCCACCATGTGACTCAGGCGAATTTCTACGCCCACT GGAACAGGGAGATTCTGCTCACCAGCAGACACTGTGAACCAAGTGCCTGGGCTGGGCTGCCTG GGAAGGGTACTTGAGAACCGGTTGATGTTCTAATGCTGAAATCCCTCTGTTGTGATGGC CAGGGTCTTTCCTGTGAGCTAGAGTCTGGGTTGAAGGGGCTAGTTGACTGACATCTGACTGG GAAGAGCGAGAAAACAGCAGATGACGTGAGGAGTGGGGTCTGGCTGCGGCCACGCGGGT TTTCCCATCTTCTTCTCATCTCCGCTCAGATCAAGGGCTTCCGGTTAAGAAGGAGGGCACCG CAGAATCAGACACTGTCTGTACCTGTAAGGAAGGACAACACTGCACCAGCAAGGATTGCGAGG CATGTGCTCAGCACACGCCCTGTATCCCTGGCTTTGGAGTTATGGAGATGGGTGAGTGGCCTG CCTGGGGAAACAGCTCTGTGGTGGGAGAGCTGGGGTGAAGTCTTGGTCTCTGGCCTCCAGA AGCTGAGGGCAGAGAAAAGTCCACCTGGGCTGGGATCTTTCATTTGGATTGGACCTGGGCTC

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		ACTAATGTCATCTGTGGTGAGTCCAGGGGAGAATGGCCTTGCCAAGTCTTTGGGAAGCAGGGA ACTGGGGAGAGACTGAGGCACGCAGGAACACTGACTGGGATAGGAGTGAGACCAAGAGGCAG CTTGGGGTACAGTACCTTAGCTCCCGTCTTGGGAGCTGGGTAAGTCACATCCCTTGTCTGAGC CTCAGTTTCTTCAATTGTGAAATAGGCCACAGCAGCTCCTTCCCTACGCACAAACACAGCA AGTGGCATTGGAATTTTGCAGTTTGAAGCTGCTGCCCCCTTGCTTGAGGTTTCAAGTTCACTGTG ACAGTGTACCTGGTAACCCAGTTTGGATGCTAGGATGTAAACTTGACCATCCCTAATGGA TCACAATCTCAGATAACAATAGAGACCAGGCCACCTTTGAATGAGTGAAGACAGAGAAGGGTAA GAGAGCTAGGTCTGATGAGCGGGCCTGTCAGCGCAGCTAATTAGAGGCAAGAGCTTTGTAAGT TCAAGGCTAGCCTGGGCAGCTTAGAAAAGATACTGGTTCAACATAGAAAAGGGCTGCTGAGATG GGTGAGTAAGTTAAGCTCTTGCCTGATGGCCAGCTTCCATCCCCAGCATCCATGGAAAAGGTA GAAGGAGAGGATCAGTCTCTAAAAGTTGTCCTGTGACCGCTTCCATGTACAGCACAACACAGCA CAGCACATGTGAGTGTGCATACATCATGCACACACATCATACATACATCATGTGCACATATCAT GCACACACATCATGTGCACACATCATACACACATCATGCACACACATCATGTGCACACATCATG CACACACATCATACATACATCATGTGCACATATCATGCACACACATCATGTGCACACATCATGCA TACACATCATACACACACATCATGCACACACATCATGTGCACATATCATACTCACATCACGTACA CACATCATGCACACACATCATACACACATATCATGTGCACCCATCATGTGCACACATCATACACA CATCATGTACATGCACACCCACAATAGTGATAAAATAAAAGTTTAAATATGTTTCTAGGGCTGGG AGGTGGCACTTGCTGTTCTGACAGAGGACCTGGTTGAGTTTCCATGTACCGCTGCTATTGCTAG TATAAACTGTCCATGACTCCAGCTCCTGGTGATCTGATATCTCTGCTGGCGCTAGGCACATAC ATGATGCACGTACATACCTCTAGCACTTTCTGATATACATAAAATAAAATAGATACAAATTAAG ACATTAATAAAAAAGTAAGAAGATAGCTGGGGGTGGGGCTCAGTGGTGGAGCAGTTGTCCAGC ATGCTGAGGTCCTGGGTTAGACCCCCACACTTGCCCTAGCATATGTGAGGTCCTGGGTTAGACC CCCCACTTGCCCTAGCATATGTAAGGTCCTGGGTTAGACCCCCACACTTGTCAGGATATATGA GGTCTGGGTTAGACCCCCACACTTGCCCTAGTATGTGAGGTCCTGGGTTAGACCCCCACAC TTGTCCAGGATATGTGAGGTCCTGGGTTAGACCATAGTCCACAAAGTAAAAAGAAAAATAAAGT GCAGTCTCTTTGCTTTTTCTCAAGATTGCTTTTTGTCTTGAAAGGCGTGAGTGGATGGAGTG TGAAGCACTTGAGTTTATGTGAACATTTAAGAAGACATCGAGAAGGGACAGTAAGCAAGAG AAAACATTTTGAGATGATGCTAGAAAAACCAAAACACTTTTAAAAATCTGAAACCAAAAAACAT GTTGATGCACAACATAGCAAAATTTTAAATTAAGTAAGAGCTGGGCTGGCGAGATGGCTCAGCG GGTAAGAGCACTGACTGCTCTTCCAGAGGTCCTGAGTTCAAATCCAGCAACCACATGGTGGC TCACAACCCATAATGAGATCTGATGCCCTCTTCTGGTGTGTCTGAAGTCAGCTACAATGTA CTTACATATAATAATAAAATCTTTAAAAAGTAAGAGCTGACCTTGCCAGACTCGAGACCTTG CCTACCTTCTTCATCCTAACCTTGCCCTGGTTCCTCAATACAAGTTTATCTAAAGGCCAGGTAGCC ACCTATGGACCTGTGTTTGTTCCTGCTGCTGCCCTCCCGACCCATGAGCTGGGTGTGGTGG TACAGGCATGAGAGCCCCAAGTTTAAAGGCCAGCCTGAACCACTTAGTGAGACCCTGTCTCAA GAAGACTTACCTTCATGACAGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGA GTGTATACACACCCCTTGCCCTCAAGAGTACCTGCCCTTTTGGTCTTTTCAATTTGTCACTTAGAAT AGCAGTGCAGACACAGAGATACTTTGTTTCTCTCCGCCATACTTCCATAGCAGATGTAACAATT CGGGGGCTAGTGTAGGGAGGGGTAAAGCAGGCAGGCAGGCTGTCCCTTCAAGACACAGCTGA CGAACACACAGATGGGAAGTTGGCTCACTGGGGCTTCTCATTCCAAGTCAATCTCCAGA GCTAGAGACTGCTCAAAGGGTAGGAGTATTTACTATGCAAGCATGAGCTAGGTGTGGCCACAC CACATGTGACTGTGACCTTAATACCATGTGTAGGGGGGTGGCACTAGAAGGAGCATTGTAGGG TGTTGCCCTCCAGACTAGCTCCAGGCTCAAGAGACTCTGTCTCAGCAGAAAGGTGCAATATGGT GGATCAGGATACCGGCACCTCCTTTGGCCTCTGCATGTATGCACAGATGCACACATCTAAACA CATACCAGGCCCCCATACACCGTACCCACACACACAAAGATGGACTCTCTCAATTTATAAA CACTGGCAAATACCATAAAGCGTTTTAAAAAGTTCCCTAGTGACCACCTAACCCACAATGCTACT GCTTAAAAAGCTCTGACCTGGATTCTGGGAGTGTACAGATGCTTTGGGCAAGGAGTGGGAGCA CCTGCCAGTGAGAGCACCTGCCAGTGGGAGCACACTCCATTAGGAGCACCCGCCAGTGGGAG CATCTGCCAGTGGGGTCCACTCAAGTCTGTTTCTCCAGGTTTAAAGTCCCGGATGCGAGCCCT GCTGGTCATTCTGTCTGTGATGGGCATCCTCATCACCATTTTCGGGGTGTCTCTATATCAGT GAGTGCTCAGGAGAGGAAAGGGAGGGAGGGTTCAGCCCTGTGCAACCAGCCTCCTGACTCAC CCTCGCAATGTCCACACCCCTTCTTCTTCTCACTAGAAAAGGTGGTCAAGAAACCAAGGATA ATGAGGTAAGCCATCCCTGAGGGAGAGATGCTGGAAAGAGTGAAGTGGTGGCAGGGAGGGAG GCTCACGGCGTAGGGAGACAGACTCAGT
6	Cd79a-CreERT2 knock-in region	AAATAACCGACTCACTGGGAGACGCCAGGGGGTCTAGAAGCCCTCAGAGCCCTGCCTCTCCT CCTCTTCTTGTACATACGCTGTTTGGGTAATGTGGCCAAAGGGAAGGTGCGGGTGGGGCGGG GGTGTTGCGGCTGACAGGAGAGGTGCAGAGTGGAGTCAGAAATGGCCAGGCAGGGTGATGCA TAACGTGGCATCTCTTAAAGTAACGCCAGAATCTAGGAAACTGAGGAAGGAGGAACACTGCA TTTGAGGCCAGCCTGGGCTACATAATGAGTTCTATCTAGGCCAACCTGGGCAAGTTACTGATTA AAATGTCTCAAAAAGAAAAGGAGTGTGGGGAGGGAATCAGGGAAAGCCGTCTGTAGTACGCC ACAGGCTTGAGTAAGTCCACTGTTAGGCAACACTACCTTGCCCCACGACACAGCCTTAG CTCCACAGGTCAGAAATGAGAAGTGAAGTAAAGTGTGACTGAACACACACACAGTGGAGGTGA GCAATGGGTGTGGTGAGGGAACGGGTTTTATTTGTTTGTGTTTGTGTTTGTGTTTGTGTTTGTGTT TTTTCTCTGCCTTACATCTGGAGGGCAGGCTTGCCATGTGACCACATAGACCCAGGTTTGCAG TGGAGGTCTTAGGAGCAGGGACTGGGGCCCTGCTCTGTGTCTTAAGCCTGTGTGACCAGGA ACTGGCTGGGGCTCTTTCCCTCCCTTTCTTTCTTCTCACTTCCGACCTTCTTCTTTCTGTCA TGTCCACAAAAGGGAGCTACAAGCCAGTGTGGAGCTGGGGAGGCTGAGAACAGGGTGGGGG GTGGGGGGCGCGTTCAAGATAAATAACAAGAGCAGACACCATAGCCACCAAGTCCACCTCGG CTCTAGCCTCCTCCTCAGCCTAGGGGTAAACCGAAGTTCTATACTTTCTAGAGAATAGGAAC

	TTCGGAATAGGAACTTCGGCGCGCCGATGCCCCAGACAGAACTCAGTCCCAGTGCCTAGGTTA ATACTGGTTTCTCTGTTTTGTTTTGTTTTTCGAGACAGGGCTTCTGTGTGCAACTGGAACCTACT CTGTTAGACCAGGCAGGCCTCAAACCTCCAGAGATCTGCCTGACTCTCTCTCTGAGTGCTGG GATTAAAGGCATGTACCACTACTGCCTGGCAGGTGCGCACATTTAAATTACAGAGATGAGAAAC AGCCTAGGCACAAAAAACAAAAAACAAACAAACAAAAACAAAAACAAAAACAAAAACAAAA CAAGCCAAGCGCAGTAAAGGAGAGATTGAGGAAGGTGGGAATTGGGCTCTACTATCTTTTCCA ATAGCCTGGTTACTGCTGAGCTGAGCCCCACCGCTGCATCTAAGGTTCTGCCCCACCTTCACC CCCTCCCCGTGGGGGTTCTAAGAGTTGTAAGGAATGGGGACCAATGGGATGGTTTTTCATGAA AGAGTCCCTGGGCTGTTTGGGGAACATCAAAATACAAAGGGGAAAGGGAAGAAAGAGGGCCATA GGGTGTGCCCCCCCCCAAGTCTCCCATCATCTTTTTCTAGGTACCATTACCATTGGTACGGCTC CACTCCTGATGTGAAGGGTCTGGGCTAGAGCAATCATCTCCATCTCCAGGCTCTGACCCATC TGTCTCCTCTCCTCTCTCCACAGGTCCCGGGCCACCATGTCCAATTTACTGACCGTACACCAAA ATTTGCCTGCATTACCGGTGATGCAACGAGTGATGAGGTTGCAAGAACCTGATGGACATGTT CAGGGATCGCCAGGCGTTTTCTGAGCATACCTGGAAAATGCTTCTGTCCGTTTCCGGGTGCTG GGCGGCATGGTGCAAGTTGAATAACCGGAAATGGTTTCCCGCAGAACCTGAAGATGTTGCGCA TTATCTTCTATATCTTCAGGCGCGCGGTCTGGCAGTAAAACTATCCAGCAACATTTGGGCCAG CTAAACATGCTTCATCGTCCGTCCGGGTGCCACGACCAAGTGACAGCAATGCTGTTTCACTG GTTATGCGGCGGATCCGAAAAGAAAAACGTTGATGCCGGTGAACGTGCAAAACAGGCTCTAGCG TTCGAACGCACTGATTTGACCAAGTTGTTCACTCATGGAAAATAGCGATCGCTGCCAGGATA TACGTAATCTGGCATTCTGGGGATTGCTTATAACACCCTGTTACGTATAGCCGAAATTGCCAG GATCAGGGTTAAAGATATCTCACGTAAGTGGGAGAAATGTTAATCCATATTGGCAGAACG AAAACGCTGGTTAGCACCGCAGGTGTAGAGAAGGCACTTAGCCTGGGGGTAACATAAAGTGGTC GAGCGATGGATTTCCGTCTCTGGTGTAGCTGATGATCCGAATAACTACCTGTTTTGCCGGGTCA GAAAAAATGGTGTGCGCGCCATCTGCCACCAAGGCACTATCAACTCGCGCCCTGGAAGGGA TTTTGAAGCAACTCATCGATTGATTTACGGCGCTAAGGATGACTCTGGTCAGAGATACCTGGC CTGGTCTGGACACAGTGCCCGTGTGCGAGCGCGCGAGATATGGCCGCGCTGGAGTTTCAA TACCGGAGATCATGCAAGCTGGTGGCTGGACCAATGTAATATTGTCATGAAGTATATCCGTAA CCTGGATAGTGAAACAGGGGCAATGGTGGCCTGCTGGAAGATGGCGATTCTGCTGGAGACA TGAGAGCTGCCAACCTTTGGCCAAGCCCGCTCATGATCAAACGCTCTAAGAAGAAGAGCCTGG CCTTGTCCTGACGGCCGACAGATGGTCAGTGCCCTGTTGGATGCTGAGCCCCCATACTCT ATTCGAGTATGATCCTACCAGACCCTTCAGTGAAGCTTCGATGATGGGCTTACTGACCAACCT GGCAGACAGGGAGCTGGTTCACATGATCAACTGGGCGAAGAGGGTGCCAGGCTTTGTGGATT TGACCCTCCATGATCAGGTCCACCTTCTAGAATGTGCCTGGCTAGAGATCTGATGATTGGTCT CGTCTGGCGCTCCATGGAGCACCCAGTGAAGCTACTGTTTGTCTCTAACTTGCTCTTGGACAG GAACCAGGGAAAATGTGTAGAGGGCATGGTGGAGATCTTCGACATGCTGCTGGCTACATCATC TCGGTTCCGCATGATGAATCTGCAGGGAGAGGAGTTTGTGTGCCTCAAATCTATTATTTGCTT AATTCTGGAGTGATACACATTTCTGTCCAGCACCTGAAGTCTCTGGAAGAGAAGGACCATATCC ACCGAGTCTGGACAAGATCACAGACACTTTGATCCACCTGATGGCCAAGGCAGGCCTGACCC TGACAGCAGCAGCACCAGCGGCTGGCCAGCTCCTCCTCATCCTCTCCACATCAGGCACATGA GTAACAAAGGCATGGAGCATCTGTACAGCATGAAGTGCAAGAACGTGGTGCCCTCTATGACC TGCTGCTGGAGGCGGCCGACGCCCACCGCTACATGCGCCCACTAGCCGTGGAGGGGCATC CGTGGAGGAGACGGACCAAGCCACTTGGCCACTGCGGGCTCTACTTCATCGCATTCTTGGCA AAAGTATTACATCACGGGGGAGGCAGAGGGTTTCCCTGCCACAGTCTGACTGACATGTCCAC CCTTCCCTGCTGCCATATGTCTGACTCCAGCATCCCTGCCTCCCCACTCTTCCCTGGAGTCC CCCCTTCCAGTGCTTGTCCAGCCACTCGAGTGGCCTACCCTTGCTCCAGGCTGCCCCACC CTATTCTCCACCTAATCTTGTCTTCCCCAAGTCCAGCTCCCTCTCTAGTGGGTAATGAGCCC TTAATCGCTGCCTTAGGGGAGCTGATTACAGCAGCCTCGTTAGTGTACCCCCCTCTCTCC AGATCTGTCATGGCCACTTAAGTGATAATAAATCCTTCCCAAAGCA
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The invention includes the combination of the aspects and preferred features described except where such a combination is clearly impermissible or expressly avoided.

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The features disclosed in the foregoing description, or in the following claims, or in the accompanying drawings, expressed in their specific forms or in terms of a means for performing the disclosed function, or a method or process for obtaining the disclosed results, as appropriate, may, separately, or in any combination of such features, be utilized for realizing the invention in diverse forms thereof.

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For the avoidance of any doubt, any theoretical explanations provided herein are provided for the purposes of improving the understanding of a reader. The inventors do not wish to be bound by any of these theoretical explanations.

Any section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

- 5 Throughout this specification, including the claims which follow, unless the context requires otherwise, the word “comprise” and “include”, and variations such as “comprises”, “comprising”, and “including” will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.
- 10 Where a nucleic acid sequence is disclosed herein, the reverse complement thereof is also expressly contemplated. Also, where a polypeptide-encoding nucleic acid sequence is disclosed herein equivalent polypeptide-encoding sequences as a result of degeneracy of the genetic code are also expressly contemplated.
- 15 It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Ranges may be expressed herein as from “about” one particular value, and/or to “about” another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by the use of the antecedent “about,” it
- 20 will be understood that the particular value forms another embodiment. The term “about” in relation to a numerical value is optional and means for example +/- 10%.

Methods disclosed herein may be performed, or products may be present, *in vitro*, *ex vivo*, or *in vivo*. The term “*in vitro*” is intended to encompass experiments with materials, biological substances, cells and/or

25 tissues in laboratory conditions or in culture whereas the term “*in vivo*” is intended to encompass experiments and procedures with intact multi-cellular organisms. In some embodiments, methods performed *in vivo* may be performed on non-human animals. “*Ex vivo*” refers to something present or taking place outside an organism, e.g. outside the human or animal body, which may be on tissue (e.g. whole organs) or cells taken from the organism.

30 For standard molecular biology techniques, see Sambrook, J., Russel, D.W. Molecular Cloning, A Laboratory Manual. 3 ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press

Aspects and embodiments of the present disclosure will now be discussed. Further aspects and

35 embodiments will be apparent to those skilled in the art. All documents mentioned in this text are incorporated herein by reference in their entirety. While the invention has been described in conjunction with the exemplary embodiments described below, many equivalent modifications and variations will be apparent to those skilled in the art when given this disclosure. Accordingly, the exemplary embodiments of the invention set forth above are considered to be illustrative and not limiting. Various changes to the

40 described embodiments may be made without departing from the spirit and scope of the invention.

Brief Description of the Figures

Embodiments and experiments illustrating the principles of the invention will now be discussed with reference to the accompanying figures.

5 **Figures 1A and 1B.** Schematic representations of (1A) the genomic mouse locus targeted, the targeting vector, the targeted allele (after integration of the targeting vector insert), and the constitutive knock-in allele, for the production of the *Cd79a*-CreERT2 knock-in mouse as described in Example 8.1; and (1B) the targeting vector, the nucleotide sequence of which is shown in SEQ ID NO:1.

10 **Figures 2A and 2B.** Schematic representations of (2A) the genomic mouse locus targeted, the targeting vector, the targeted allele (after integration of the targeting vector insert), conditional knockout allele and the knockout allele after Cre recombination, for the production of the *Cd40* CKO mouse as described in Example 8.2; and (2B) the targeting vector, the nucleotide sequence of which is shown in SEQ ID NO:2.

15 **Figure 3.** Immunization, dosing and sampling timeline for experiments to assess the inhibition of a primary immune response via blocking stimulation of naïve B cells using anti-CD40L antibody.

20 **Figure 4.** (4A) Graphs showing immune responses to antigens. All mice received two initial administrations of EGFR-hFc, followed by a third administration of either PBS (left panels), EGFR-hFc (middle panels) or CD33-mFc (right panels). Data shown as mean +/- sem. n = 9 for Days 7, 14 & 26. n = 6 for Days 31, 35 and 40. n = 2 for Day 50. * p < 0.05, multiple unpaired t-test with Holm-Sidak correction.

25 **Figure 5.** Graphs showing the percentage of B cells in which CreERT2-mediated recombination of the ZsGreen allele is detected in B cells isolated from the bone marrow, spleen and lymph nodes, and a graph showing the percentage of non-B cells comprising CreERT2-mediated recombination of the ZsGreen allele in tissues, of mice homozygous for a lox-Stop-lox ZsGreen allele (*Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}*) and heterozygous for *Cd79a^{CreERT2}*, at the indicated number of days following treatment with 100 mg/kg bodyweight or 200 mg/kg bodyweight of tamoxifen.

30 **Figure 6.** Graphs showing the percentage of B cells in which CreERT2-mediated recombination of the ZsGreen allele is detected in B cells isolated from the bone marrow, spleen and lymph nodes of mice homozygous for a lox-Stop-lox ZsGreen allele (*Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}*) and heterozygous for *Cd79a^{CreERT2}*, at the indicated number of days following treatment with tamoxifen at 100 mg/kg bodyweight, or vehicle control (corn oil).

40 **Figures 7A and 7B.** Schematic and images illustrating CreERT2-mediated deletion of *Cd40* exons 2-5 *in vivo* in the B cells of *Cd40^{fllox/fllox}; Cd79a^{+/-CreERT2}* mice 48 h after treatment with tamoxifen at 200 mg/kg bodyweight. (7A) Schematic illustrating the architecture of the *Cd40^{fllox}* locus before (upper) and after (lower) tamoxifen-induced, CreERT2-mediated deletion of *Cd40* exons 2-5. The location of the forward and reverse primers for PCR amplification of the control and *Cd40^{KO}* amplicons are indicated. (7B) Images of the products of PCR reactions performed using primers for amplification of the *Cd40^{KO}* (left)

and control (right) amplicons using genomic DNA obtained from cells isolated from the bone marrow of *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* mice 48 h after treatment with tamoxifen at 200 mg/kg bodyweight (TAM) or vehicle control (corn oil; Oil), following separation by agarose gel electrophoresis. The expected positions of the control and *Cd40^{KO}* amplicons are indicated.

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Figure 8. Histograms and bar chart showing the proportion of Cd45^{r+} cells expressing Cd40 obtained from the spleens and lymph nodes of *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* mice, 48 h after treatment with tamoxifen at 200 mg/kg bodyweight, or corn oil (vehicle control), as determined by flow cytometry.

Figures 9A and 9B. Schematic and graphs illustrating maintenance of CreERT2-mediated recombination of the ZsGreen allele in B cells isolated from the bone marrow, spleen and lymph nodes of mice homozygous for a lox-Stop-lox ZsGreen allele (*Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}*) and heterozygous for *Cd79a^{CreERT2}*, achieved by repeated administration of tamoxifen. **(9A)** Schematic illustrating the schedule for administration of tamoxifen and vehicle (corn oil) to the mice in Arm 1 and Arm 2. **(9B)** Graphs showing the percentage of B cells in which CreERT2-mediated recombination of the ZsGreen allele is detected in B cells isolated from the bone marrow, spleen and lymph nodes, at the indicated number of days following the administration of the initiating dose of tamoxifen, for mice in Arm 1 and Arm 2.

Figure 10. Schematic illustrating the schedule for administration of tamoxifen to *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* mice for maintaining B cell-specific, CreERT2-mediated deletion of *Cd40* exons 2-5.

Figure 11. Bar charts showing the diversity of VH and VL gene usage among B cells within samples collected from *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* (CD40 cKO) mice and BALB/c mice, as determined by NGS sequencing.

Figure 12. Graphs showing sera binding profiles and titers against full length or epitope-deleted protein in BALB-c mice boosted once or twice with antigen with or without anti-CD40L treatment, as analyzed by flow cytometry. MFI = mean fluorescence intensity.

Figures 13A to 13C. **(13A)** Graph showing quantification of IgG⁺ B cells in the spleen and lymph nodes of BALB/c mice and *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* (CD40 cKO) after tamoxifen-induced gene deletion. **(13B)** Graph showing quantification of B220⁺ B cells in the lymph node (LN), spleen (SP) and bone marrow (BN) of BALB/c mice and CD40 cKO mice after tamoxifen-induced gene deletion. **(13C)** Graph showing quantification of IgG⁺ B cells in the spleen and lymph nodes of BALB/c mice and CD40 cKO mice after tamoxifen-induced gene deletion.

Figures 14A to 14C. **(14A)** Image of the products of PCR reactions performed using primers for amplification of a 815 bp wild-type (wt) or a 713 bp flox (fl) amplicon detectable in the absence of CreERT2-mediated recombination of the *CD40^{flox}* locus and a 467 bp CD40cKO amplicon detectable following CreERT2-mediated recombination of the *CD40^{flox}* locus, using genomic DNA obtained from cells isolated from the spleen and ear of BALB/c or *CD40^{flox/flox}; Cd79a^{+/CreERT2}* 10 days after the last treatment

with tamoxifen at 200 mg/kg bodyweight (tamoxifen) in a treatment regime consisting of 5 doses of tamoxifen over a course of 40 days, following separation by agarose gel electrophoresis. (14B and 14C) Flow cytometry and quantification of CD40 protein expression in B cells collected from lymphoid tissues (spleen and lymph nodes) in tamoxifen-induced transgenic mice (CD40 cKO) compared with wild-type mice.

Figure 15. Immunization strategy for the generation of targeted epitope specific antibodies for each protein of interest. 'Antigen 1' refers to a peptide comprising an amino acid sequence for the targeted epitope within the protein of interest. 'Antigen 2' refers to the full-length protein of interest containing the targeted epitope.

Figures 16A to 16D. (15A) Graphs showing binding of antibodies in sera generated in CD40 cKO mice and wild-type mice, for full-length or target epitope-deleted protein of interest 1 as defined in Example 17, as determined by ELISA. (15B) FACS analysis of binding profiles for antibodies raised against protein of interest 1 as defined in Example 17, present in the sera generated in CD40 cKO and wild-type mice. (15C) Graphs showing binding of antibodies in sera generated in CD40 cKO mice and wild-type mice, for the targeted domain or non-targeted domain in protein of interest 2 as defined in Example 16, as determined by ELISA. (15D) FACS analysis of binding profiles for antibodies raised against protein of interest 2 as defined in Example 17, in sera generated in CD40 cKO and wild-type mice.

Figure 17. FACS analysis showing the binding of four representative recombinant antibodies to either the full-length extracellular domain (ECD) or the epitope-deleted (Δ Ep) extracellular domain of protein of interest 1 as defined in Example 17.

Figures 18A to 18D. Graphs showing the dose-response curve for binding to full-length extracellular domain of protein of interest 3 as defined in Example 17, as determined by ELISA, for four representative antibody clones obtained from the *CD40^{flox/flox}; Cd79a^{+/CreERT2}* mouse.

Figure 19. Graph showing binding of antibodies produced in the sera in response to immunizing BALB/c or NZBWF1 mice with recombinant human DLL3-Fc tagged protein, as determined by ELISA. Sera were collected on Day 38 post-immunization.

Figure 20. Graph showing binding of antibodies produced in the sera in response to immunization with two doses of a 13-mer peptide conjugated to KLH in BALB/c or NZBWF1 mice, as determined by ELISA. Sera were collected on Day 25 post-immunization.

Examples

Example 1: Transgenic mice providing for inducible knockout of CD40

Transgenic mice providing for inducible knockout of mouse homologs of the CD40 gene are produced.

Briefly, mouse embryonic stem cells are modified by CRISPR/Cas9-mediated gene editing (as described in Lee *et al.*, Drug Discovery Today: Disease Models (2016) 20: 13-20) to comprise *loxP* target sequences flanking exons of the CD40 gene, and to encode CreERT under the control of a promoter providing for expression in B cell lineage cells. The modified embryonic stem cells are used to generate transgenic mice via introduction of into a blastocyst, and subsequent intrauterine implantation of the blastocyst for gestation.

The expression of mouse CD40 can be inducibly knocked-out in the resulting transgenic mice by administration of tamoxifen.

Example 2: Transgenic mice encoding human immunoglobulin genes providing for inducible knockout of CD40

Transgenic mice encoding human immunoglobulin genes providing for inducible knockout of mouse homologs of the CD40 gene are produced.

Briefly, embryonic stem cells derived from transgenic mice encoding human immunoglobulin genes are modified by CRISPR/Cas9-mediated gene editing (as described in Lee *et al.*, Drug Discovery Today: Disease Models (2016) 20: 13-20) to comprise *loxP* target sequences flanking exons of the CD40 gene, and to encode CreERT under the control of a promoter providing for expression in B cell lineage cells.

The modified embryonic stem cells are used to generate transgenic mice via introduction of into a blastocyst, and subsequent intrauterine implantation of the blastocyst for gestation.

The resulting transgenic mice produce antibodies having fully human variable region (*i.e.* VH and VL) sequences, in which expression of mouse CD40 can be inducibly knocked-out by administration of tamoxifen.

Example 3: Production of antibodies using transgenic mice providing for inducible knockout of CD40

The mice produced as described in Examples 1 and 2 are used for the production of antibodies capable of binding to an amino acid sequence of interest in its native presentation.

Peptide comprising a sequence of interest of the extracellular domain of a protein of interest is prepared synthetically using standard methods. The extracellular domain of the protein of interest is recombinantly expressed in and purified from Chinese Hamster Ovary (CHO) cells or HEK293 cells.

Peptides are conjugated to KLH and hepatitis B core antigen (HBc) carriers for immunization. Conjugation uses succinimidyl 6-((beta-maleimidopropionamido) hexanoate) (SMPH) as a linker between the peptide and protein carriers. Successful conjugation to protein carriers is confirmed by SDS-PAGE analysis.

Mice are first immunized with 50 µg of peptide (representing a small fragment of an extracellular domain of the protein of interest [First Injection]).

Subsequent to immunization with the peptide [First Injection]:

(a) one group of mice is administered with tamoxifen (by intraperitoneal injection) to induce knockout of CD40; and

(b) another group of mice is not treated to induce knockout of CD40.

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Both groups of mice are subsequently immunized with 50 µg of polypeptide representing the full-length amino acid sequence of the extracellular domain of the protein of interest [Second Injection].

Subsequent to the second injection, mice are immunized a further two times with the same peptide or polypeptide that they were immunized with at the second injection [Third and Fourth Injections].

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Subsequent to the third and fourth injections, the mice are given one to three booster injections, of the same peptide or polypeptide that they were immunized with at the second injection [Booster Injections].

15 Sites of injection include the armpits, groin, foot, back and abdomen.

Different formulations are used for the different injections, as follows:

First Injection: peptide + Freund's complete adjuvant

Second Injection: ECD + Freund's incomplete adjuvant

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Third and Fourth Injections: ECD + Freund's incomplete adjuvant

Booster Injections: ECD (with or without adjuvant)

Example 4: Analysis of antibody production by ELISA

After the final Booster Injection, serum is collected from mice and analyzed for antibody binding to

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(1) the peptide, and

(2) the extracellular domain of the protein of interest (ECD).

Analysis is performed by Enzyme Linked Immunosorbent assay (ELISA).

30 Briefly, ELISA plates are coated overnight with the peptide or ECD (1 µg/ml in PBS) at 4°C After coating, ELISA plates are washed with sashing buffer (0.05% Tween 20 in 1x PBS) and then blocked with 1% BSA in 1x PBS for 1 hour at room temperature, and subsequently washed thrice with washing buffer.

35 Serum is collected from mice, serial dilutions are applied to wells of ELISA plates, and plates are incubated at room temperature for 1 hour. After washing with washing buffer, a Horseradish Peroxidase (HRP)-conjugated antibody is added to wells and plates are incubated at room temperature for 1 hour

ELISA plates are then developed using TMB substrate solution at room temperature for 10 mins.

40 Development is terminated by addition of 2M sulfuric acid (H₂SO₄), and plates are read at O.D. 450 nm, within 30 min of termination.

Example 5: Hybridoma production

Hybridomas are produced, and antibody production by hybridomas is analyzed as follows.

Fusion (Day 1): Mice are dissected under a sterile environment to obtain spleen and lymph nodes, and the single cell suspensions of cells of these tissues were prepared.

Cells are fused with myeloma cells either by polyethylene glycol (PEG) fusion or by electrofusion,

For PEG fusion, ClonaCell-HY Hybridoma Cloning Kit is used and cells are fused in accordance with the manufacturer's instructions (Stemcell Technologies, Canada). Fused cells are cultured in ClonaCell-HY Medium C (Stemcell Technologies, Canada) overnight at 37°C in a 5% CO₂ incubator. The next day, fused cells are centrifuged and resuspended in 10 ml of ClonaCell-HY Medium C and then gently mixed with 90 ml of semisolid methylcellulose-based ClonaCell-HY Medium D (StemCell Technologies, Canada) containing HAT components and plated into 96 well plates. Cells are allowed to grow at 37 °C in a 5% CO₂ incubator. After 7–10 days, single hybridoma clones are identified and antibody producing hybridomas are selected by screening the supernatants by Enzyme-linked immunosorbent assay (ELISA).

Alternatively, for Electrofusion, a NEPA21 Super Electroporator is used and cells are fused according to manufacturer's protocol (Nepagene). Fused cells are let to recover in ClonaCell-HY Medium C (Stemcell Technologies, Canada) overnight at 37°C in a 5% CO₂ incubator. The next day, fused cells are centrifuged and resuspended in 1 ml of ClonaCell-HY Medium C and then gently mixed with 90 ml of semisolid methylcellulose-based ClonaCell-HY Medium D (StemCell Technologies, Canada) containing HAT components and 500ug of FITC-labelled anti-mouse antibody (Jackson Immunoresearch). The cells are then plated into 8 to 16 x 6-well plates. Colonies are allowed to grow at 37 °C in a 5% CO₂ incubator for 7 days. Colonies are scanned for FITC fluorescence and picked using Clonepix (Fortebio) device and transferred into a 96 well plate containing to AOF Media. Picked colonies were allowed to grow for 5 days after which supernatants were screened by Enzyme-linked immunosorbent assay (ELISA).

Second round ELISA: At Day 13 a second round ELISA is performed on cell culture supernatant of wells of the 96 well plate.

Expansion/storage: At Day 14, cells from wells which give a positive result in the second round ELISA are transferred to wells of a 24 well plate for culture and expansion. When cultures are almost confluent, cells are harvested and frozen for storage.

Example 6: Peptide- and ECD-binding antibodies and hybridomas

Mice immunized according to the protocol wherein the mice are administered tamoxifen for inducible knockout of CD40 prior to the Second Injection (*i.e.* (a) group mice – See Example 3) produce a much higher titer of antibody capable of binding to the extracellular domain of the protein of interest as compared to mice in which CD40 is not knocked-out prior to the Second Injection (*i.e.* (b) group mice).

No difference is observed between (a) and (b) group mice in terms of titer of antibody capable of binding to the peptide.

The proportion of mice having sera containing antibodies capable of binding to both the peptide and the extracellular domain of the protein of interest is greater in the (a) group as compared to the (b) group.

The proportion of mice producing hybridomas which produce antibody displaying binding to both the peptide and the extracellular domain of the protein of interest is greater in the (a) group as compared to the (b) group.

Example 7: Hybridomas producing antibody binding peptide and ECD

Cells from wells giving positive signal in ELISA are diluted to ~1 cell per well of a multiwell plate, and cultured *in vitro* for 1-2 weeks.

The cell culture supernatant from the wells is then analyzed by ELISA as described in Example 4 for binding to:

- (1) Recombinant Fc-tagged protein of interest;
- (2) Optionally, Fc alone; and
- (3) The peptide with which the antibody was raised.

Cells from wells producing antibody capable of binding (1) and (3) only are diluted to ~1 cell per well of a multiwell plate, and cultured *in vitro* for 1-2 weeks.

The cell culture supernatant from the wells was then analyzed by ELISA as above. Cells from these wells are considered to be monoclonal.

Mice immunized according to the protocol wherein the mice are administered tamoxifen for inducible knockout of CD40 prior to the Second Injection (*i.e.* (a) group mice – See Example 3) generate hybridomas producing monoclonal antibodies capable of binding to both of the peptide and the extracellular domain comprising the peptide with a success rate which is greater than the success rate mice in which CD40 is not knocked-out prior to the Second Injection (*i.e.* (b) group mice).

Cells from the wells are transferred to T25 flasks for culture and expansion. After a period of 3-4 weeks, cells are frozen for storage in liquid nitrogen, or used for ascites production.

Example 8: Exemplary transgenic mice

8.1 Constitutive Cd79a-CreERT2 knock-in mouse

A mouse comprising an endogenous nucleotide sequence providing for constitutive knock-in of CreERT2 at *Cd79a* is produced.

A targeting vector was designed based on the mouse *Cd79a* transcript NCBI Ref: NM_007655.4. The targeting vector insert is shown schematically in Figure 1A, and comprises (from 5' to 3'):

A ~3 kb short homology arm, comprising exon 1 of *Cd79a* having a mutated translation initiation codon;

A puromycin resistance gene *PuroR*, flanked by *FRT* sites (providing for its flippase-mediated excision);

5 The first 7 nucleotides of *Cd79a* exon 2, followed by the Kozak sequence and ORF of CreERT2;
The *Cd79a* 3' UTR and human growth hormone polyadenylation signal (hGHpA; in order to prevent transcriptional read-through);

A ~6 kb long homology arm, including exons 3 to 5 of *Cd79a* and *Arhgef1*; and
Nucleic acid encoding thymidine kinase.

10

The targeting vector is represented schematically in Figure 1B, and the nucleotide sequence of the targeting vector is shown in SEQ ID NO:1.

15

The targeting vector is transfected into cells of a Balb/c embryonic stem cell line. Embryonic stem cell clones comprising a successfully integrated sequence are identified by positive selection for puromycin resistance, and negative selection for thymidine kinase activity. The selected cells are subsequently treated to effect flippase-mediated excision of *PuroR*, yielding the mature knock-in allele.

20

Embryonic stem cells comprising the knock-in allele are used to produce transgenic mice by microinjection into blastocysts from Balb/c mice (as described in Sumiyama *et al.*, PLoS One (2018) 13(9):e0203056), which are then implanted into female Balb/c mice for gestation.

25

The *Cd79a*-CreERT2 mice constitutively express CreERT2 in B cell lineage cells, under the control of the *Cd79a* promoter.

8.2 Conditional *Cd40* knockout mouse

A mouse comprising an endogenous nucleotide sequence providing for inducible knockout of *Cd40* is produced.

30

A targeting vector was designed based on the mouse *Cd40* transcript NCBI Ref: NM_011611.2. The targeting vector insert is shown schematically in Figure 2A, and comprises (from 5' to 3'):

A ~3 kb short homology arm;

loxP sites flanking a region comprising: puromycin resistance gene *PuroR*, flanked by *FRT* sites (providing for its flippase-mediated excision); and exons 2 to 5 of *Cd40*;

35

A ~6 kb long homology arm; and

Nucleic acid encoding thymidine kinase.

40

The targeting vector is represented schematically in Figure 2B, and the nucleotide sequence of the targeting vector is shown in SEQ ID NO:2.

The targeting vector is transfected into cells of a Balb/c embryonic stem cell line. Embryonic stem cell clones comprising a successfully integrated sequence are identified by positive selection for puromycin

resistance, and negative selection for thymidine kinase activity. The selected cells are subsequently treated to effect flippase-mediated excision of *PuroR*, yielding the mature conditional knockout allele.

Cre recombinase-mediated excision of exons 2 to 5 of *Cd40* is predicted to delete approximately half of the amino acid sequence of the target protein, to introduce a frameshift in downstream exons 6 to 9, and introduce a premature stop codon in exon 6. The transcript of the nucleic acid encoded after Cre recombinase-mediated excision of exons 2 to 5 is predicted to be non-functional, and may moreover be the subject of nonsense-mediated RNA decay.

Embryonic stem cells comprising the conditional knockout allele are used to produce transgenic mice by microinjection into blastocysts from Balb/c mice (as described in Sumiyama *et al.*, PLoS One (2018) 13(9):e0203056, which is hereby incorporated by reference in its entirety), which are then implanted into female Balb/c mice for gestation.

The *Cd40* CKO mice provide for Cre-conditional knockout of expression of Cd40.

Cd40 CKO mice are crossed with *Cd79a*-CreERT2 knock-in mice described in Example 8.1, yielding mice that are homozygous for the *Cd40^{flox}* allele, and heterozygous for the *Cd79a^{CreERT2}* allele, and providing for knockout of expression of Cd40 in B cells in response to treatment with tamoxifen. The resulting transgenic mice had the following genotype: *Cd40^{flox/flox}; Cd79a^{+/CreERT2}*.

Example 9: Inhibition of a primary immune response by blocking CD40-CD40L signalling

Experiments were performed to test the ability of anti-CD40L antibody treatment to restrict the primary B cell response to a second antigen.

The anti-CD40L antibody binds to CD40L which is expressed on activated T cells. Treatment with anti-CD40L blocks the interaction between the CD40 receptor on B cells with its CD40L ligand on activated T cells. Treatment with anti-CD40L immediately after immunization with antigens was found to inhibit the initiation of adaptive humoral immunity by blocking the co-stimulation of naïve B cells by activated T cells leading to the failure to form functional germinal centers.

The immunization, dosing and sampling timelines are shown in Figure 3.

8-9 week old mice were immunized with the first antigen, EGFR-hFc (50µg in complete Freund's adjuvant) on Day 1 to induce a primary response to EGFR. Mice were injected again with EGFR-hFc (50µg in incomplete Freund's adjuvant) on Day 7 to augment the primary response to EGFR. On day 30, mice were either immunized for a third time, either with the same antigen (EGFR-hFc, 50µg in incomplete Freund's adjuvant) or a new antigen (CD33-mFc, 50µg in incomplete Freund's adjuvant), or injected with equal volume of PBS in incomplete Freund's adjuvant.

Immunized mice were injected intra-peritoneally with 3 doses of either 10mg/kg of anti-CD40L (anti-mouse CD154; clone MR-1) or isotype antibody (IgGaK). The 3 doses were given 1, 3 and 6 days after the third immunization (Days 31, 33 and 36 of the full experimental timeline).

5 Sera was collected from the mice on days 7, 14, 26, 31 (to assess the primary response to EGFR) and days 36, 40 and 50 (to assess the secondary response to EGFR and the primary response to CD33). Sera was assessed for antibody binding to EGFR-His and CD33-His proteins, followed by detection with anti-mouse IgG-HRP secondary antibody and development of colorimetric substrate 3,3',5,5'-tetramethylbenzidine in ELISA assays. Samples were first diluted 1:100, then serially diluted 3-fold to
 10 yield 11 dilutions for the ELISA assays. Responses to antigens were plotted as the dilution factor at 50% of maximum binding.

Results

Figure 4A shows the immune responses observed after two immunizations with EGFR-hFc to generate a
 15 primary immune response, followed by a third immunization with either PBS (left panels), EGFR-hFc (middle panels) or CD33-mFc (right panels), then subsequent treatment with anti-CD40L antibody.

Mice treated with anti-CD40L antibody demonstrated a secondary response to EGFR (first antigen) and an extremely limited immune response to CD33 (second antigen).

20 A secondary immune response was only observed after the third administration of EGFR (top middle panel). Maximum effect of anti-CD40L treatment on the secondary response to EGFR was observed by Day 40: anti-CD40L treated mice exhibited a reduced EGFR response at 75% of isotype control treated mice. At Day 50, recovery of the secondary response to 91% of control was observed in anti-CD40L
 25 treated mice. Sustained effects of anti-CD40L treatment on primary response to CD33 was observed at Days 40 and 50.

The CD33 response in anti-CD40L treated mice was 5% at Day 40 and 2% at Day 50 of control (bottom right panel). Inhibition of the primary response upon anti-CD40L treatment was significant at Day 50 ($p =$
 30 0.02, multiple unpaired t-test with Holm-Sidak correction)

Thus, short-term treatment with anti-CD40L antibody can inhibit primary response to new antigens without severely impacting secondary responses. The effects of anti-CD40L treatment on the primary response can be sustained for up to 14 days post injection.

35 **Example 10: Characterization of CreERT2 activity in transgenic mice carrying the *Cd79a*^{CreERT2} constitutive knock-in allele**
 Mice homozygous for a lox-Stop-lox ZsGreen allele (*Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}*) and heterozygous for *Cd79a*^{CreERT2} were used to assess induction of B cell-specific cre/lox recombination *in vivo*. In such mice,
 40 tamoxifen-induced expression of CreERT2 is expected to result in B cell-specific expression of ZsGreen.

Briefly, 6-8 week-old mice were injected intra-peritoneally with single dose of tamoxifen at 100 mg/kg or 200 mg/kg body weight. Mice were euthanized at 1-, 2-, 4- and 5- days post-treatment for analyzes. Cells isolated from bone marrow, spleen, lymph nodes and blood were stained with Zombie NIR reagent and anti-mouse Cd45r antibody, and assessed by flow cytometry to determine the proportions of Cd45r⁺ B cells and Cd45r⁻ non-B cells that were ZsGreen-fluorescent.

The results are shown in Figure 5. Cre-mediated recombination giving rise to ZsGreen fluorescence was detected specifically in Cd45r⁺ B cells in all lymphoid tissues. ZsGreen fluorescence was detected in 60-90% of total Cd45r⁺ B cells in the bone marrow, spleen, lymph nodes and blood, while less than 5% of Cd45r⁻ non-B cells in these tissues exhibited ZsGreen fluorescence. Effective B cell-specific cre-mediated recombination was achieved within 2 days of tamoxifen treatment in a dose-dependent manner. These data indicate that transgenic mice carrying a single *Cd79a*^{CreERT2} allele can achieve effective, tamoxifen-induced, B cell-specific, cre/lox-mediated genetic knockout, in a dose-dependent manner.

In further experiments, the kinetics of cre/lox-mediated genetic knockout in the mice was evaluated.

Briefly, 6-8 week-old mice were injected intra-peritoneally with a single dose of tamoxifen at 100 mg/kg body weight or corn oil (vehicle control). Mice were euthanized at 1-, 2-, 6-, 11- and 17- days post-treatment for analyzes. Cells isolated from bone marrow, spleen, lymph nodes and blood were stained with Zombie NIR reagent and anti-mouse Cd45r antibody, and assessed by flow cytometry to determine the proportions of Cd45r⁺ B cells that were ZsGreen-fluorescent.

The results are shown in Figure 6. Cre-mediated recombination giving rise to ZsGreen fluorescence was efficiently induced by tamoxifen in Cd45r⁺ B cells in the spleen, bone marrow, lymph nodes and blood. The highest proportions of recombined, ZsGreen-fluorescing Cd45r⁺ B cells were detected in all tissue types at the Day 2 timepoint. Slight declines (~10%) in the total number of recombined Cd45r⁺ B cells were observed in the spleen, lymph nodes and blood by Day 17 post-treatment. The proportion of recombined Cd45r⁺ B cells in the bone marrow decreased rapidly from 2 days post tamoxifen injection. By Day 17, B cells in the bone marrow consisted predominantly of ZsGreen-negative, non-recombined cells. These data indicate that transgenic mice carrying a single *Cd79a*^{CreERT2} allele can achieve effective, tamoxifen-induced, B cell-specific, cre/lox-mediated genetic knockout. While all lymphoid tissues are highly responsive to tamoxifen-induced CreERT2 activity, high proportions of recombined B cells persist in the peripheral lymphoid tissues for more than 10 days post tamoxifen treatment, but the proportion of recombined cells among cells of the bone marrow decrease more rapidly.

Example 11: Characterization of CreERT2-induced knockout of *Cd40* in *Cd40*^{flox/flox}; *Cd79a*^{+/-CreERT2} transgenic mice

Tamoxifen-induced deletion of *Cd40* in *Cd40*^{flox/flox}; *Cd79a*^{+/-CreERT2} mice (described in Example 8) was evaluated. Briefly, 6 week-old mice were injected intra-peritoneally either with a single dose of tamoxifen at 200 mg/kg bodyweight or corn oil (vehicle control). Mice were euthanized 48 h post-treatment for analyzes. Genomic DNA was isolated from bone marrow cells and used in genotyping PCRs. Two sets of primers were used to detect deletion of exons 2-5 on the *Cd40*^{KO} allele, and a control locus present in

both $Cd40^{flox}$ and $Cd40^{KO}$ alleles (represented schematically in Figure 7A). A first primer set provides for the amplification of a 473bp control amplicon, detectable in both in the presence and absence of the CreERT2-mediated recombination of the $Cd40^{flox}$ locus (control). A second primer set provides for the amplification of a 269bp amplicon detectable following CreERT2-mediated recombination of the $Cd40^{flox}$ locus ($Cd40^{KO}$ amplicon).

The results are shown in Figure 7B. The control locus was intact and detected in both tamoxifen- and vehicle- treated mice. The amplicon for the $Cd40^{KO}$ allele was detected only in cells isolated from tamoxifen-treated mice. These data indicate that deletion of floxed exons 2-5 of $Cd40$ was achieved by tamoxifen treatment in $Cd40^{flox/flox}; Cd79a^{+}/CreERT2$ mice.

In further experiments, tamoxifen-induced knockout of $Cd40$ in $Cd40^{flox/flox}; Cd79a^{+}/CreERT2$ mice was evaluated at the cellular level, by analysis of $Cd40$ expression on B cells in peripheral lymphoid tissues.

Briefly, 6 week-old $Cd40^{flox/flox}; Cd79a^{+}/CreERT2$ mice were injected intra-peritoneally with a single dose of tamoxifen at 200 mg/kg body weight or corn oil (vehicle control). Mice were euthanized 48 h post-tamoxifen treatment for analyzes. Cells isolated from the spleen and lymph nodes were stained with Zombie NIR reagent, anti-mouse $Cd45r$ antibody and anti-mouse $Cd40$ antibody, and the proportions of $Cd40^{+}Cd45r^{+}$ B cells and $Cd40^{-}Cd45r^{+}$ B cells were determined by flow cytometry.

The results are shown in Figure 8. High percentages (>75%) of the $Cd45^{+}$ B cells isolated from the spleen and lymph nodes of tamoxifen-treated mice were $Cd40^{-}$. By contrast, < 7% of $Cd45^{+}$ B cells isolated from the spleen and lymph nodes of vehicle-treated mice were $Cd40^{-}$. These data indicate that tamoxifen treatment efficiently induces knockout of $Cd40$ in $Cd40^{flox/flox}; Cd79a^{+}/CreERT2$ mice, thereby preventing $Cd40$ expression on B cells.

Example 12: Maintenance of high levels of CreERT2-induced knockout

The ability of repeated administration of tamoxifen to maintain high levels CreERT2-mediated knockout of floxed loci was investigated.

Briefly, 6-8 week-old mice homozygous for a lox-Stop-lox ZsGreen allele ($Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}$) were injected intra-peritoneally with tamoxifen at 200 mg/kg body weight. The animals were then split into 2 groups (represented schematically in Figure 9A). In Arm 1, mice were injected with 4 additional doses of tamoxifen at 100 mg/kg body weight, at 5 to 9-day intervals. In Arm 2, mice were injected with corn oil (vehicle control) according to the same schedule. Mice were euthanized for analyzes at Days 3, 10, 17 and 32 after the initial tamoxifen administration. Cells isolated from bone marrow, spleen and lymph nodes were stained with Zombie NIR reagent and anti-mouse $Cd45r$ antibody, and assessed by flow cytometry to determine the proportions of $Cd45r^{+}$ B cells that were ZsGreen-fluorescent.

The results are shown in Figure 9B. Mice that received only a single, initiating dose of tamoxifen displayed a ~ 90% decrease in recombined B cells in the bone marrow and ~ 25% decrease in

recombined B cells in the spleen and lymph nodes over the course of experiment. Mice in Arm 1 (which received repeated injections of tamoxifen at intervals of 5-9 days) maintained high proportions of recombined B cells in all lymphoid tissues throughout the course of experiment. These data indicate that high proportions of B cells with CreERT2-mediated knockout of floxed loci can be maintained over time by repeated administration of tamoxifen at regular intervals.

In further experiments, the ability of repeated administration of tamoxifen to maintain high levels of *Cd40* knockout in B cells of *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* mice (described in Example 8) is investigated (see Figure 10).

Briefly, 6 week-old *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* mice are injected intra-peritoneally with tamoxifen at 200 mg/kg body weight. The mice are injected with additional doses of tamoxifen at 100 mg/kg body weight, at intervals from 5 to 10 days. Mice are euthanized for analyzes at Days 3, 10, 17 and 32 after the initial tamoxifen administration. Cells isolated from the spleen and lymph nodes are stained with Zombie NIR reagent, anti-mouse Cd45r antibody and anti-mouse Cd40 antibody, and the proportions of Cd40⁺Cd45r⁺ B cells and Cd40⁻Cd45r⁺ B cells are determined by flow cytometry.

High percentages (>75%) of the Cd45⁺ B cells isolated from the spleen, lymph nodes and bone marrow of tamoxifen-treated mice are Cd40⁻, across all time points, indicating that high levels of CreERT2-mediated knockout of *Cd40* expression in B cells can be maintained over time by repeated administration of tamoxifen at regular intervals.

Example 13: Application of transgenic mice providing for inducible knockout of *Cd40* to obtain antibodies directed to a particular region of a protein of interest

The inventors employed the transgenic mice providing for inducible knockout of *Cd40* to obtain antibodies that bind to the membrane-proximal protease cleavage site within the extracellular domain of a protein of interest.

8-9 week old *Cd40^{flox/flox}; Cd79a^{+/CreERT2}* mice (described in Example 8) or BALB/c mice were immunized with a peptide comprising the protease cleavage site ('Antigen 1'; 50µg in complete Freund's adjuvant) on Days 0 and 7 to induce a primary response. On day 26, mice were injected intra-peritoneally with tamoxifen at 200 mg/kg body weight to induce *Cd40* knockout in B cells. On days 30 and 44, mice were immunized with the full extracellular domain of the protein (comprising the protease cleavage site, 'Antigen 2'; 50µg in incomplete Freund's adjuvant). Mice were injected with 3 additional doses of tamoxifen at 100 mg/kg body weight on Days 34, 42 and 52, to maintain *Cd40* knockout in B cells.

Peripheral lymphoid samples were collected from the mice at day 28 ('first timepoint', prior to induction of *Cd40* knockout and immunization with the full extracellular domain of the protein) and day 63 ('second timepoint', after induction of *Cd40* knockout and immunization with the full extracellular domain of the protein), for analysis of V-gene usage of IgG⁺ B cells that bound to the extracellular domain of the protein of interest, and for analysis of binding of antibodies produced in the mice to (i) the soluble, cleaved form

of the extracellular domain (*i.e.* lacking the protease cleavage site), and (ii) the non-cleaved form of the extracellular domain (*i.e.* lacking the protease cleavage site).

Briefly, antibody gene sequences from B cells within the samples collected at days 28 and 63 were obtained by NGS sequencing, and clustered into clonotypes using Cell Ranger (which computationally groups B cells belonging to a common lineage based on their antibody gene sequences). Antibody sequences were then aligned to an in-house antibody database to determine V(D)J gene usage. Sequences were processed to remove all clones with non-functional or multiple functional heavy and/or light chain sequences. The resulting antibody amino acid sequences were then aligned to their closest germline sequence to quantify the number of somatic hypermutations (SHMs), based on the number of distinct residues from the identified germline sequences. For simplicity, the analysis assumed no backmutations to germline residues.

The following tables illustrate that *Cd40* knockout did not prevent somatic hypermutation. Indeed, the number of somatic hypermutations in VH and VL genes of representative antibody clones expressing antibodies that bind specifically the protease cleavage site of the protein of interest, produced from the *Cd40^{flox/flox}; Cd79a^{+/-CreERT2}* mice was found to be increased at the second timepoint (*i.e.* after *Cd40* knockout).

The following tables illustrate that *Cd40* knockout did not prevent somatic hypermutation.

Mouse	No. of clonotypes	
	1 st Time point	2 nd Time point
<i>Cd40^{flox/flox}; Cd79a^{+/-CreERT2}</i>	113	14
BALB/c	47	43

*Only clonotypes >1 included

Protease cleavage site-specific clone	VH gene	VL gene	Average no. of SHM	
			1 st Time point	2 nd Time point
Abv1-01	IGHV1-50	IGKV1-132	12.3	18.5

*Representative clone

Mouse genotype	Average #SHM in clonotypes with >3 members
Wild-type	13.9
CD40 cKO	12.6

The antibody gene sequences from the ~20 most represented clonotypes obtained from the *Cd40^{flox/flox}; Cd79a^{+/-CreERT2}* and BALB/c mice were cloned into plasmids for expression as chimeric mouse/human

antibodies (comprising murine VH and VL regions, and human IgG1 CH1, hinge, CH2 and CH3 regions (heavy chain), and human κ CL (light chain)) expressed in ExpiCHO (ThermoFisher) cells and Protein A-purified for subsequent characterization.

5 The antibodies were evaluated for their ability to bind to the protease cleavage site by ELISA. Briefly, wells of a polypropylene plate were coated with 1 μ g/ml Neutravidin (Invitrogen), incubated overnight at 4°C, washed and blocked with 1x phosphate-buffered saline (PBS) with 1% BSA for 2 hours at room temperature. The plate was then washed two times with 1x PBS with 0.05% Tween 20 before the addition of 1 μ g/ml of (i) biotinylated, His-tagged soluble extracellular domain of the protein of interest (lacking the
10 protease cleavage site) or (ii) biotinylated, His-tagged full-length extracellular domain of the protein of interest (comprising the protease cleavage site). Plates were washed three times with 1x PBS with 0.05% Tween 20 and dried in between each step. Nine points of a four-fold dilution series (serially diluted with 1x PBS + 1% BSA, starting from 10 μ g/ml) of the antibodies were then added and incubated for 1 hr at room temperature. After washing three times with 1x PBS with 0.05% Tween 20 and dried in between each
15 step, HRP-conjugated goat anti-human secondary antibody (Invitrogen) was prepared at 1:7000 dilution with 1x PBS with 1% BSA and added to plates. Plates were incubated in the dark, at room temperature, for 1 hr. Plates were developed with colorimetric detection substrate 3,3',5,5'-tetramethylbenzidine (Turbo-TMB; Pierce, USA). The reaction was stopped with Invitrogen ELISA Stop Solution (Cat. No. #SS04), and OD was measured at 450 nM using a BioTek PowerWave HT.

20 Among antibodies from the most represented clonotypes, 21% of those obtained from *Cd40^{flox/flox}*; *Cd79a^{+/-CreERT2}* mice were found to bind specifically to the full-length extracellular domain of the protein of interest (comprising the protease cleavage site) and not to the soluble extracellular domain of the protein of interest (lacking the protease cleavage site), whereas none of the antibodies produced from the most
25 represented clonotypes obtained from the BALB/c mice displayed such specific binding (*i.e.* only to the protease cleavage site-containing antigen, and not to the other).

Figure 11 shows that diversity of VH and VL gene usage was found to be preserved in the *CD40^{flox/flox}*; *Cd79a^{+/-CreERT2}* mice (CD40cKO) (*i.e.* relative to usage in their wild-type counterparts).

30 These data demonstrate that transgenic mice providing for inducible knockout of *Cd40* are useful to obtain a diverse, antibodies focused to regions of interest of a given protein of interest.

Example 14: Characterisation of response to anti-CD40L antibody

35 Experiments were carried out to investigate the effect of treating wild-type mice with one or two doses of an anti-CD40L antibody, in combination with immunization with antigens. Briefly, adult BALB/c mice were first immunized with a peptide comprising the protease cleavage site ('Antigen 1'; 50 μ g in complete Freund's adjuvant) on Days 0 and 7 to induce a primary response. On days 30 and 44, mice were immunized with the full extracellular domain of the protein (comprising the protease cleavage site,
40 'Antigen 2'; 50 μ g in incomplete Freund's adjuvant). Mice were injected intra-peritoneally with 3 doses of 10mg/kg of anti-CD40L (anti-mouse CD154; clone MR-1) on Days 31, 33 and 36 following immunization on Day 30 and on Days 45, 47 and 50 following immunization on Day 44. Sera was collected from the

mice on days 42 and 55 to assess epitope-specific response to the protease cleavage site driven by Antigen 1 and new primary responses to non-specific regions in the extracellular domain of the protein, driven by Antigen 2. The sera binding profiles and titers to cells with surface expression of either the full-length or epitope-deleted protein were analyzed by FACS. Briefly, CHO cells were transiently transfected with (i) a plasmid encoding the membrane-bound version of the protein of interest (comprising the protease cleavage site), or (ii) a plasmid comprising the soluble version of the extracellular domain of the protein of interest (lacking the protease cleavage site) and the native transmembrane domain (for membrane localization and surface expression). Non-transfected CHO cells served as negative controls. After 18 hr, cells were harvested, and 3×10^4 cells/well of each cell type were incubated with 50 μ L of 3-fold serial dilutions (starting from a 1:200 dilution in PBS) of sera obtained from the mice on the indicated days, and incubated at 4°C for 1 hr. Cells were then washed twice with 150 μ L of FACS buffer (1xPBS + 1% FBS) and resuspended in 50 μ L of Alexa Fluor 647 AffiniPure Goat Anti-Mouse IgG (Jackson Immuno, 1 in 1000 dilution) antibody for 20 minutes at 4°C. Cells were washed twice with FACS buffer and resuspended in a final volume of 40 μ L DAPI solution except the unstained well in 40 μ L of FACS buffer without DAPI. Samples were then run on iQue3 flow cytometry and data were analyzed using FlowJo v10.8.1 software.

The results are shown in Figure 12. These results show that the first dose of anti-CD40L is effective in blocking new primary responses to non-specific domains outside of the targeted epitope (the protease cleavage site) but subsequent dose(s) of anti-CD40L is not. These results indicate a need to develop genetic-based methods to control CD40 signaling to achieve a long-term and sustained effect in controlling new primary responses in animals.

Example 15: Characterisation of B cell properties following tamoxifen-induced conditional gene deletion

The inventors employed the transgenic mice providing for inducible knockout of *CD40* to investigate whether the IgG class-switched response is maintained following tamoxifen-induced conditional gene deletion.

8-9 week old *Cd40^{flox/flox}; Cd79a^{+/-CreERT2}* mice (described in Example 8) or BALB/c mice were immunized with a peptide comprising the protease cleavage site ('Antigen 1'; 50 μ g in complete Freund's adjuvant) on Days 0 and 7 to induce a primary response. On day 26, mice were injected intra-peritoneally with tamoxifen at 200 mg/kg body weight to induce *Cd40* knockout in B cells. On days 30 and 44, mice were immunized with the full extracellular domain of the protein (comprising the protease cleavage site, 'Antigen 2'; 50 μ g in incomplete Freund's adjuvant). Mice were injected with 3 additional doses of tamoxifen at 100 mg/kg body weight on Days 34, 42 and 52, to maintain *Cd40* knockout in B cells.

Peripheral lymphoid samples were collected from the mice at day 28 ('first timepoint', prior to induction of *CD40* knockout and immunization with the full extracellular domain of the protein) and day 63 ('second timepoint', after induction of *CD40* knockout and immunization with the full extracellular domain of the protein). Cells isolated from lymph nodes, spleen and bone marrow were stained with Zombie NIR

reagent, anti-mouse CD45R, IgG1, IgG2a, and IgG2b, and assessed for the proportion of IgG⁺, CD45R⁺ B cells by flow cytometry.

The results are shown in Figure 13A. The proportion of IgG⁺ B cells in lymph nodes and spleen are decreased in CD40 cKO mice compared with wild-type BALB/c mice. The total number of CD45R⁺ B cells is decreased in the spleen of CD40 cKO mice after tamoxifen-induced gene deletion, compared with wild-type, but not in the lymph node or bone marrow (Figure 13B; LN = lymph node; SP = spleen; BM = bone marrow).

The above experiments were repeated with a second protein of interest. The results are shown in Figure 13C. The proportion of IgG⁺ B cells in the lymph nodes and spleen are decreased in CD40 cKO mice compared with wild-type BALB/c mice.

Taken together, these results show that disruption of CD40 signaling has an inhibitory effect on IgG⁺ B cell expansion during new immune responses and the antibodies that can be isolated from the CD40cKO mice are derived from a smaller pool of class-switched B cells

Example 16: Additional characterisation of transgenic mice providing for inducible knockout of CD40

Tamoxifen-induced deletion of *CD40* in *CD40^{fllox/fllox}; Cd79a^{+/CreERT2}* mice (described in Example 8.2) was further evaluated. Briefly, 8-9 week old *Cd40^{fllox/fllox}; Cd79a^{+/CreERT2}* mice (described in Example 8) or BALB/c mice were immunized with a peptide comprising the protease cleavage site ('Antigen 1'; 50µg in complete Freund's adjuvant) on Days 0 and 7 to induce a primary response. On day 26, mice were injected intra-peritoneally with tamoxifen at 200 mg/kg body weight to induce *Cd40* knockout in B cells.

On days 30 and 44, mice were immunized with the full extracellular domain of the protein (comprising the protease cleavage site, 'Antigen 2'; 50µg in incomplete Freund's adjuvant). Mice were injected with 3 additional doses of tamoxifen at 100 mg/kg body weight on Days 34, 42 and 52, to maintain *Cd40* knockout in B cells.

Mice were euthanized 10 days after the last tamoxifen treatment for analysis. Genomic DNA was isolated from cells in the spleen and ear and used in genotyping PCRs. A first primer set provided for the amplification of a 713 bp wildtype or / a 815 bp *CD40^{fllox}* amplicon detectable in the absence of CreERT2-mediated recombination of the *CD40^{fllox}* locus. A second primer set provided for the amplification of a 497 bp CD40cKO amplicon detectable following CreERT2-mediated recombination of the *CD40^{fllox}* locus.

Genotyping revealed B-cell-specific, tamoxifen-induced conditional deletion of *CD40* (Figure 14A). This is demonstrated by the presence of the band at 467 bp in B cells isolated from the spleen of tamoxifen-induced transgenic mice (CD40 cKO (fl/fl + tamoxifen)). The 467 bp band is not present in B cells isolated from the spleens of mice not treated with tamoxifen. In addition, the 467 bp band was not detected in ear cells taken from *CD40^{fllox/fllox}; Cd79a^{+/CreERT2}* (CD40 cKO) mice, which demonstrates the tissue specificity of the inducible knockout to B cells.

Flow cytometry of B cells collected from lymphoid tissues demonstrated a 80-90% deletion of CD40⁺ B cells in the lymphoid tissues in tamoxifen-induced transgenic mice (CD40 cKO) compared with wild-type mice (Figures 14B and 14C).

- 5 Taken together, these results demonstrate that the tamoxifen-induced conditional deletion of *CD40* is specific to B cells.

Example 17: Rate of targeted epitope antibody discovery using transgenic mice providing for inducible knockout of CD40 is improved

- 10 Two proteins of interest were used to analyse the generation of targeted epitope specific antibodies. The results are summarized below.

The immunization strategy for the generation of targeted epitope specific antibodies (for each protein of interest ("POI") is shown in Figure 15.

- 15 The following tables show the % of targeted epitope-specific sera generated in *CD40^{flox/flox}; Cd79a^{+/CreERT2}* (CD40 cKO) mice compared with wild-type.

Protein of interest 1 (POI1)

Genotype	# of mice immunized	# with titer to POI1-FL	# with titer to POI1-delEpi	% epitope-specific
Wild-type	8	8	8	0
CD40 cKO	6	5	1	83

- 20 Protein of interest 2 (POI2)

Genotype	# of mice immunized	# with titer to POI2-FL	# with titer to POI2-delEpi	% epitope-specific
Wild-type	3	3	3	0
CD40 cKO	6	6	0	100

- 25 The antibodies were evaluated for their ability to bind to the protease cleavage site by ELISA. Briefly, wells of a polypropylene plate were coated with 1 µg/ml Neutravidin (Invitrogen), incubated overnight at 4°C, washed and blocked with 1x phosphate-buffered saline (PBS) with 1% BSA for 2 hours at room temperature. The plate was then washed two times with 1x PBS with 0.05% Tween 20 before the addition of 1 µg/ml of (i) biotinylated, His-tagged soluble extracellular domain of the protein of interest (lacking the protease cleavage site) or (ii) biotinylated, His-tagged full-length extracellular domain of the protein of interest (comprising the protease cleavage site). Plates were washed three times with 1x PBS with 0.05% Tween 20 and dried in between each step. Nine points of a four-fold dilution series (serially diluted with 1x PBS + 1% BSA, starting from 10 µg/ml) of the antibodies were then added and incubated for 1 hr at room temperature. After washing three times with 1x PBS with 0.05% Tween 20 and dried in between each step, HRP-conjugated goat anti-human secondary antibody (Invitrogen) was prepared at 1:7000 dilution with 1x PBS with 1% BSA and added to plates. Plates was incubated in the dark, at room temperature, for 1 hr. Plates were developed with colorimetric detection substrate 3,3',5,5'-tetramethylbenzidine

(Turbo-TMB; Pierce, USA). The reaction was stopped with Invitrogen ELISA Stop Solution (Cat. No. #SS04), and OD was measured at 450 nM using a BioTek PowerWave HT.

FACS analysis was also performed to assess the sera binding profiles. Briefly, CHO cells were transiently transfected with (i) a plasmid encoding the membrane-bound version of the protein of interest (comprising the protease cleavage site), or (ii) a plasmid comprising the soluble version of the extracellular domain of the protein of interest (lacking the protease cleavage site) and the native transmembrane domain (for membrane localization and surface expression). Non-transfected CHO cells served as negative controls. After 18 hr, cells were harvested, and 3×10^4 cells/well of each cell type were incubated with 50 μ L of 3-fold serial dilutions (starting from a 1:200 dilution in PBS) of sera obtained from the mice on the indicated days, and incubated at 4°C for 1 hr. Cells were then washed twice with 150 μ L of FACS buffer (1xPBS + 1% FBS) and resuspended in 50 μ L of Alexa Fluor 647 AffiniPure Goat Anti-Mouse IgG (Jackson Immuno, 1 in 1000 dilution) antibody for 20 minutes at 4°C. Cells were washed twice with FACS buffer and resuspended in a final volume of 40 μ L DAPI solution except the unstained well in 40 μ L of FACS buffer without DAPI. Samples were then run on iQue3 flow cytometry and data were analyzed using FlowJo v10.8.1 software.

The results for protein of interest 1 (POI1) are shown in Figures 16A and 16B. The results for protein of interest 2 (POI2) are shown in Figures 16C and 16D.

The following table shows the rate of obtaining epitope specific antibody clones from clonal B cell screens performed for POI1.

Mouse strain	Total # clones screened	% Epitope-specific
Wild-type	21	4.8
CD40 cKO	14	28.6

Figure 17 shows the specificity of recombinant antibodies as assayed by FACS analysis for POI1. Four representative recombinant antibodies derived from the *CD40^{flox/flox}; Cd79a^{+/-CreERT2}* (CD40cKO) mice were evaluated for their ability to bind to cells expressing the full-length protein of interest (comprising the protease cleavage site), or a version of the protein of interest that lack the protease cleavage site by FACS analysis. The results show that these antibodies are specific for the targeted protease cleavage site and do not bind to other domains in the full-length protein.

Figures 18A to 18D show the dose-response curve for binding to full-length extracellular domain of POI1 (comprising the protease cleavage site) as determined by ELISA, for four representative antibody clones obtained from the *CD40^{flox/flox}; Cd79a^{+/-CreERT2}* (CD40 cKO) mouse. The below table provides the EC₅₀ for each. These results demonstrate that all four antibodies bind to the target antigen with high affinity.

Antibody	EC ₅₀
Abv1-01	1.98E-11

Abv1-09	2.96E-10
Abv1-17	2.15E-11
Abv1-13	4.65E-11
Isotype control	-

Example 18: NZBWF1 mice respond better to immunization to antigens with high homology to self

The inventors used a highly immuno-reactive mouse strain (NZBWF1) to analyse responses to antigens with high homology to self.

5

6-12 weeks old BALB/c or NZBWF1 mice were immunized with recombinant human DLL3-His tagged protein. Sera from animals were analysed for antibody titer against human DLL3-Fc tagged protein on Day 38 post-immunization by indirect Enzyme Linked Immunosorbent Assay (ELISA).

10 The results are shown in Figure 19. Human DLL3 has 85.8 % homology to mouse DLL3 protein. NZBWF1 mice exhibited stronger titers against the antigen when compared to the BALB/c mice.

These results show that NZBWF1 mice respond to immunization with protein antigens, and develop strong antibody titers to antigens with high homology (>80%) to self.

15

Example 19: NZBWF1 mice produce a stronger antibody response to poorly immunogenic antigens

The inventors used the highly immuno-reactive mouse strain (NZBWF1) used in Example 18 to analyse responses to poorly immunogenic antigens.

20

6-10 weeks old BALB/c or NZBWF1 mice were immunized with two doses of a 13-mer peptide conjugated to KLH, seven days apart. Sera from animals were analysed for antibody titer against the peptide conjugated to biotin on Day 21 post-immunization by indirect Enzyme Linked Immunosorbent Assay (ELISA).

25

The results are shown in Figure 20. NZBWF1 mice exhibited stronger titers against peptide antigen compared to BALB/c mice. These results show that NZBWF1 mice respond to immunization with small peptides and produce antibody titers to poorly immunogenic antigens.

Example 20: Generation of an exemplary hyperimmune $CD40^{flox/flox}; Cd79a^{+/CreERT2}$ mouse

An exemplary hyperimmune mouse was generated as follows. A hyperimmune $CD40^{flox/flox}; Cd79a^{+/CreERT2}$ mouse can be generated by standard breeding strategies which may include the mating of mice carrying the $CD40^{flox/flox}$ and $Cd79a^{+/CreERT2}$ alleles (in a BALB/c background) (for example the mice generated in Example 8.2) with the autoimmune parental strains NZB or NZW, and subsequently mating the NZB and NZW mice to generate NZBWF1 progeny that carry the two alleles.

35

The expression of mouse CD40 can be inducibly knocked-out in the resulting transgenic mice by administration of tamoxifen. The resulting hyperimmune $CD40^{flox/flox}; Cd79a^{+/CreERT2}$ mouse is a highly

immuno-reactive mouse capable of a strong immune response, which is useful for producing epitope-specific antibodies. For example, such mice are capable of producing strong antibody titers to antigens with high homology (>80%) to self, or poorly immunogenic antigens.

Claims:

1. An animal comprising an endogenous nucleotide sequence providing for inducible inhibition of a primary humoral immune response.

5 2. The animal according to claim 1, wherein the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in mounting a primary humoral immune response.

10 3. The animal according to claim 1 or claim 2, wherein the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in immunoglobulin isotype switching, B cell maturation, and/or production of plasma and/or memory B cells.

15 4. The animal according to any one of claims 1 to 3, wherein the animal comprises an endogenous nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or both genes selected from CD40 and/or CD40L.

20 5. The animal according to any one of claims 1 to 4, wherein the animal comprises an endogenous nucleotide sequence providing for recombinase-mediated disruption of expression of one or more genes involved in mounting a primary humoral immune response.

25 6. The animal according to any one of claims 1 to 5, wherein the endogenous nucleotide sequence comprises target sequences for a recombinase flanking all or part of the nucleotide sequence of a gene involved in mounting a primary humoral immune response.

7. The animal according to claim 6, wherein the animal comprises an endogenous nucleotide sequence providing for inducible expression or activity of the recombinase.

30 8. The animal according to claim 7, wherein the endogenous nucleotide sequence providing for inducible expression or activity of the recombinase encodes a conditional system for controlling expression or activity of the recombinase.

35 9. The animal according to any one of claims 6 to 8, wherein the target sequences for a recombinase are *loxP* sequences, and wherein the recombinase is a Cre recombinase.

10. The animal according to any one of claims 1 to 9, wherein the animal comprises an endogenous nucleotide sequence encoding one or more human immunoglobulin genes or gene segments.

40 11. The animal according to any one of claims 1 to 10, wherein the animal is a mouse, a rat or a rabbit.

12. The animal according to any one of claims 1 to 11, wherein the endogenous nucleotide sequence comprises target sequences for a recombinase flanking one or more exons of CD40, and/or one or more exons of CD40L.

5 13. The animal according to any one of claims 1 to 12, wherein the animal comprises an endogenous nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:4.

10 14. The animal according to any one of claims 1 to 13, wherein the animal comprises an endogenous nucleotide sequence encoding a conditional system for controlling expression and/or activity of a Cre recombinase.

15 15. The animal according to claim 14, wherein expression of the Cre recombinase is under the control of a promoter driving expression in B cell lineage cells.

16. The animal according to any one of claims 1 to 15, wherein the animal comprises an endogenous nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:6.

20 17. A method for producing an antigen-binding molecule, comprising administering a peptide/polypeptide, or nucleic acid encoding a peptide/polypeptide, to an animal according to any one of claims 1 to 16.

18. The method according to claim 17, wherein the method comprises:

25 (i) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;
(ii) treating the animal to inhibit its ability to mount a primary immune response; and
(iii) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

30 19. The method according to claim 17, wherein the method comprises:

(i) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;
(ii) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest; and
(iii) treating the animal to inhibit its ability to mount a primary immune response.

40 20. The method according to claim 18 or claim 19, wherein treating the animal to inhibit its ability to mount a primary immune response comprises administering an agent for inducing expression or activity of the recombinase.

21. A method for producing an antigen-binding molecule, wherein the method comprises:

(i) administering a first peptide/polypeptide, or nucleic acid encoding the first peptide/polypeptide, to an animal, wherein the first peptide/polypeptide comprises an amino acid sequence of interest;

5 (ii) treating the animal to inhibit its ability to mount a primary immune response; and

(iii) administering a second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide, to the animal, wherein the second peptide/polypeptide comprises the amino acid sequence of interest or an amino acid sequence which is similar to the amino acid sequence of interest.

10 22. The method according to claim 21, wherein the method comprises treating the animal to inhibit its ability to mount a primary immune response before, during and/or after the administration of the second peptide/polypeptide, or nucleic acid encoding the second peptide/polypeptide.

15 23. The method according to any one of claims 18 to 22, wherein treating the animal to inhibit its ability to mount a primary immune response comprises administering an agent that inhibits the expression of, or inhibits the activity of the product of, one or more genes involved in mounting a primary humoral immune response.

20 24. The method according to any one of claims 18 to 23, wherein treating the animal to inhibit its ability to mount a primary immune response comprises administering an agent that reduces/blocks stimulation, differentiation, activation, maturation and/or proliferation of naïve B cells in the animal, reduces the number/proportion of naïve B cells in the animal, and/or inhibits immunoglobulin isotype switching and/or B cell maturation.

25 25. The method according to claim 23 or claim 24, wherein the agent inhibits the expression of, or inhibits the activity of the product of, one or both genes selected from CD40 and/or CD40L.

30 26. The method according to any one of claims 23 to 25, wherein the agent inhibits the activity of CD40 and/or CD40L.

27. The method according to any one of claims 23 to 26, wherein the agent is selected from, or comprises, an antibody, antigen-binding molecule, polypeptide, decoy receptor, aptamer, sequestering agent or small molecule.

35 28. The method according to any one of claims 23 to 25, wherein the agent inhibits the expression of CD40 and/or CD40L.

29. The method according to any one of claims 23 to 25 or claim 28, wherein the agent is, or comprises, RNAi, siRNA, an antisense nucleic acid, an antisense oligonucleotide, or a gene editing system.

40 30. The method according to any one of claims 21 to 29, wherein the animal comprises an endogenous nucleotide sequence encoding one or more human immunoglobulin genes or gene segments.

31. The method according to any one of claims 21 to 30, wherein the animal is a mouse, a rat or a rabbit.

32. The method according to any one of claims 17 to 31, wherein the method further comprises
5 generating a hybridoma producing the antigen-binding molecule capable of binding to a protein/protein complex of interest.

33. The method according to any one of claims 17 to 32, wherein the method further comprises isolating
10 one or more antigen-binding molecules capable of binding to a protein comprising the amino acid sequence of interest.

34. The method according to any one of claims 17 to 33, wherein the method further comprises
15 formulating the antigen-binding molecule capable of binding to a protein comprising the amino acid sequence of interest to a pharmaceutical composition.

35. A nucleic acid, or a plurality of nucleic acids, comprising a nucleotide sequence providing for inducible inhibition of a primary humoral immune response.

36. The nucleic acid, or a plurality of nucleic acids according to claim 35, comprising a nucleotide
20 sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in mounting a primary humoral immune response.

37. The nucleic acid, or a plurality of nucleic acids according to claim 35 or claim 36, comprising a
25 nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or more genes involved in immunoglobulin isotype switching and/or B cell maturation, or one or more genes involved in stimulation, differentiation, activation and/or proliferation of naïve B cells.

38. The nucleic acid, or a plurality of nucleic acids according to any one of claims 35 to 37, comprising a
30 nucleotide sequence providing for inducible inhibition of the expression of, or inhibition of the activity of the product of, one or both genes selected from CD40 and/or CD40L.

39. The nucleic acid, or a plurality of nucleic acids according to any one of claims 35 to 38, comprising a
35 nucleotide sequence providing for recombinase-mediated disruption of expression of one or more genes involved in mounting a primary humoral immune response.

40. The nucleic acid, or a plurality of nucleic acids according to any one of claims 35 to 39, comprising a
40 nucleotide sequence encoding all or part of the nucleotide sequence of a gene involved in mounting a primary humoral immune response, flanked by target sequences for a recombinase.

41. The nucleic acid, or a plurality of nucleic acids according to any one of claims 35 to 40, further comprising a nucleotide sequence encoding a conditional system for controlling expression or activity of the recombinase.

5 42. The nucleic acid, or a plurality of nucleic acids according to any one of claims 35 to 41, wherein the target sequences for a recombinase are *loxP* sequences, and wherein the recombinase is a Cre recombinase.

10 43. The nucleic acid or plurality of nucleic acids according to any one of claims 35 to 42, comprising a nucleotide sequence comprising target sequences for a recombinase flanking one or more exons of CD40, and/or one or more exons of CD40L.

15 44. The nucleic acid or plurality of nucleic acids according to any one of claims 35 to 43, comprising a nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:4.

20 45. The nucleic acid or plurality of nucleic acids according to any one of claims 35 to 44, comprising a nucleotide sequence encoding a conditional system for controlling expression and/or activity of a Cre recombinase.

46. The nucleic acid or plurality of nucleic acids according to claim 45, wherein expression of the Cre recombinase is under the control of a promoter driving expression in B cell lineage cells.

25 47. The nucleic acid or plurality of nucleic acids according to any one of claims 35 to 46, comprising a nucleotide sequence comprising, or consisting of, a nucleotide sequence having 60% or greater nucleotide sequence identity to SEQ ID NO:6.

30 48. A vector, or a plurality of vectors, comprising the nucleic acid or plurality according to any one of claims 35 to 47.

49. A cell comprising the nucleic acid or plurality of nucleic acids according to any one of claims 35 to 47, or the vector or plurality of vectors according to claim 48.

35 50. The cell according to claim 49, wherein the cell comprises an endogenous nucleotide sequence encoding one or more human immunoglobulin genes or gene segments.

51. The cell according to claim 49 or claim 50, wherein the cell is a mammalian cell.

40 52. The cell according to any one of claims 49 to 51, wherein the cell is an embryonic stem cell.

53. The cell according to any one of claims 49 to 52, wherein the cell is a mouse cell, a rat cell or a rabbit cell.

54. The animal according to any one of claims 1 to 16, or the method according to claims 17 to 34 wherein the animal is a hyperimmune mouse or a mouse having a hyperimmune phenotype.

55. The animal according to any one of claims 1 to 16 or 54, or the method according to claims 17 to 34,
5 wherein the animal is a humanized mouse.

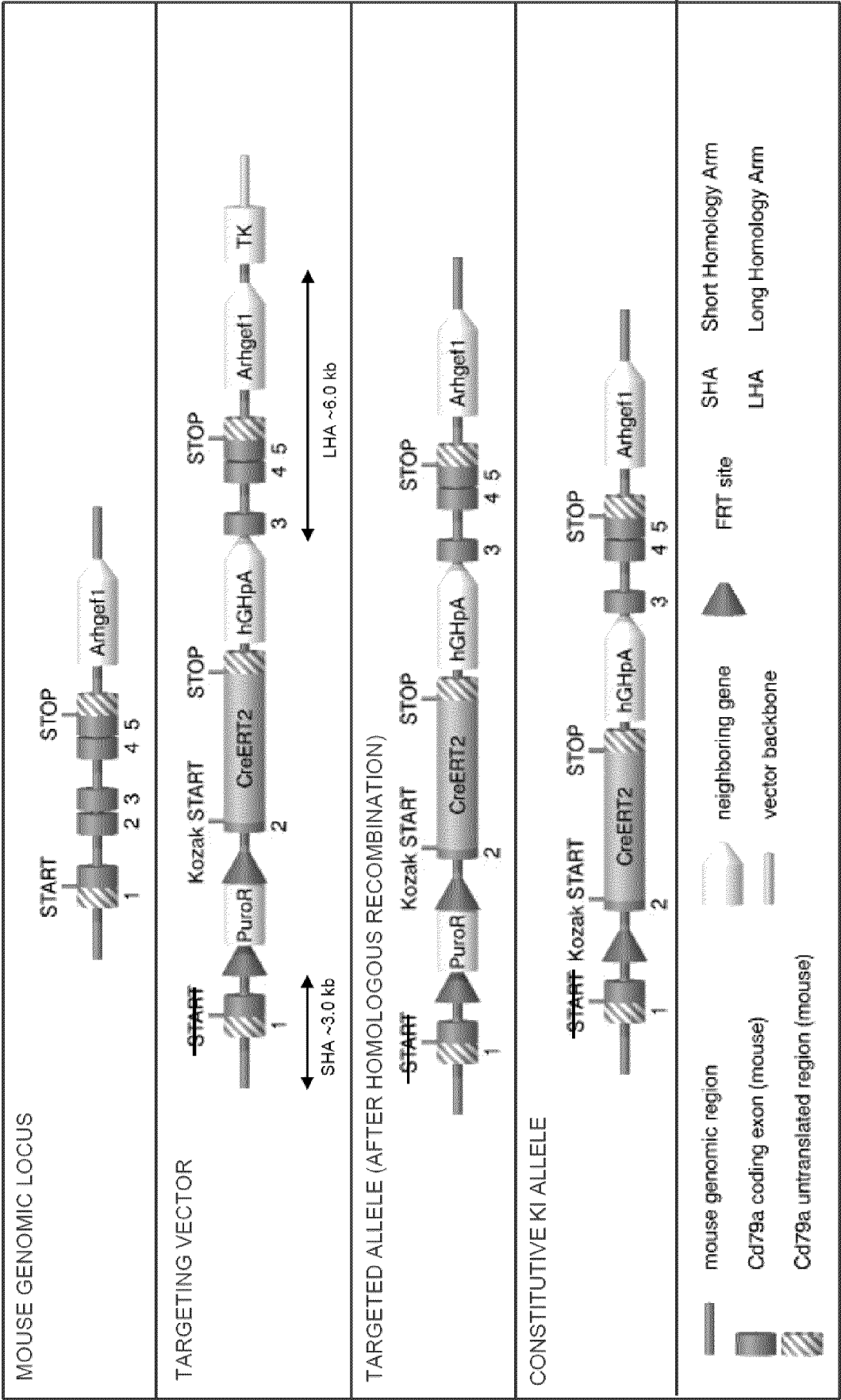


Figure 1A

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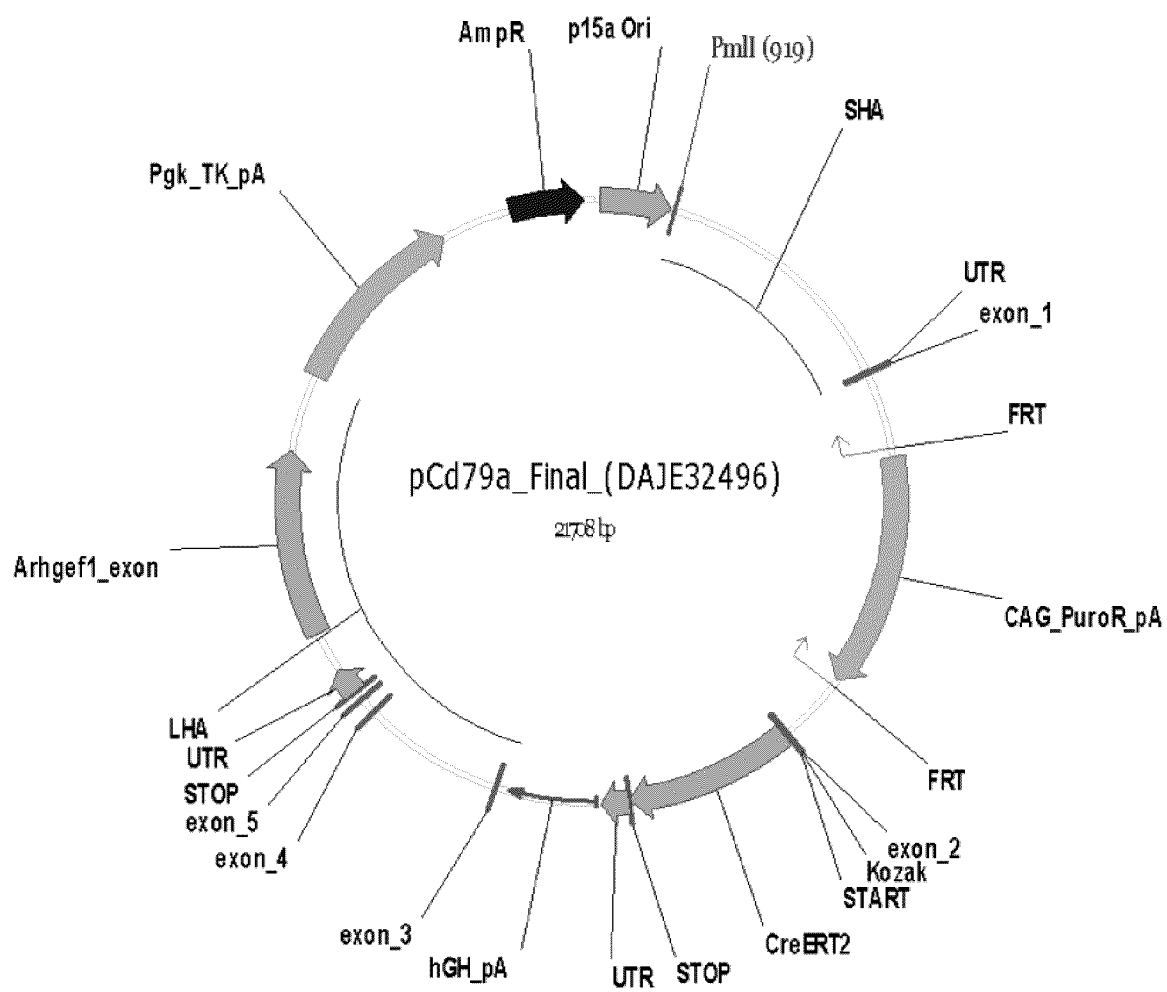


Figure 1B

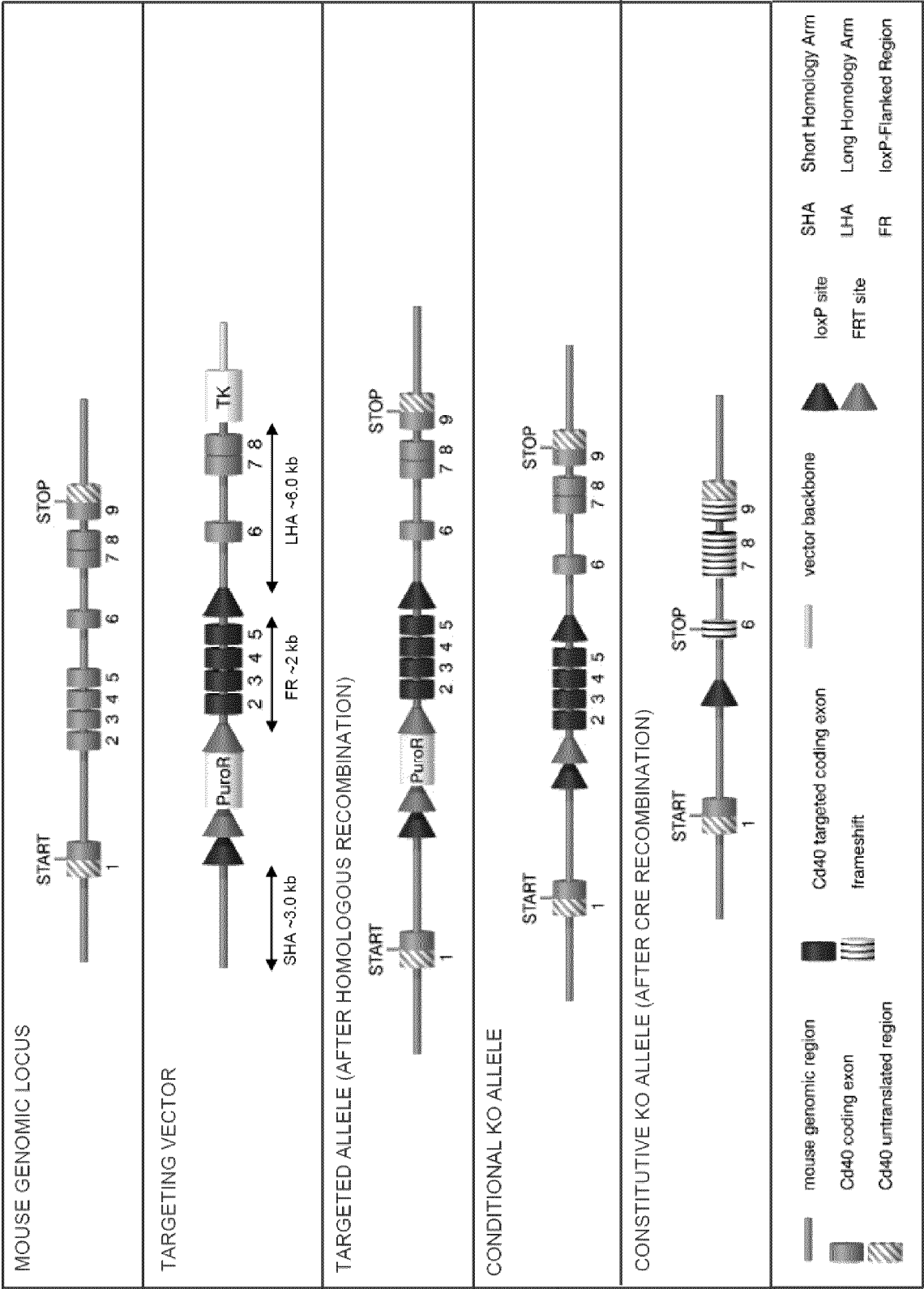


Figure 2A

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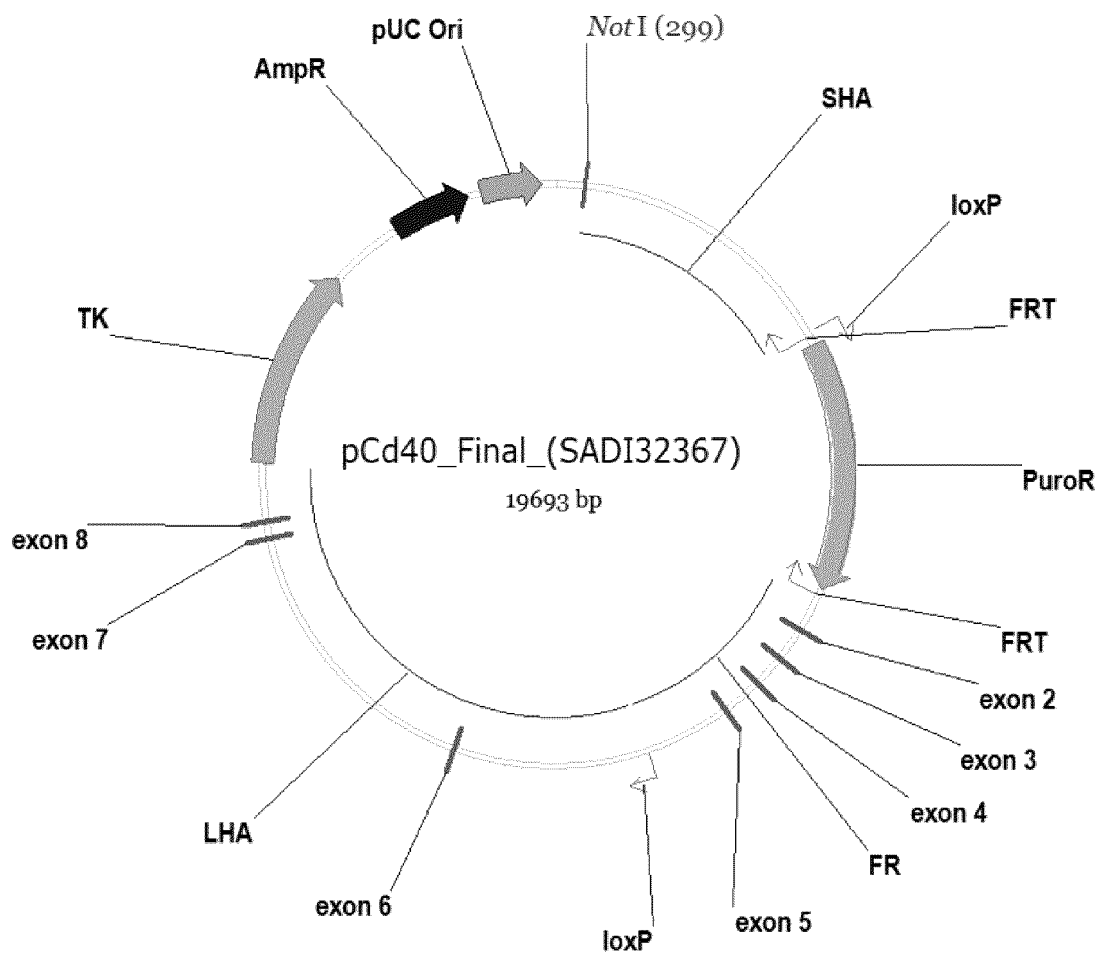


Figure 2B

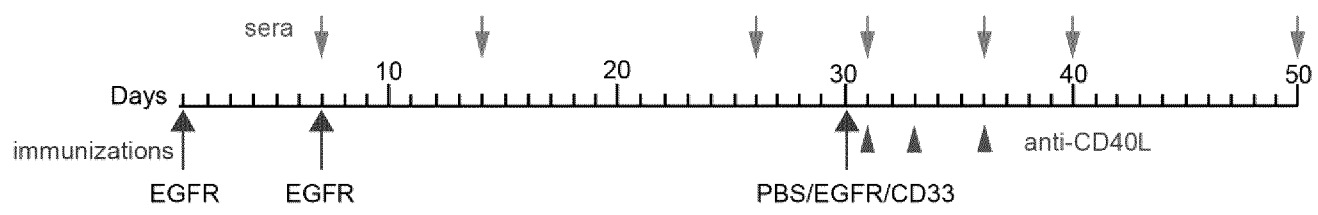


Figure 3

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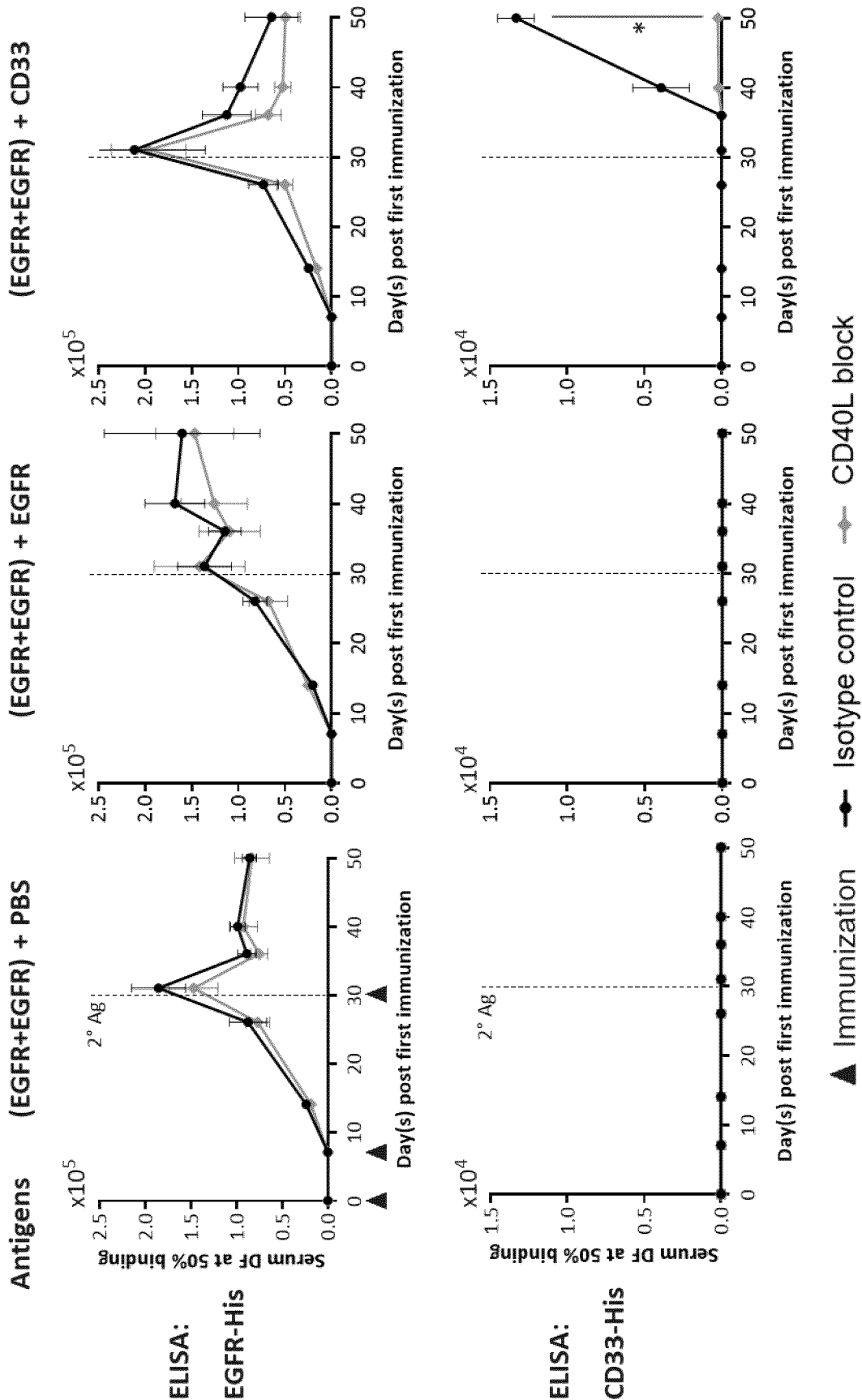


Figure 4

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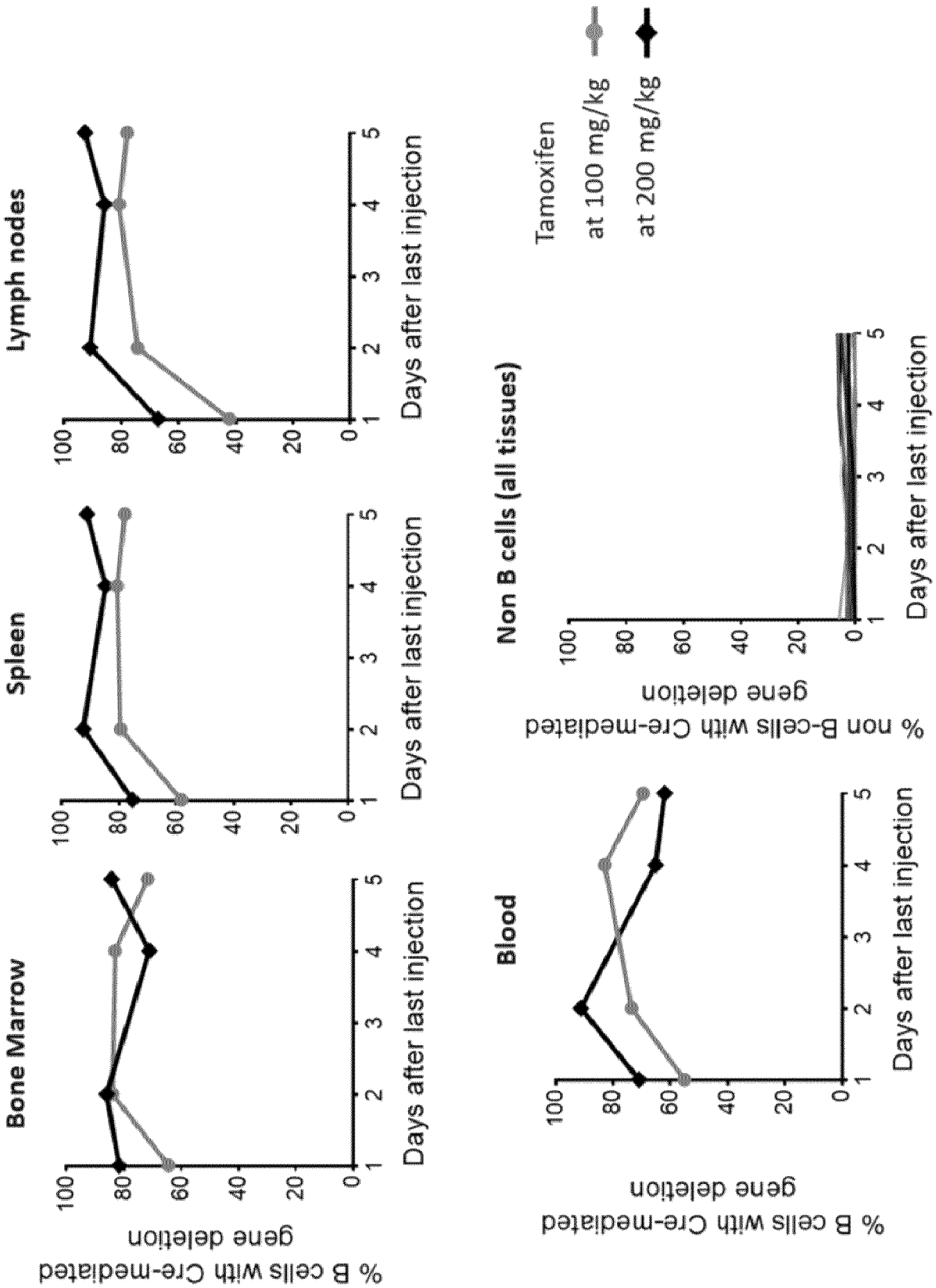


Figure 5

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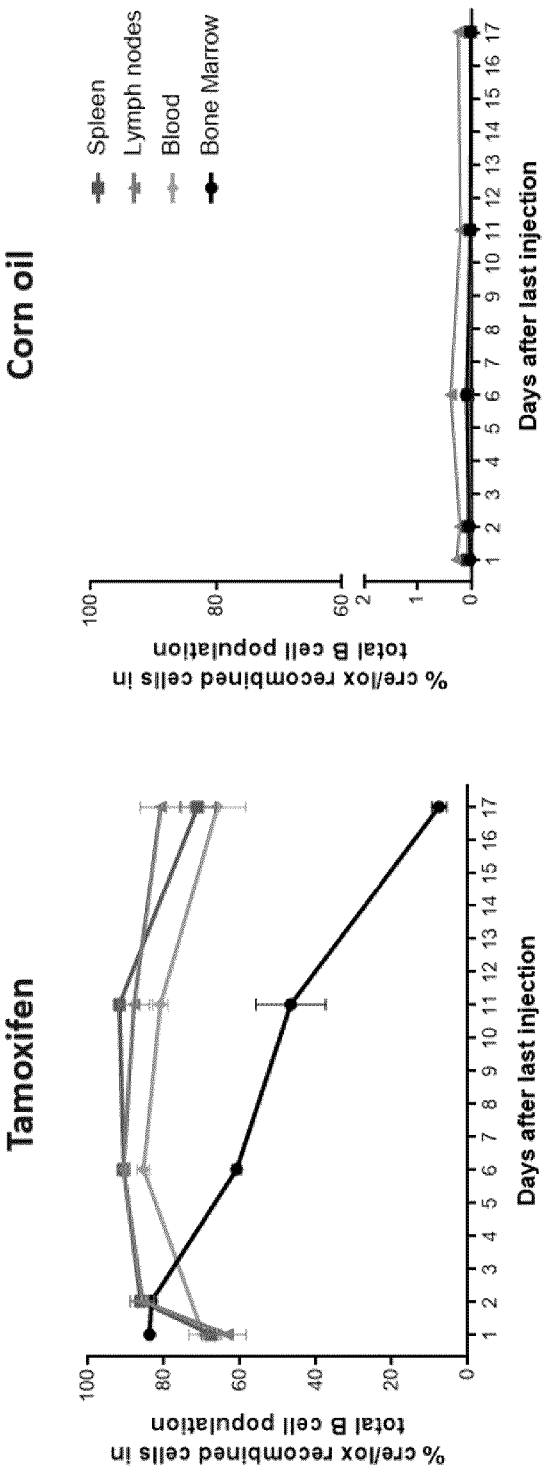


Figure 6

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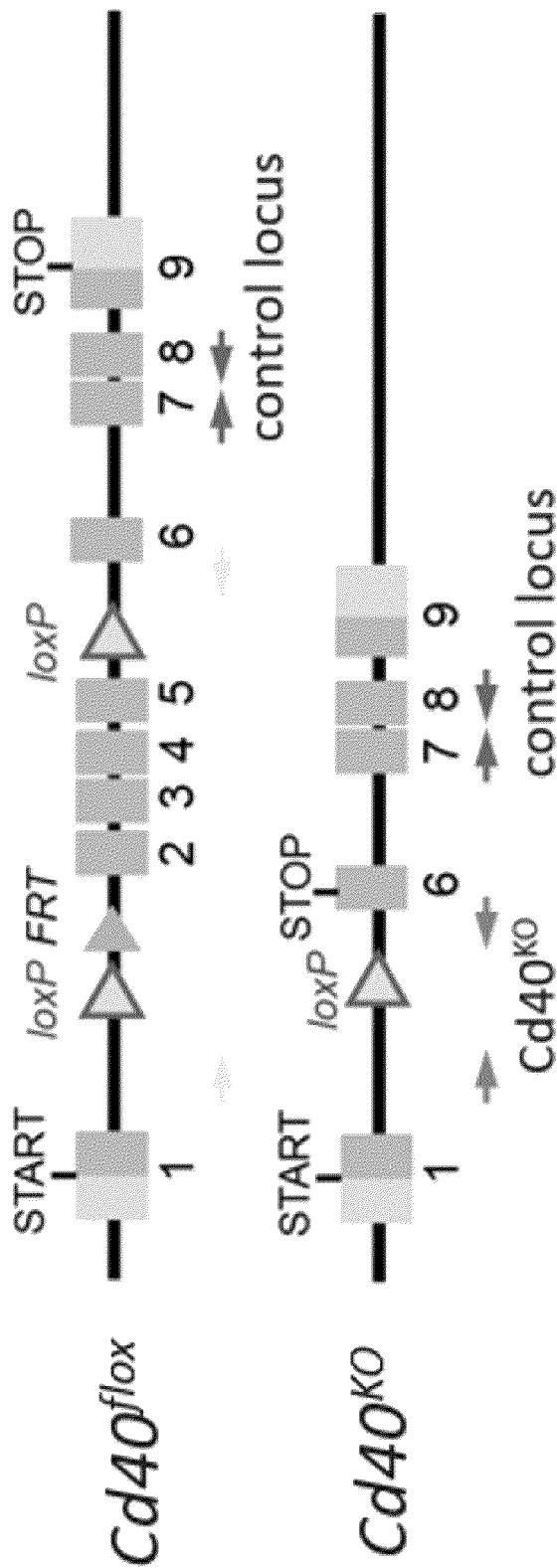


Figure 7A

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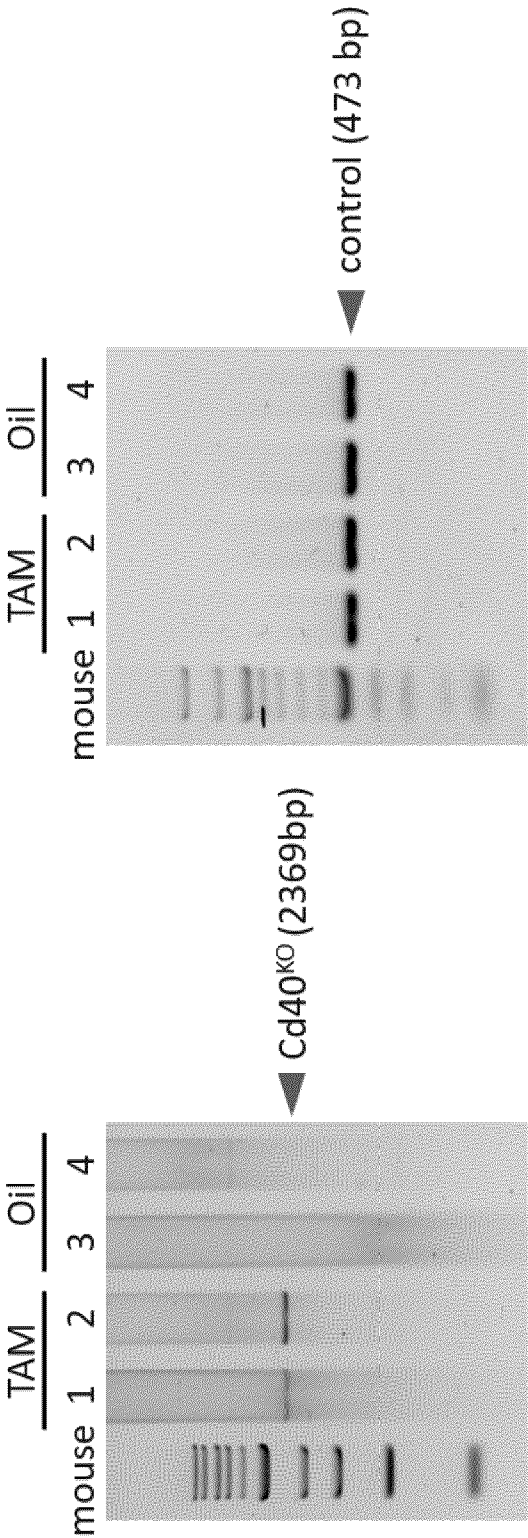


Figure 7B

10/29

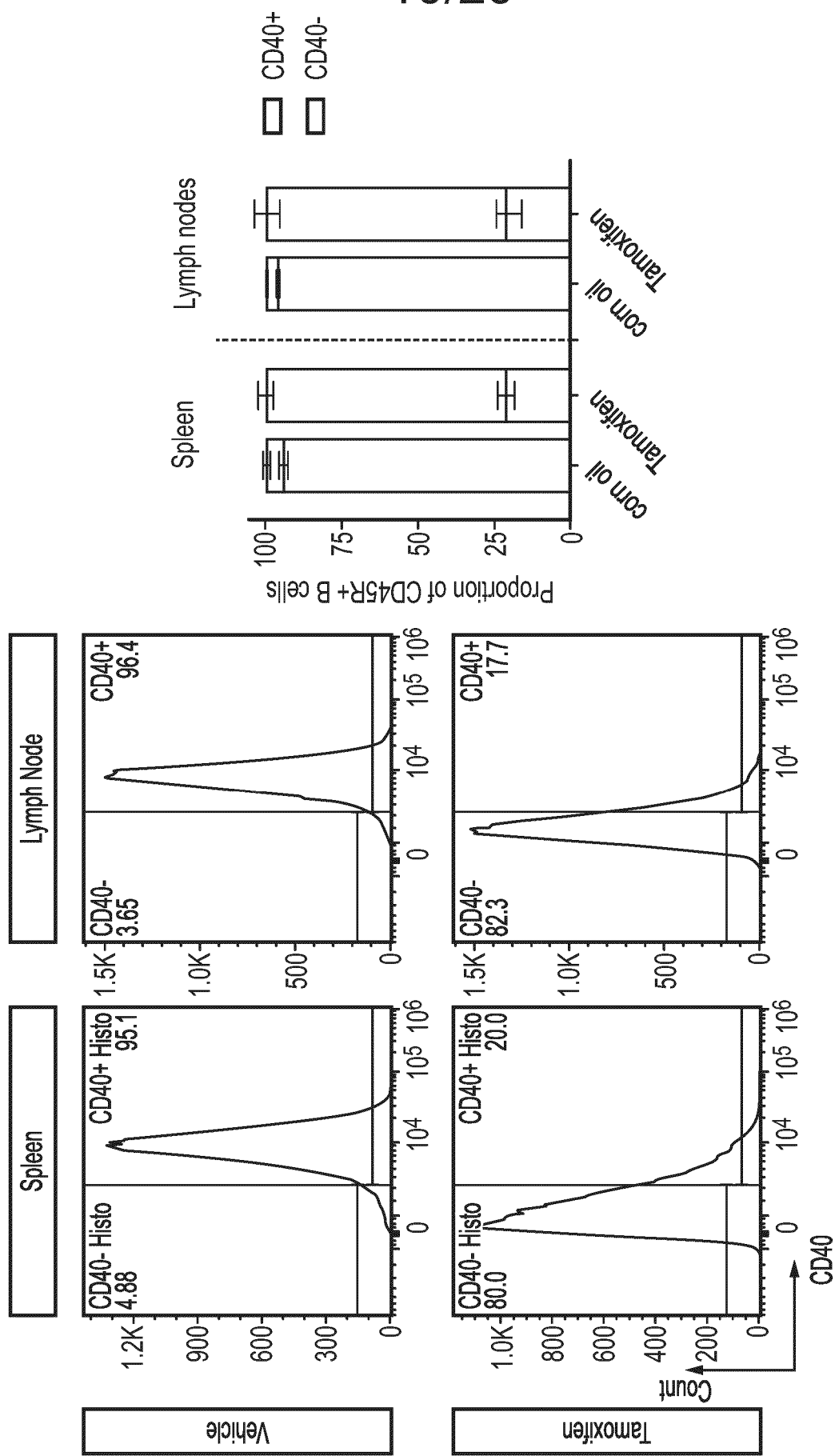


Figure 8

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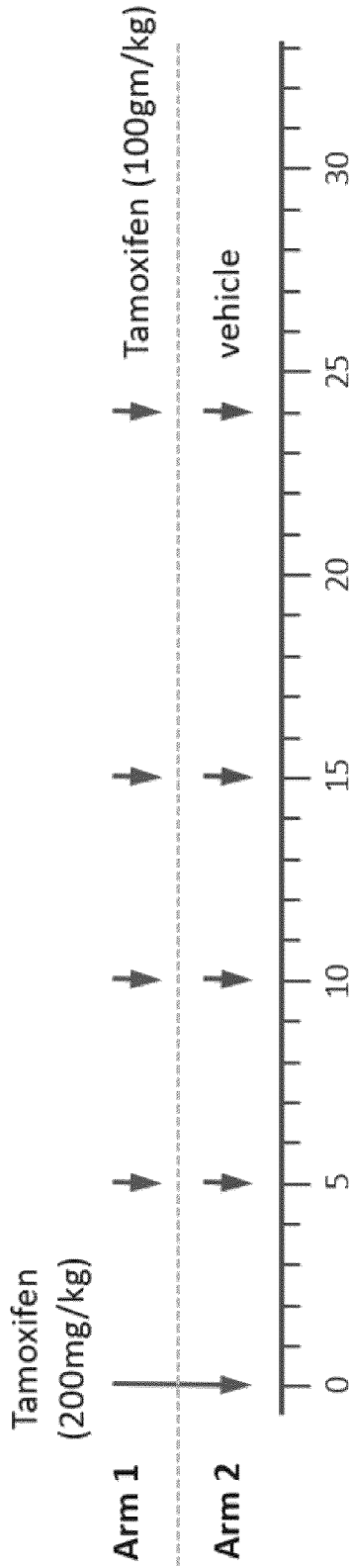


Figure 9A

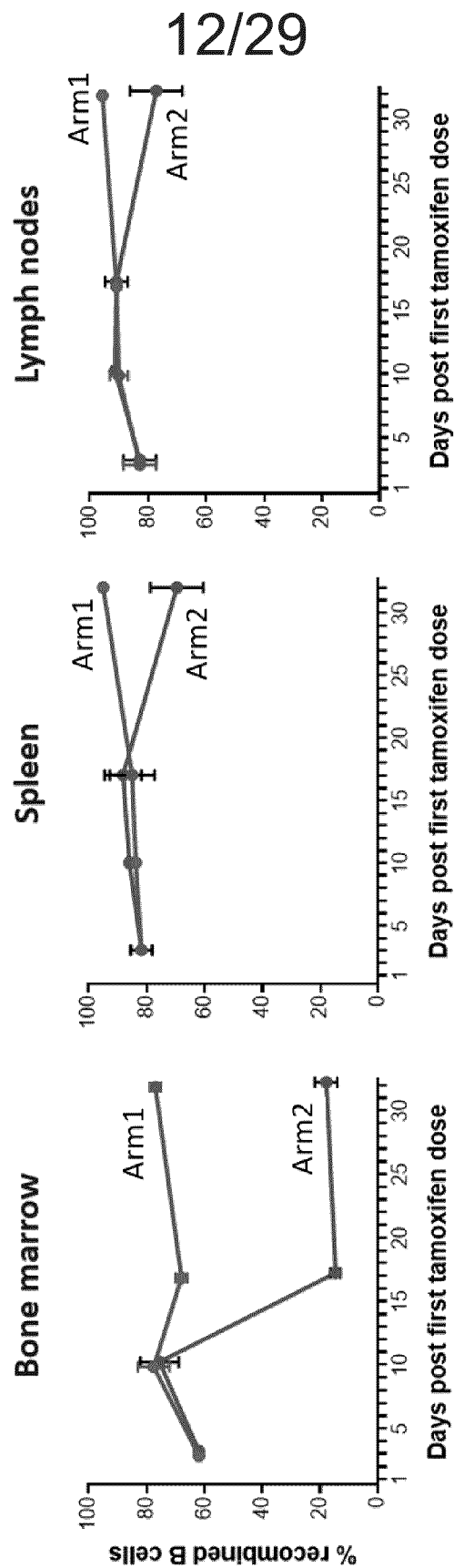


Figure 9B

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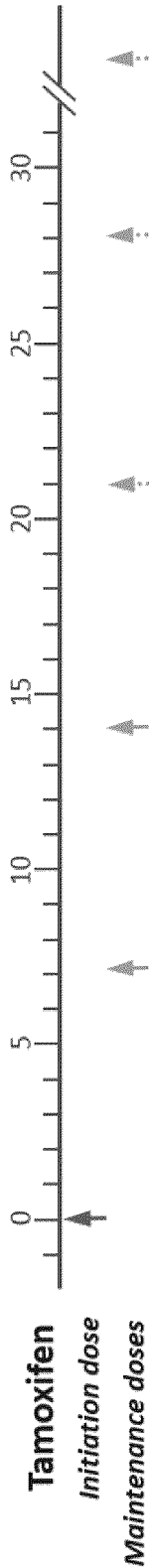


Figure 10

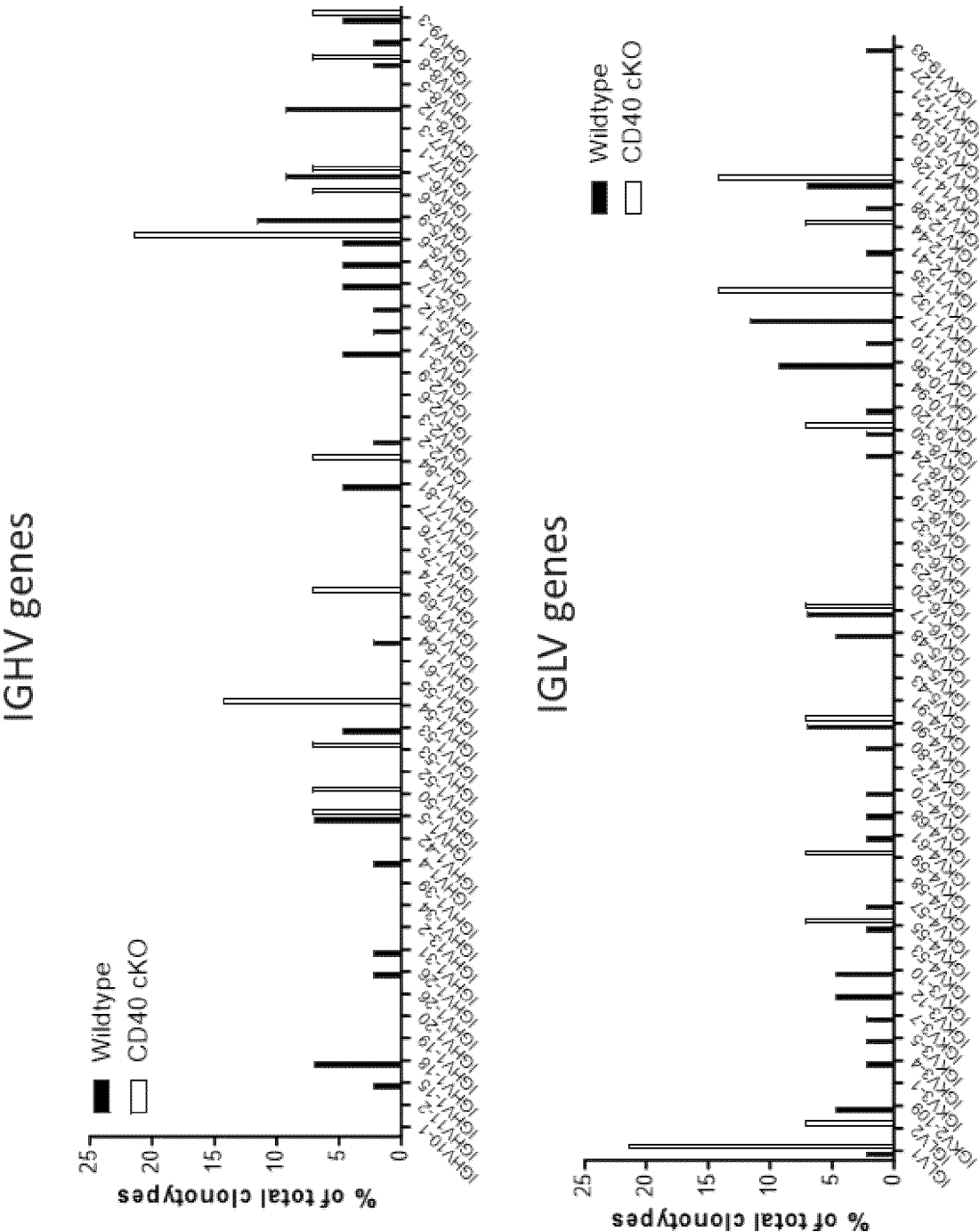


Figure 11

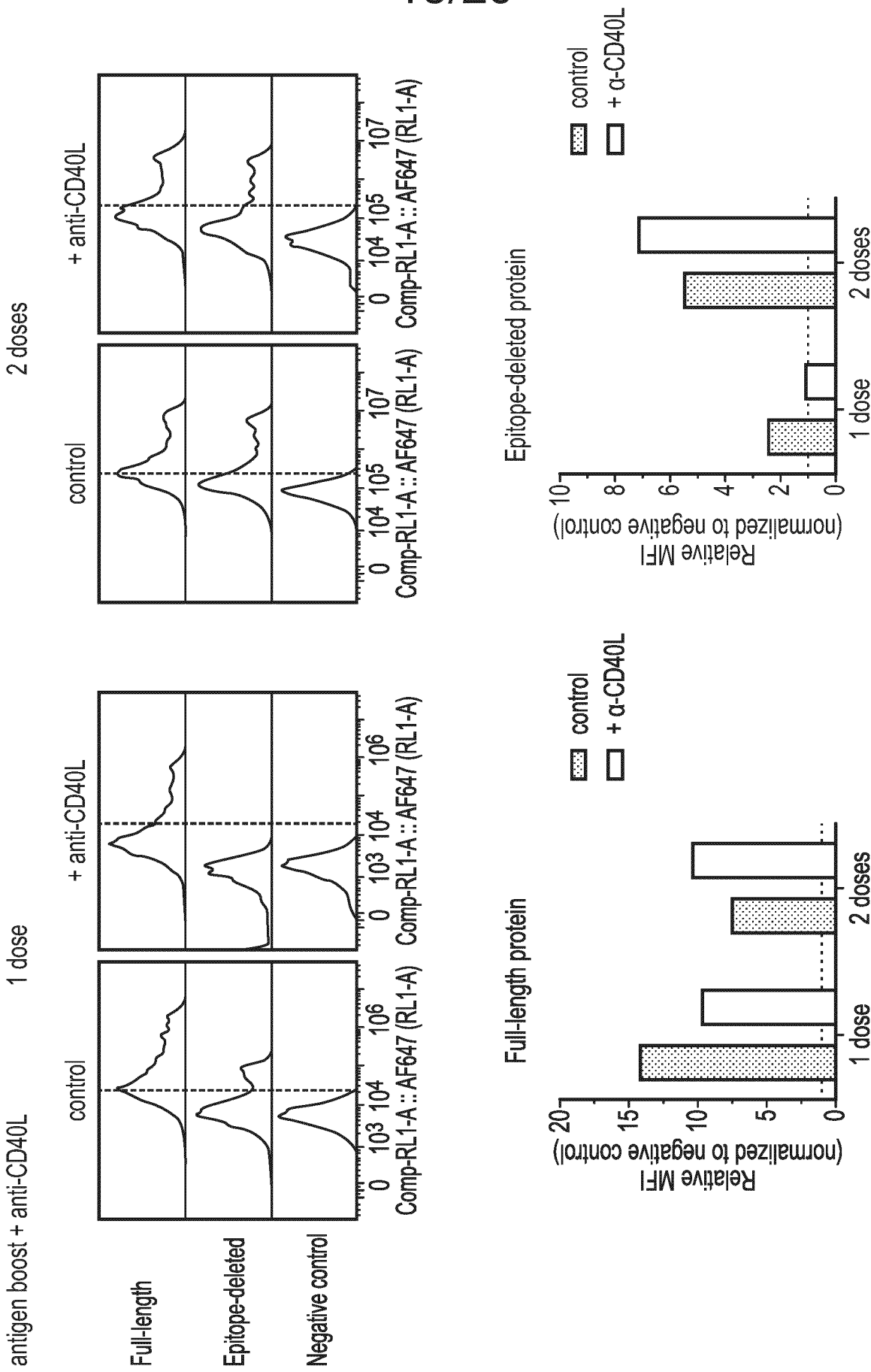


Figure 12

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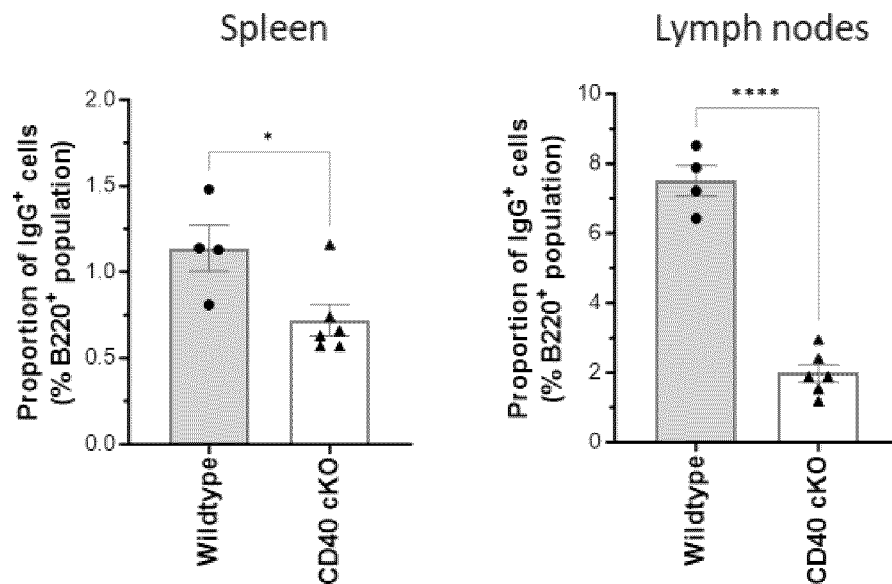


Figure 13A

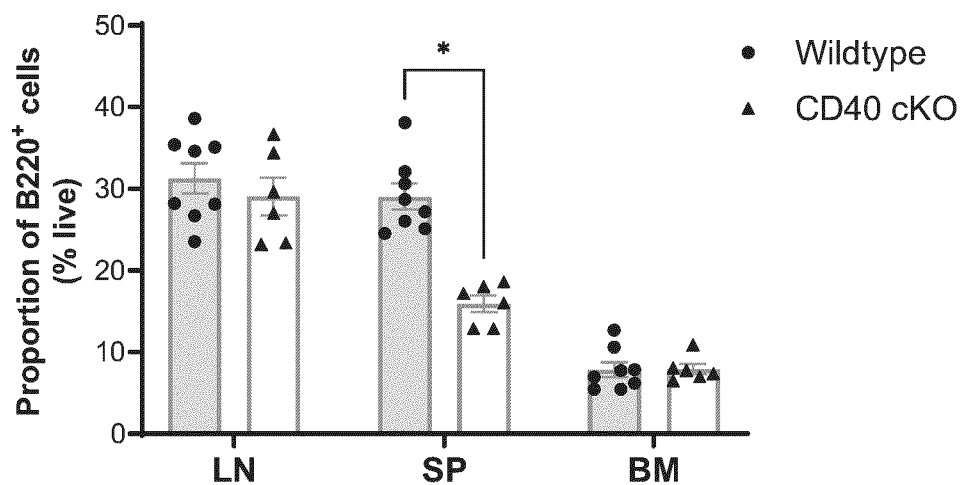


Figure 13B

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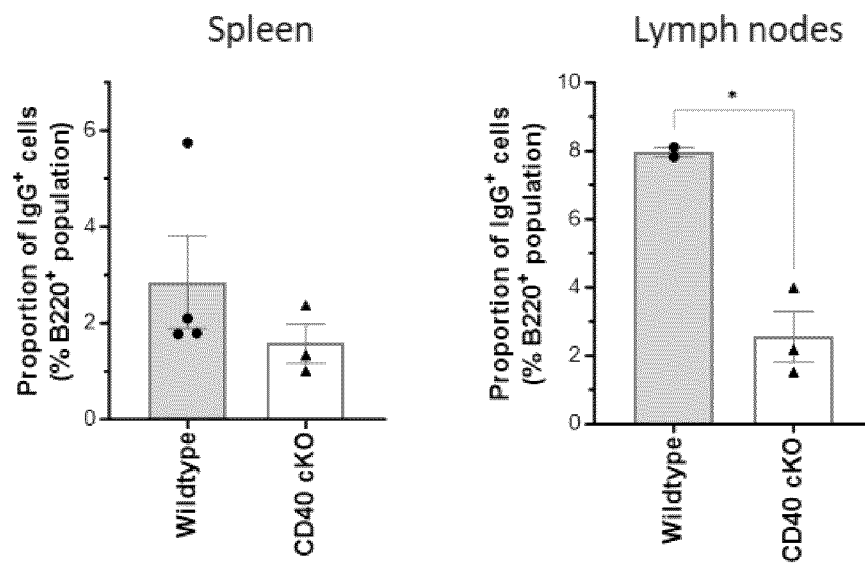


Figure 13C

18/29

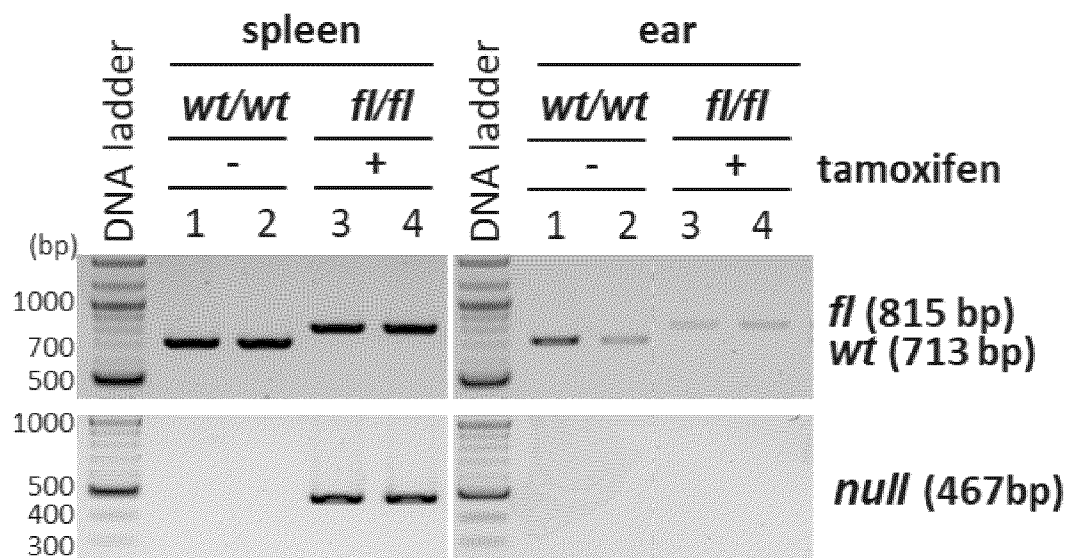


Figure 14A

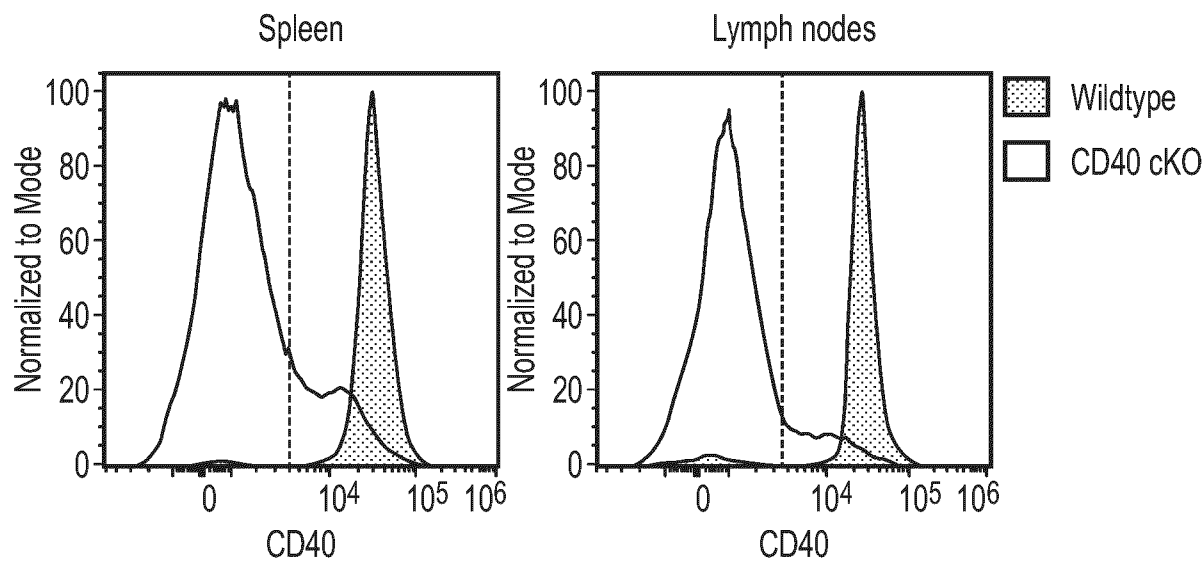


Figure 14B

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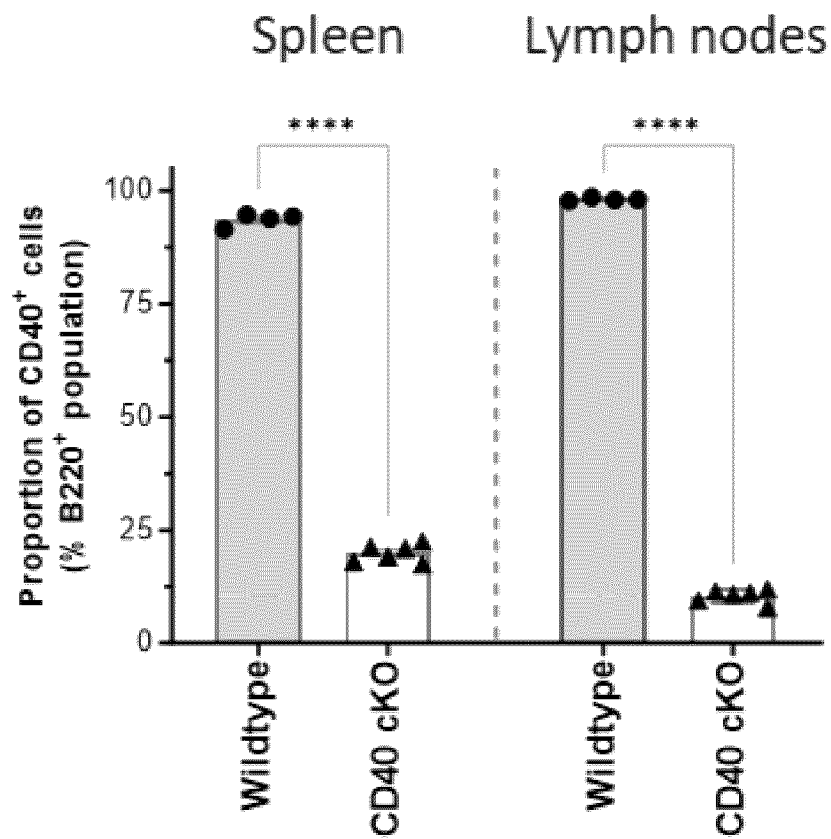


Figure 14C

20/29

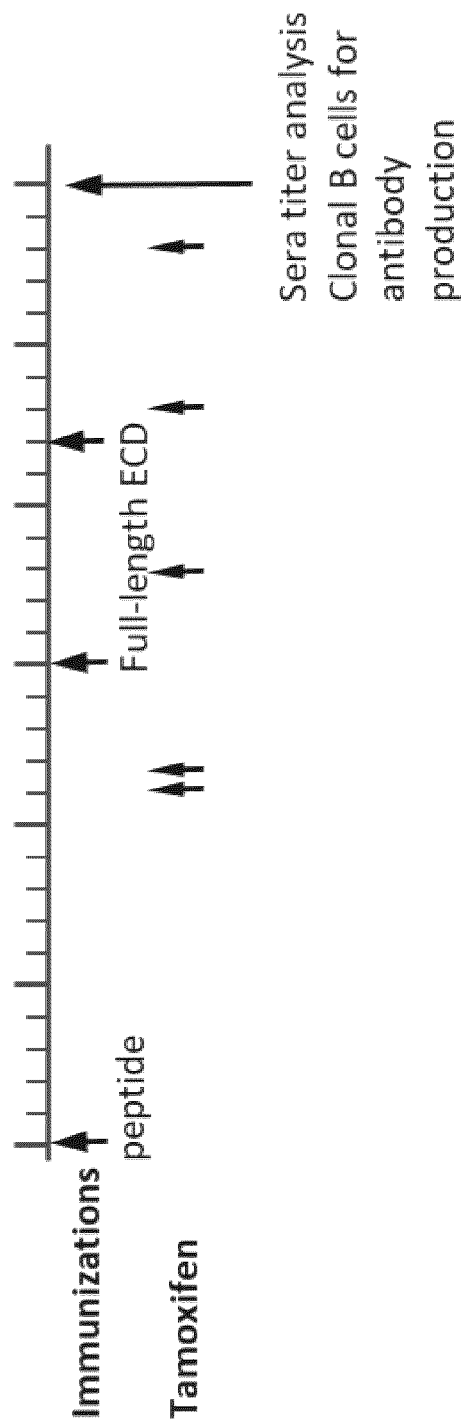


Figure 15

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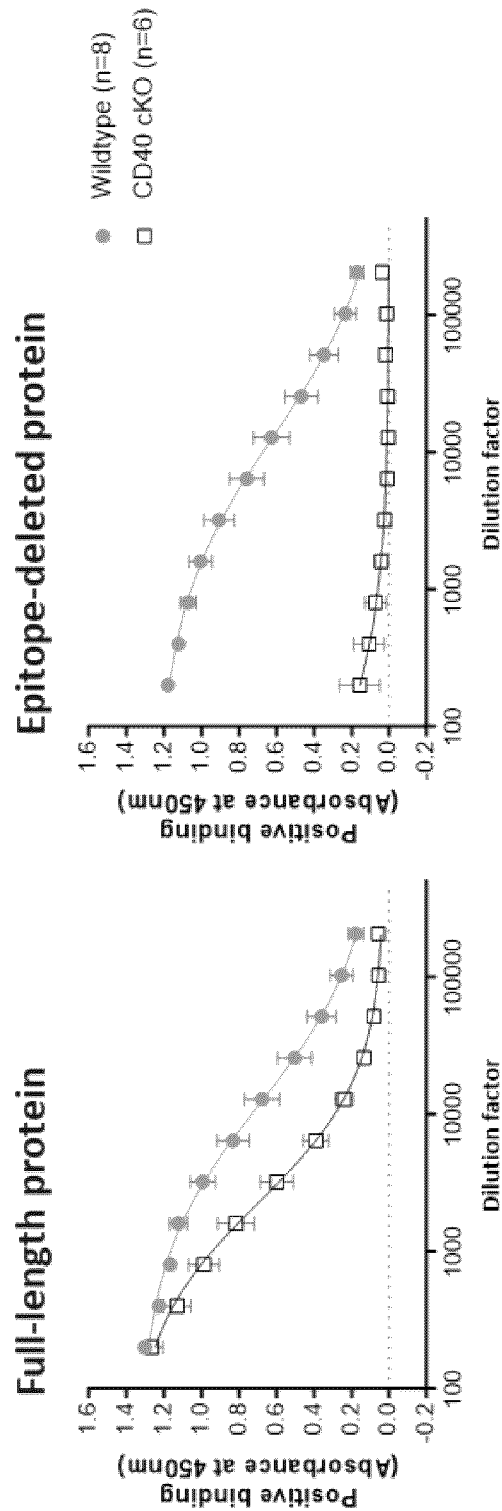


Figure 16A

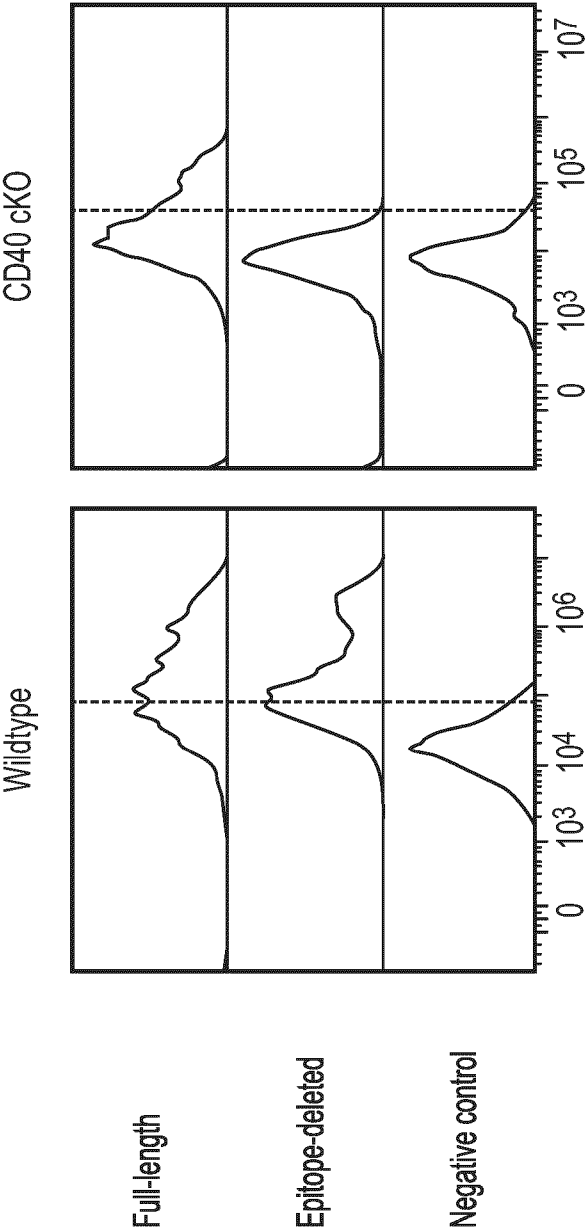


Figure 16B

23/29

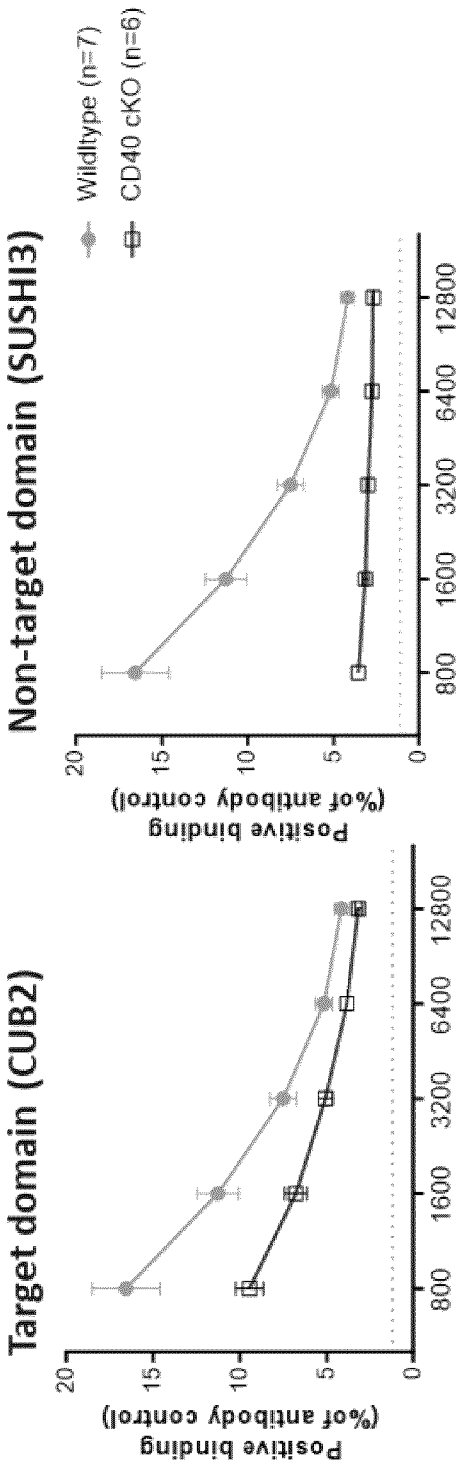


Figure 16C

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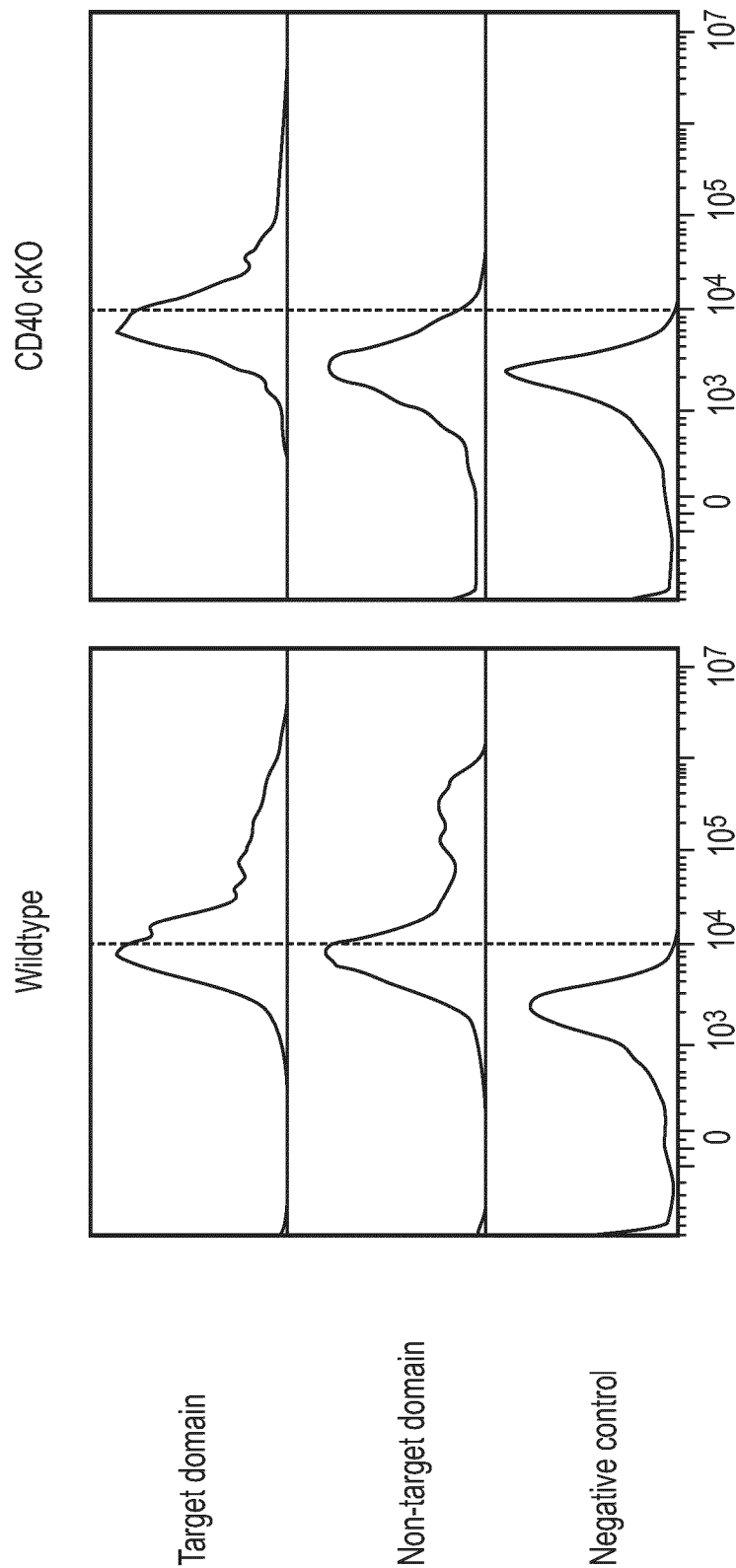


Figure 16D

25/29

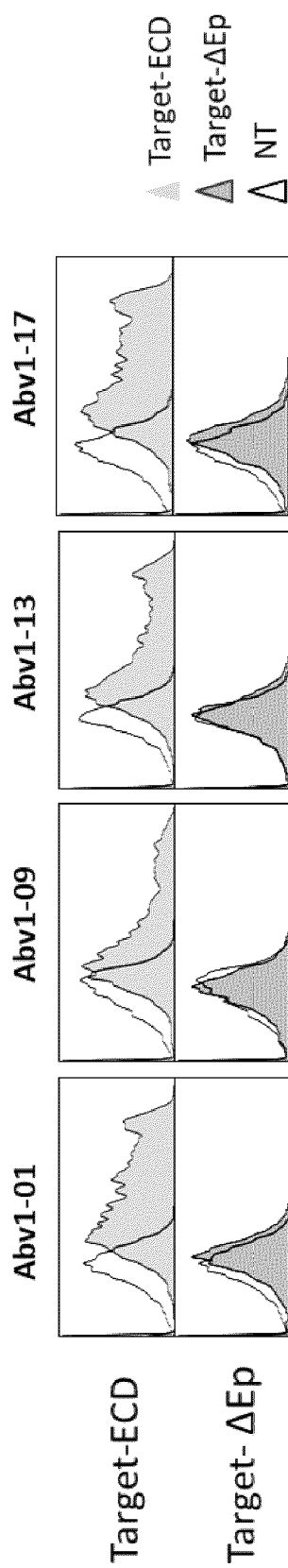


Figure 17

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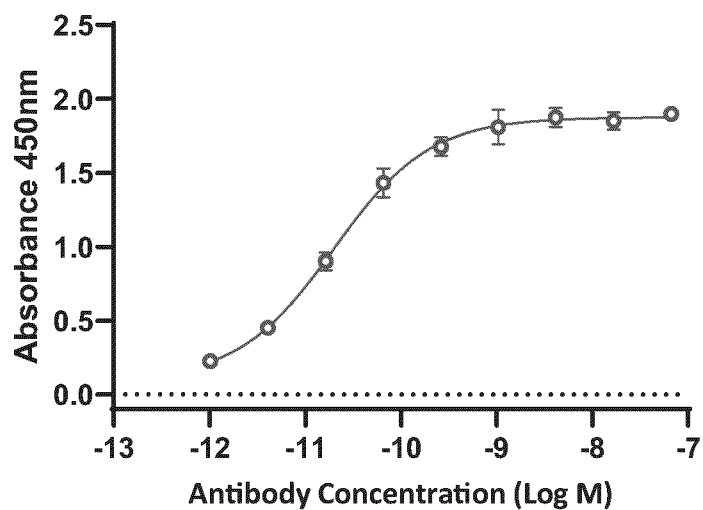
Abv1-01

Figure 18A

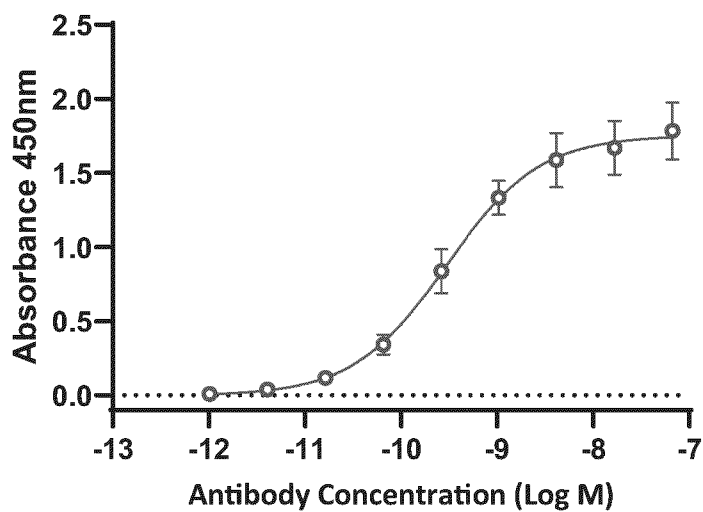
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Figure 18B

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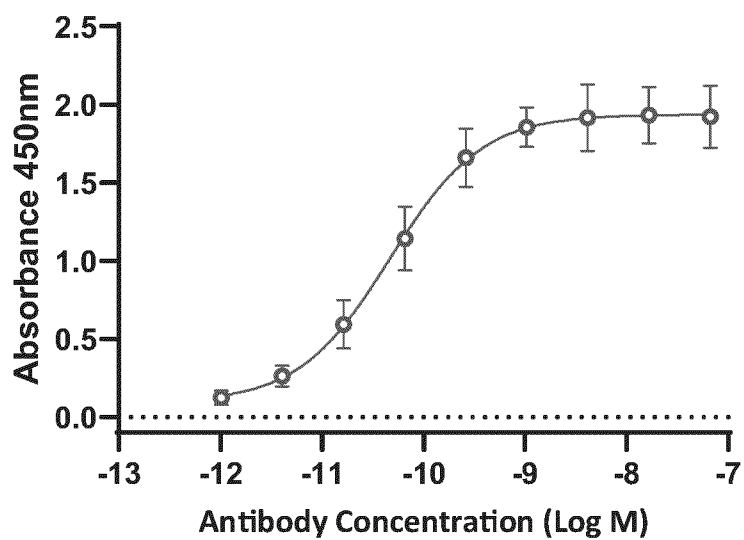
Abv1-13

Figure 18C

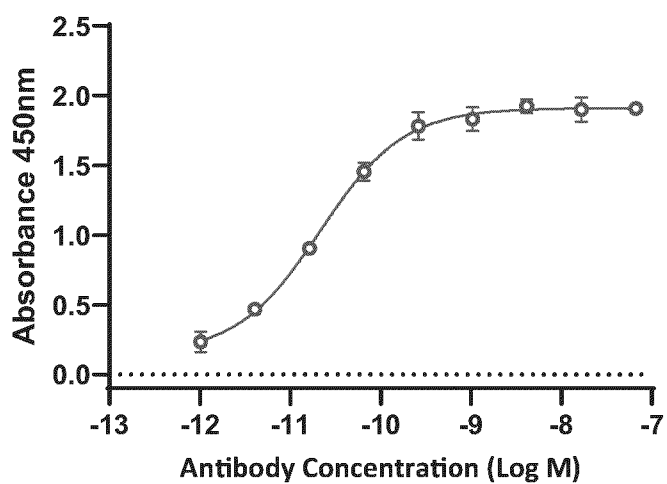
Abv1-17

Figure 18D

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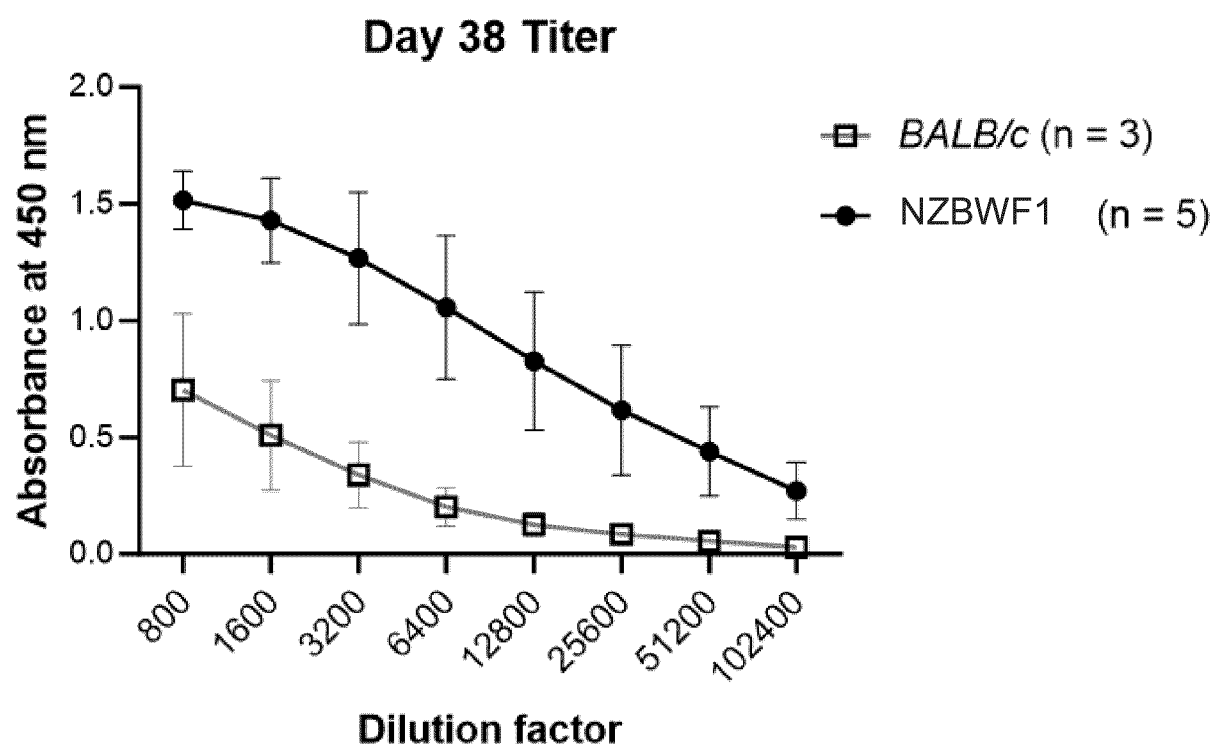


Figure 19

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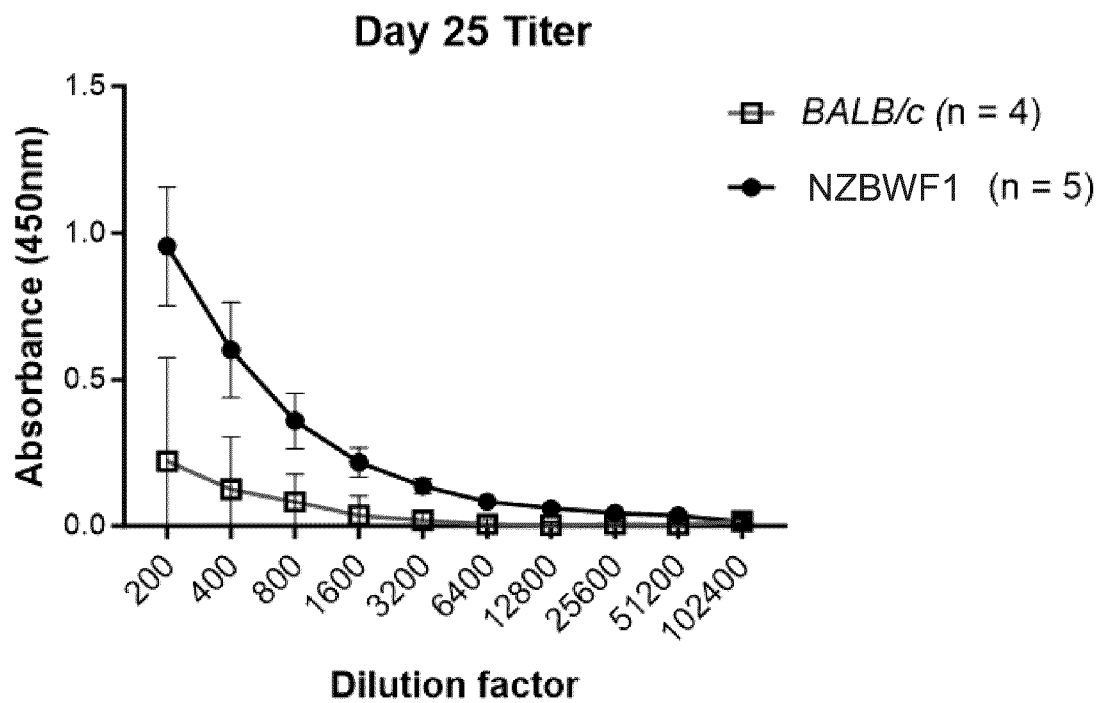


Figure 20

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/069941

A. CLASSIFICATION OF SUBJECT MATTER		
INV. A01K67/0275 A01K67/0276 A01K67/0278 C07K14/705 C07K16/00 C12N15/85		
ADD. According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) A01K C07K C12N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, CHEM ABS Data, Sequence Search, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LU YING ET AL: "CD40 Drives Central Nervous System Autoimmune Disease by Inducing Complementary Effector Programs via B Cells and Dendritic Cells", JOURNAL OF IMMUNOLOGY, vol. 209, no. 11, December 2022 (2022-12), pages 2083-2092, XP093213371, ISSN: 0022-1767, DOI: 10.4049/jimmunol.2200439 Retrieved from the Internet: URL:https://watermark.silverchair.com/ji2200439.pdf>	1-9,11, 12,14, 15, 35-43, 45,46, 48,49, 51-53
Y	page 2084 ----- -/-	16,47
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 10 October 2024		Date of mailing of the international search report 21/10/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Deleu, Laurent

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/069941

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HORRILLO ANGÉLICA ET AL: "Generation of mice with conditional ablation of the Cd40lg gene: new insights on the role of CD40L", TRANSGENIC RESEARCH, vol. 23, no. 1, 13 September 2013 (2013-09-13), pages 53-66, XP093213322, NL ISSN: 0962-8819, DOI: 10.1007/s11248-013-9743-2 Retrieved from the Internet: URL:http://link.springer.com/article/10.1007/s11248-013-9743-2/fulltext.html> page 54 - page 55 -----	1-9,11, 12,14, 35-43, 45,48, 49,51-53
Y	Donocoff Rachel S ET AL: "Optimization of tamoxifen-induced Cre activity and its effect on immune cell populations", Scientific reports, 17 September 2020 (2020-09-17), page 15244, XP055944631, England DOI: 10.1038/s41598-020-72179-0 Retrieved from the Internet: URL:https://www.nature.com/articles/s41598-020-72179-0.pdf [retrieved on 2022-07-20] abstract -----	16,47
Y	CHAKRABORTY ARUP K ET AL: "Variant proteins stimulate more IgM+ GC B-cells revealing a mechanism of crossreactive recognition by antibody memory", ELIFE, May 2018 (2018-05), pages 1-14, XP093212776, DOI: 10.7554/eLife.26832.001 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5959717/pdf/elif-26832.pdf> page 2 - page 7 ----- -/-	10,13, 17-34, 44,50, 54,55

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/069941

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SUNDLING CHRISTOPHER ET AL: "Positive selection of IgG+ over IgM+ B cells in the germinal center reaction", IMMUNITY, vol. 54, no. 5, May 2021 (2021-05), pages 988-1001.e5, XP093212782, AMSTERDAM, NL ISSN: 1074-7613, DOI: 10.1016/j.immuni.2021.03.013 Retrieved from the Internet: URL:https://www.cell.com/action/showPdf?pii=S1074-7613(21)00125-4> figure 1 -----	10,13, 17-34, 44,50, 54,55
Y	RANDALL TROY D ET AL: "Arrest of B Lymphocyte Terminal Differentiation by CD40 Signaling: Mechanism for Lack of Antibody-Secreting Cells in Germinal Centers", IMMUNITY, vol. 8, June 1998 (1998-06), pages 733-742, XP093213355, Retrieved from the Internet: URL:https://pdf.sciencedirectassets.com/272197/1-s2.0-S1074761300X00398/1-s2.0-S1074761300805786/main.pdf> page 739 - page 740 -----	10,13, 17-34, 44,50, 54,55
Y	CALLARD ROBIN E ET AL: "CD40 cross-linking inhibits specific antibody production by human B cells", INTERNATIONAL IMMUNOLOGY JUN 2010, vol. 7, no. 11, November 1995 (1995-11), pages 1809-1815, XP093213347, ISSN: 1460-2377, DOI: 10.1093/intimm/7.11.1809 page 1811 - page 1814 -----	10,13, 17-34, 44,50, 54,55
Y	LONBERG NILS: "Human antibodies from transgenic animals", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP US, NEW YORK, vol. 23, no. 9, September 2005 (2005-09), pages 1117-1125, XP002442149, ISSN: 1087-0156, DOI: 10.1038/NBT1135 page 1119 - page 1120 ----- -/-	10,30,50

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/069941

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HUSE KANUTTE ET AL: "Mechanism of CD79A and CD79B Support for IgM+ B Cell Fitness through B Cell Receptor Surface Expression", JOURNAL OF IMMUNOLOGY, vol. 209, no. 10, 15 November 2022 (2022-11-15), pages 2042-2053, XP093212983, ISSN: 0022-1767, DOI: 10.4049/jimmunol.2200144 Retrieved from the Internet: URL:https://watermark.silverchair.com/ji2200144.pdf> page 2042 - page 2043 -----	15,16, 46,47
A	RAUL ELGUETA ET AL: "Molecular mechanism and function of CD40/CD40L engagement in the immune system", IMMUNOLOGICAL REVIEWS, vol. 229, no. 1, May 2009 (2009-05), pages 152-172, XP055425214, US ISSN: 0105-2896, DOI: 10.1111/j.1600-065X.2009.00782.x page 158 - page 160 -----	1-55
A	SUZUKI MOTOHIKO ET AL: "A novel allergen-specific therapy with CD40-silenced B cells and dendritic cells", JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 142, no. 6, 23 August 2018 (2018-08-23), page 1994, XP085549843, ISSN: 0091-6749, DOI: 10.1016/J.JACI.2018.07.031 page 1994 -----	21-29
A	WO 2005/112620 A2 (MASSACHUSETTS INST TECHNOLOGY [US]; VENTURA ANDREA [US] ET AL.) 1 December 2005 (2005-12-01) paragraph [0122]; claims 1-6, 12 -----	21-29

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2024/069941

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☐ forming part of the international application as filed.
 - b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13~~ter~~.1(a)).
☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2024/069941

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2005112620 A2	01-12-2005	US 2005289659 A1	29-12-2005
		WO 2005112620 A2	01-12-2005
