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**B cells for in vivo delivery of therapeutic agents and dosages thereof**

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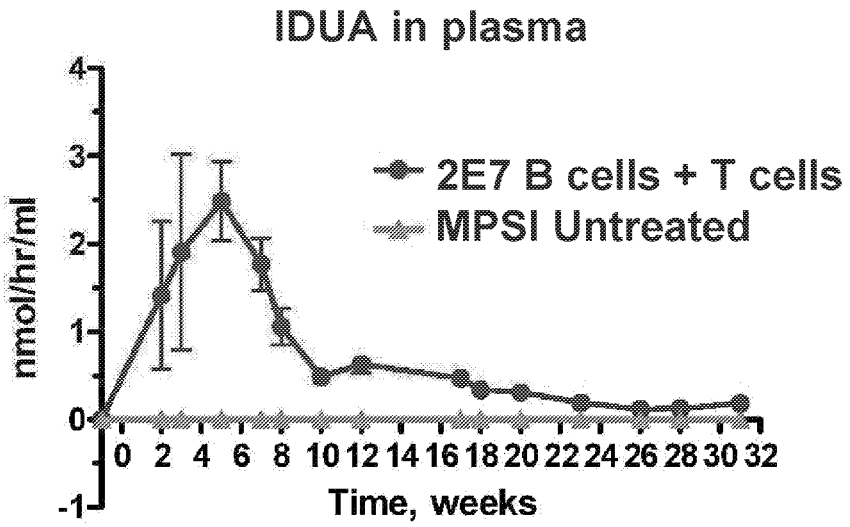
(54) Title: B CELLS FOR *IN VIVO* DELIVERY OF THERAPEUTIC AGENTS AND DOSAGES THEREOF

Figure 10




(57) Abstract: The present invention relates to methods for administering autologous and/or allogeneic B cells genetically modified to produce a therapeutic agent, such as a therapeutic protein. Specifically disclosed are methods for administering a single, maximally effective dose of genetically modified B cells and for administering multiple doses of genetically modified B cells. The compositions and methods disclosed herein are useful for the long-term, in vivo delivery of a therapeutic agent.

*[Continued on next page]*

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# B CELLS FOR *IN VIVO* DELIVERY OF THERAPEUTIC AGENTS AND DOSAGES THEREOF

## CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/491,151, filed on April 27, 2017, which application is incorporated by reference herein in its entirety.

## STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is IMCO-006_01WO_ST25.txt. The text file is 10KB, was created on April 26, 2018, and is being submitted electronically via EFS-Web.

## BACKGROUND

### Technical Field

The present disclosure relates to the use of B cells for long term *in vivo* delivery of a therapeutic agent, such as an antigen-specific antibody or protein (*e.g.*, an enzyme), and in particular to administering single and multiple dosages of the B cells.

### Description of the Related Art

Current methods for treating chronic diseases and disorders include direct infusion of a therapeutic agent (e.g., enzyme replacement therapy), gene therapy via a viral vector, and adoptive transfer of stem cells (e.g., hematopoietic stem cell transfer). However, each of these methods have disadvantages. Injection of a recombinant therapeutic protein suffers from the finite half-life of the protein, and all three methods provide sub-optimal tissue penetration by the therapeutic agent. Altering endogenous tissues to produce a therapeutic agent, such as via injection of recombinant adeno-associated virus (AAV) and lentiviral vectors, generally results in the therapeutic agent being produced from a centralized location. Production of the therapeutic agent from one

location increases the chances for localized toxicity in the producing tissues. Additionally, as recombinant viruses are viewed as foreign, it is unlikely viral vectors can be administered multiple times without causing an adverse reaction, meaning that there is a single injection opportunity to achieve the correct dosage of the therapeutic agent. Given the biological variation inherent in a procedure such as *in vivo* introduction of nucleic acids into cells using a virus, it would be very tenuous to achieve a desired dosage under the constraints of a single injection.

Recently, the use of differentiated B cell compositions for long term *in vivo* expression of a transgene has been identified as a promising strategy for the treatment of various diseases and disorders. However, methods for administering modified B cells for delivery of therapeutic agents have not yet been described in order to achieve therapeutically effective levels of the agents *in vivo*.

Accordingly, there still remains a need in the art for the long-term treatment for many chronic diseases and disorders. The present disclosure provides methods for administering and dosing genetically modified B cell compositions for treating chronic diseases and disorders. The present disclosure provides these and other advantages as described in the detailed description.

## SUMMARY OF THE INVENTION

One aspect of the present invention provides a method for administering genetically modified B cells to a subject for *in vivo* production of a therapeutic agent comprising administering two or more sequential doses of genetically modified B cells to a subject.

One aspect of the invention provides a method for delivering a therapeutic agent to multiple tissues *in vivo* comprising administering two or more doses of genetically modified B cells to a subject.

One aspect of the invention provides a method for treating MPS I comprising administering two or more sequential doses of B cells genetically modified to produce IDUA to a subject with MPS I.

One aspect of the invention provides a method for reducing an amount of glycosaminoglycan (GAG) in a subject with MPS I comprising administering two or more sequential doses of B cells genetically modified to produce IDUA to the subject.

One aspect of the invention provides a method for delivering a therapeutic agent to one or more tissues *in vivo* comprising administering one or more doses of genetically modified B cells to a subject, wherein the genetically modified B cells are migratory.

One aspect of the invention provides a method of administering genetically modified B cells to a subject to enable synergistic *in vivo* production of a therapeutic agent comprising: determining an optimal single-dose concentration of the modified B cells for inducing the greatest *in vivo* production of the therapeutic agent; decreasing the optimal single-dose concentration of the modified B cells to obtain a sub-optimal single-dose concentration of the modified B cells; and administering two or more doses of the sub-optimal single-dose concentration of the modified B cells to the subject.

One aspect of the invention provides a genetically modified B cell that has been engineered to produce a therapeutic agent. In some embodiments, the therapeutic agent is IDUA. In some embodiments, the therapeutic agent is FIX, LPL, or LCAT.

One aspect of the invention provides a composition comprising a population of genetically modified B cells that have been engineered to produce a therapeutic agent, wherein the genetically modified B cells are at optimal migratory capacity. In some embodiments, the therapeutic agent is IDUA. In some embodiments, the therapeutic agent is FIX, LPL, or LCAT.

One aspect of the invention provides a composition comprising a population of genetically modified B cells that have been engineered to produce a therapeutic agent, wherein the genetically modified B cells in the composition are harvested from culture at a time-point when do not produce significant amounts inflammatory cytokines. In some embodiments, the therapeutic agent is IDUA. In some embodiments, the therapeutic agent is FIX, LPL, or LCAT.

One aspect of the invention provides a method of administering genetically modified B cells to a subject for *in vivo* production of a therapeutic agent comprising administering an optimal single dose of genetically modified B cells to a subject. In some embodiments, the therapeutic agent is IDUA. In some embodiments, the therapeutic agent is FIX, LPL, or LCAT.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of *Sleeping Beauty* (SB) transposon and transposase constructs/map for transposition and expression of human IDUA. IDUA is regulated by the EEK promoter (see Example 1). A bidirectional promoter that incorporates an EF1a promoter upstream of EEK regulates transcription of a drug resistant human L22Y-F31S dihydrofolate reductase (DHFR) in the opposite direction. A CMV-regulated SB100x provides SB transposase activity. Capped and polyadenylated SB100x-encoding mRNA was generated by *in vitro* transcription, provided from TriLink. Arrows: direction of transcription. Green boxes with dark triangles are T2 SB inverted repeat / direct repeat (IR/DR) sequences. pA, polyadenylation signal. FIG. 1A shows the construct designs. FIG. 1B shows the plasmid map for the pKT2/EEK-IDUA-DHFR construct shown in FIG. 1A, which comprises human DHFR with L22Y; F31S mutations.

Figure 2 shows SB-mediated expression of human IDUA in primary human B cells. Human CD19+ primary B cells were cultured for 2 days, then electroporated with pKT2/EEK-IDUA and pCMV-SB100x as a transposase source using the Lonza 4D system. Cell lysate on day 8 post electroporation was assayed for IDUA enzyme activity.

Figure 3 is a series of histograms that show MTX selective enrichment of IDUA+ cells during large-scale B cell expansion. B cells from two separate donors (19009 and 2764) were electroporated with pKT2/EEK-IDUA-DHFR transposon, incubated in medium with (bottom two histograms) or without MTX (top two histograms) from days 2-4, and then further expanded for a total of 7 days. Cells from each population were collected on day 7 and assayed for the % IDUA positive cells by intracellular staining for human IDUA followed by flow cytometry (cell count / IDUA).

Figure 4 shows iduronidase expression in NSG mice infused with IDUA-DHFR transposed B cells. NSG IDUA+ mice were infused i.p. with CD4+ T cells at days -30 and -4 and then on day 0 infused via either i.p. or i.v. injection with $10^7$ pKT2/EEK-IDUA-DHFR B cells that had been selected in MTX. As a control some mice where infused via i.p. injection with B cells expressing GFP. Plasma samples were assayed for IDUA at the indicated time points. Mice 1 through 8 received i.p. infusions of IDUA expressing B cells. Mice 9, 10, 12 and 42 received i.v. infusions of IDUA expressing B cells. Mice 43 and 48 received i.p. infusions of GFP expressing B cells.

Figure 5 shows the amount of iduronidase (IDUA) present in the plasma using a mouse model of MPS I. Mice received, from top to bottom of the key, 3 x $10^6$ B cells transduced with IDUA (IDUA+ B cells) in the presence of CD4+ memory T cells, 1 x $10^7$ IDUA+ B cells in the presence of CD4+ memory T cells, 3 x $10^7$ IDUA+ B cells in the presence of CD4+ memory T cells, CD4+ memory T cells only, or no cells on day 0 and IDUA enzyme activity levels were measured in serum through day 38 post administration.

Figure 6 shows the amount of IDUA present in the plasma in a mouse model of MPS I with multiple doses of B cells transduced with IDUA (IDUA+ B cells). Human B cells were CD19-enriched from apheresis product of a normal donor and electroporated with pKT2/EEK-IDUA transposon plus SB100x-encoding mRNA during the expansion process. CD4+ T cells were isolated from the same donor and infused into NSG MPS I animals intraperitoneally (i.p) one week prior to infusion of IDUA transposed B cells. Control groups included untreated NSG MPS I mice ("No B Cells"), and NSG MPS I mice infused with IDUA+ B cells i.v. only (i.e. no CD4+ T cells). NSG MPS I mice pre-treated with autologous CD4+ T cells were subsequently infused with IDUA+ B cells either i.v. or i.p. on Days 0, 21, and 42 (arrows). IDUA enzyme activity levels were measured in serum through day 56. N = 4.

Figure 7 shows plasma IgG from the same NSG MPS I mice that are described in Figure 6. N = 4.

Figure 8 shows IDUA activity in various tissues from MPS I mice. MPS I mice were given three dosages of 1 x $10^7$ B cells engineered to produce IDUA (or no cells as a control) on days 0, 21, and 42 in the presence of CD4+ T cells (or no cells as a control), and IDUA enzyme activity levels were measured in the indicated organs on day 60 post the first B cell infusion. N = 4.

Figure 9 shows the amount of glycosaminoglycans (GAGs) in various tissues from MPS I mice. MPS I mice were given three dosages of 1 x $10^7$ B cells engineered to produce IDUA (or no cells as a control) on days 0, 21, and 42 in the presence of CD4+ T cells (or no cells as a control) on day 0, and GAG levels were measured in the indicated organs on day 60 post the first B cell infusion. Additionally, the red horizontal bars indicate the average IDUA enzyme activity in plasma for each of the groups of mice. N = 4.

Figure 10 shows that IDUA activity is detectable long-term in plasma from MPSI NSG mice that were treated with two doses of 2 x $10^7$ B cells engineered to produce IDUA. The first dosage of B cells were given one week after administration of CD4+ T cells and the second dosage of B cells were administered 30 days after the first B cell dosage. (IDUA+ B cells). The color coded key on the right indicates the mouse groups. The X-axis indicates the time in weeks. The Y-axis indicates the amount of IDUA enzyme activity detected in mouse plasma samples.

Figure 11 shows the amount of IDUA activity present in multiple tissues in MPSI NSG mice treated with two doses of 2 x $10^7$ B cells engineered to produce IDUA according to the same protocol as in Figure 10. The color coded key on the right indicates the mouse groups and time points. The X-axis lists the tissue being surveyed and the Y-axis lists the levels of IDUA enzymatic activity that were detected.

Figure 12 shows the amount of glycosaminoglycans (GAGs) in various tissues from MPS I NSG mice treated with two doses of 2 x $10^7$ B cells engineered to produce IDUA according to the same protocol as in Figures 10 and 11. Treatment with the B cell product results in long-term reductions in the levels of GAGs in multiple tissues. The color coded key on the right indicates the organ that the GAGs were assessed in. The X-axis indicates the mouse group and cell dosage. The Y-axis indicates the amount of GAGs detected.

Figure 13 shows the migration of B cells engineered to express IDUA toward a chemoattractant in a two-chamber Transwell assay. FIG. 13A shows day 0 migration of engineered B cells towards the chemoattractant CXCL12. FIG. 13B shows migration of engineered B cells towards the chemoattractant CXCL12 after 4, 5, 6, 7, 8, or 9 days in culture after engineering. FIG. 13C shows migration of engineered B cells towards the chemoattractant CXCL13 after 4, 5, 6, 7, 8, or 9 days in culture after engineering. For both FIG. 13B and 13C, please note that the no-chemokine control was only implemented for the day 4 timepoint. FIG. 13D shows a schematic diagram of the Transwell assay utilized to generate the data in FIGS 13A-13C.

Figure 14 shows summary data of deep sequencing analysis of clonality of B cells engineered to express IDUA.

Figure 15 shows Luminex analysis of inflammatory cytokine production by B cells engineered to produce IDUA. FIG. 15A shows IL6, IFN alpha, and IFN gamma production on day 2 (D2), day 7 (D7) and day 0 (D0) base medium with and without IL6

(50ng/ml). FIG. 15B shows sFAS, TNFRp75, BAFF, HGF, and IL5 production on D2, D7, and D0 base medium.

Figure 16 shows expression of human LCAT (lecithin-cholesterol acyltransferase), human LPL (Lipoprotein Lipase), and human FIX (coagulation factor IX) in human B cells engineered according to the present invention. FIG. 16A shows LCAT activity in engineered plasmablasts / plasma cells. FIG. 16B shows LPL activity in engineered plasmablasts / plasma cells. FIG. 16C shows FIX protein expression by ELISA in engineered primary B cells.

## DETAILED DESCRIPTION

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of molecular biology, recombinant DNA techniques, protein expression, and protein / peptide / carbohydrate chemistry within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, e.g., Sambrook, et al., Molecular Cloning: A Laboratory Manual (3rd Edition, 2000); DNA Cloning: A Practical Approach, vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed., 1984); Oligonucleotide Synthesis: Methods and Applications (P. Herdewijn, ed., 2004); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., 1985); Nucleic Acid Hybridization: Modern Applications (Buzdin and Lukyanov, eds., 2009); Transcription and Translation (B. Hames & S. Higgins, eds., 1984); Animal Cell Culture (R. Freshney, ed., 1986); Freshney, R.I. (2005) Culture of Animal Cells, a Manual of Basic Technique, 5th Ed. Hoboken NJ, John Wiley & Sons; B. Perbal, A Practical Guide to Molecular Cloning (3rd Edition 2010); Farrell, R., RNA Methodologies: A Laboratory Guide for Isolation and Characterization (3rd Edition 2005). The publications discussed above are provided solely for their disclosure before the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

### DEFINITIONS AND ABBREVIATIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. As used in the specification and appended claims, unless specified to the contrary, the following terms have the meaning indicated. With regard to

this specification, any time a definition of a term as defined herein, differs from a definition given for that same term in an incorporated reference, the definition explicitly defined herein is the correct definition of the term.

The words "a" and "an" denote one or more, unless specifically noted.

By "about" is meant a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by as much as 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1% to a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length. In any embodiment discussed in the context of a numerical value used in conjunction with the term "about," it is specifically contemplated that the term about can be omitted.

A "composition" can comprise an active agent and a carrier, inert or active, e.g., a pharmaceutically acceptable carrier, diluent or excipient. In particular embodiments, the compositions are sterile, substantially free of endotoxins or non-toxic to recipients at the dosage or concentration employed.

Unless the context requires otherwise, throughout the present specification and claims, the word "comprise" and variations thereof, such as, "comprises" and "comprising" are to be construed in an open and inclusive sense, that is, as "including, but not limited to".

By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

Reference throughout this specification to "biological activity" or "bioactivity" refers to any response induced in an *in vitro* assay or in a cell, tissue, organ, or organism, (e.g., an animal, or a mammal, or a human) as the result of administering any compound, agent, polypeptide, conjugate, pharmaceutical composition contemplated herein. Biological activity may refer to agonistic actions or antagonistic actions. The biological activity may be a beneficial effect; or the biological activity may not be

beneficial, i.e. a toxicity. In some embodiments, biological activity will refer to the positive or negative effects that a drug or pharmaceutical composition has on a living subject, e.g., a mammal such as a human. Accordingly, the term "biologically active" is meant to describe any compound possessing biological activity, as herein described. Biological activity may be assessed by any appropriate means currently known to the skilled artisan. Such assays may be qualitative or quantitative. The skilled artisan will readily appreciate the need to employ different assays to assess the activity of different polypeptides; a task that is routine for the average researcher. Such assays are often easily implemented in a laboratory setting with little optimization requirements, and more often than not, commercial kits are available that provide simple, reliable, and reproducible readouts of biological activity for a wide range of polypeptides using various technologies common to most labs. When no such kits are available, ordinarily skilled researchers can easily design and optimize in-house bioactivity assays for target polypeptides without undue experimentation; as this is a routine aspect of the scientific process.

Reference to the term "e.g." is intended to mean "e.g., but not limited to" and thus it should be understood that whatever follows is merely an example of a particular embodiment, but should in no way be construed as being a limiting example. Unless otherwise indicated, use of "e.g." is intended to explicitly indicate that other embodiments have been contemplated and are encompassed by the present invention.

Reference throughout this specification to "embodiment" or "one embodiment" or "an embodiment" or "some embodiments" or "certain embodiments" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" or "in certain embodiments" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

An "increased" or "enhanced" amount is typically a "statistically significant" amount, and may include an increase that is 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, or 50 or more times (e.g., 100, 500, 1000 times) (including all integers and decimal points in between and above 1, e.g., 2.1, 2.2, 2.3, 2.4, etc.) an amount or level described herein. Similarly, a "decreased"

or "reduced" or "lesser" amount is typically a "statistically significant" amount, and may include a decrease that is about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6 1.7, 1.8, 1.9, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, or 50 or more times (e.g., 100, 500, 1000 times) (including all integers and decimal points in between and above 1, e.g., 1.5, 1.6, 1.7. 1.8, etc.) an amount or level described herein.

The terms "*in vitro*", "*ex vivo*", and "*in vivo*" are intended herein to have their normal scientific meanings. Accordingly, e.g., "*in vitro*" is meant to refer to experiments or reactions that occur with isolated cellular components, such as, e.g., an enzymatic reaction performed in a test tube using an appropriate substrate, enzyme, donor, and optionally buffers / cofactors. "*Ex vivo*" is meant to refer to experiments or reactions carried out using functional organs or cells that have been removed from or propagated independently of an organism. "*In vivo*" is meant to refer to experiments or reactions that occur within a living organism in its normal intact state.

"Mammal" includes humans and both domestic animals such as laboratory animals and household pets, (e.g., cats, dogs, swine, cattle, sheep, goats, horses, and rabbits), and non-domestic animals such as wildlife and the like.

"Optional" or "optionally" means that the subsequently described event, or circumstances, may or may not occur, and that the description includes instances where said event or circumstance occurs and instances in which it does not.

"Pharmaceutical composition" refers to a formulation of a compound (e.g. a therapeutically useful polypeptide) and a medium generally accepted in the art for the delivery of the compound to an animal, e.g., humans. Such a medium may include any pharmaceutically acceptable carriers, diluents or excipients therefore.

"Pharmaceutically effective excipients" and "pharmaceutically effective carriers" are well known to those of skill in the art, and methods for their preparation are also readily apparent to the skilled artisan. Such compositions, and methods for their preparation, may be found, e.g., in Remington's Pharmaceutical Sciences, 19th Edition (Mack Publishing Company, 1995, incorporated herein).

The terms "polynucleotide", "nucleotide", "nucleotide sequence", and "nucleic acid" are used interchangeably. They refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides, or analogs thereof. Polynucleotides may have any three dimensional structure, and may perform any function known or unknown. The following are non-limiting examples of polynucleotides: coding

or non-coding regions of a gene or gene fragment, loci (locus) defined from linkage analysis, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure may be imparted before or after assembly of the polymer. The sequence of nucleotides may include non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component.

A "subject," as used herein, includes any animal that exhibits a disease or symptom, or is at risk for exhibiting a disease or symptom, which can be treated with an agent of the invention. Suitable subjects include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients, are included.

"Substantially" or "essentially" means of ample or considerable amount, quantity, size; nearly totally or completely; for instance, 95% or greater of some given quantity.

"Therapeutic agent" refers to any compound that, when administered to a subject, (e.g., preferably a mammal, more preferably a human), in a therapeutically effective amount is capable of effecting treatment of a disease or condition as defined below.

"Therapeutically effective amount" or "Therapeutically effective dose" refers to an amount of a compound of the invention that, when administered to a subject, (e.g., preferably a mammal, more preferably a human), is sufficient to effect treatment, as defined below, of a disease or condition in the animal. The amount of a compound of the invention that constitutes a "therapeutically effective amount" will vary depending on the compound, the condition and its severity, the manner of administration, and the age of the animal to be treated, but can be determined routinely by one of ordinary skill in the art having regard to his own knowledge and to this disclosure.

"Treating" or "treatment" as used herein covers the treatment of the disease or condition of interest in a subject, preferably a human, having the disease or condition of interest, and includes: (i) preventing or inhibiting the disease or condition from occurring in a subject, in particular, when such subject is predisposed to the

condition but has not yet been diagnosed as having it; (ii) inhibiting the disease or condition, i.e., arresting its development; (iii) relieving the disease or condition, i.e., causing regression of the disease or condition; or (iv) relieving the symptoms resulting from the disease or condition. As used herein, the terms "disease," "disorder," and "condition" may be used interchangeably or may be different in that the particular malady, injury or condition may not have a known causative agent (so that etiology has not yet been worked out), and it is, therefore, not yet recognized as an injury or disease but only as an undesirable condition or syndrome, wherein a more or less specific set of symptoms have been identified by clinicians.

**OVERVIEW**

The present invention utilizes autologous and/or allogeneic B cells that have been altered through introduction of nucleic acids to produce a therapeutic agent and relates to methods of administering the modified B cells. In some embodiments, the terms "engineered B cell", "genetically engineered B cell", "modified B cell" and "genetically modified B cell" are used interchangeably herein to refer to such altered B cells that comprises one or more nucleic acids (e.g., a transgene) to produce a therapeutic agent (e.g., a transgene that enables expression of a polypeptide such as a therapeutic polypeptide). Specifically, the modified B cells can be administered as a single dosage or multiple dosages. Unexpectedly, it was found that certain B cell dosages produce greater than expected levels of therapeutic agent in comparison to other dosages. Additionally, it was surprisingly found that use of multiple dosages of B cells delivered over the course of the multi-dose regimen results in greater levels of therapeutic agent than is achieved by a single dosage containing the same number of cells. Additionally, it was surprisingly found that modified B cells have windows of optimal migratory capacity towards chemoattractants, and their migratory capacity may decline after certain time-periods in culture. Additionally, if was unexpectedly found that while the starting population of engineered B cells produced IL6, the levels of production declined to near background levels by the end of culture and most inflammatory cytokines tested were not produced by the engineered B cells. Moreover, it was shown that the final engineered B cell population was significantly polyclonal, as no particular B cell clone in the final population of engineered B cells was found to comprise more than about 0.2% of the total

B cell population. Finally, it was discovered that the modified B cells are able to effectively deliver drug to a wide range of tissues, such as lung, heart and intestine that are difficult to target using other modalities.

Accordingly, the methods for administering modified B cell compositions described herein are useful for long term *in vivo* delivery and expression of therapeutic agents. The present disclosure relates generally to methods for achieving sufficient enrichment and number of cells producing a therapeutic agent and sufficient levels of the therapeutic agent *in vivo* while ensuring product safety.

As used herein, the phrases "long term *in vivo* survival" and "long term survival" refer to the survival of the modified B cells described herein for 10 or more days post administration in a subject. Long term survival may be measured in days, weeks, or even years. In one embodiment, a majority of the modified B cells survive *in vivo* for 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more days post-administration. In one embodiment, a majority of the modified B cells survive *in vivo* for 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52 or more weeks post-administration. In another embodiment, the modified B cells survive *in vivo* for 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30 or more years. Additionally, while the modified B cells described herein may survive *in vivo* for 10 or more days, it is understood that a majority of the modified B cells survive *in vivo* for 1, 2, 3, 4, 5, 6, 7, 8, 9 or more days post-administration. Accordingly, it is contemplated that modified B cells described herein are useful for short-term treatment (e.g., 4 days) and long-term treatment (e.g., 30 or more days) methods.

**B cells**

After leaving the bone marrow, a B cell acts as an antigen presenting cell (APC) and internalizes antigens. Antigen is taken up by the B cell through receptor-mediated endocytosis and processed. Antigen is processed into antigenic peptides, loaded onto MHC II molecules, and presented on the B cell extracellular surface to CD4+ T helper cells. These T cells bind to the MHC ll/antigen molecule and cause activation of the B cell. Upon stimulation by a T cell, the activated B cell begins to differentiate into

more specialized cells. Germinal center B cells may differentiate into long-lived memory B cells or plasma cells. Further, secondary immune stimulation may result in the memory B cells giving rise to additional plasma cells. The formation of plasma cells from either memory or non-memory B cells is preceded by the formation of precursor plasmablasts that eventually differentiate into plasma cells, which produce large volumes of antibodies (see *e.g.*, Trends Immunol. 2009 June; 30(6): 277-285; Nature Reviews, 2005, 5:231 - 242). Plasmablasts secrete more antibodies than B cells, but less than plasma cells. They divide rapidly, and they continue to internalize antigens and present antigens to T cells. Plasmablasts have the capacity to migrate to sites of chemokine production (*e.g.* in bone marrow) whereby they may differentiate into long-lived plasma cells. Ultimately, a plasmablast may either remain as a plasmablast for several days and then die or irrevocably differentiate into a mature, fully differentiated plasma cell. Specifically, plasmablasts that are able home to tissues containing plasma cell survival niches (*e.g.*, in bone marrow) are able to displace resident plasma cells in order to become long lived plasma cells, which may continue to secrete high levels of proteins for years.

The B cells used in the methods described herein include pan B cells, memory B cells, plasmablasts, and/or plasma cells. In one embodiment, the modified B cells are memory B cells. In one embodiment, the modified B cells are plasmablasts. In one embodiment, the modified B cells are plasma cells.

Terminally differentiated plasma cells typically do not express common pan-B cell markers, such as CD19 and CD20, and express relatively few surface antigens. Plasma cells express CD38, CD78, CD138 and interleukin-6 receptor (IL-6R) and lack expression of CD45, and these markers can be used, *e.g.*, by flow cytometry, to identify plasma cells. CD27 is also a good marker for plasma cells as naive B cells are CD27-, memory B cells are CD27+ and plasma cells are CD27++. Memory B cell subsets may also express surface IgG, IgM and IgD, whereas plasma cells do not express these markers on the cell surface. CD38 and CD138 are expressed at high levels on plasma cells (See Wikipedia, The Free Encyclopedia., "Plasma cell" Page Version ID: 404969441 ; Date of last revision: 30 December 2010 09:54 UTC, retrieved January 4, 2011 ; See also: Jourdan et al. Blood. 2009 Dec 10;114(25):5173-81; Trends Immunol. 2009 June; 30(6): 277-285; Nature Reviews, 2005, 5:231 - 242; Nature Med. 2010, 16:123-129; Neuberger, M. S.; Honjo, T.; Alt, Frederick W. (2004). Molecular biology of B cells. Amsterdam: Elsevier, pp. 189-191 ; Bertil Glader; Greer, John G.; John

Foerster; Rodgers, George G.; Paraskevas, Frixos (2008). Wintrobe's Clinical Hematology, 2-Vol. Set. Hagerstwon, MD: Lippincott Williams & Wilkins. pp. 347; Walport, Mark; Murphy, Kenneth; Janeway, Charles; Travers, Paul J. (2008). Janeway's immunobiology. New York: Garland Science, pp. 387-388; Rawstron AC (May 2006). "Immunophenotyping of plasma cells". Curr Protoc Cytom).

"Quiescent", as used herein, refers to a cell state wherein the cell is not actively proliferating.

"Activated", as used herein, refers to a cell state wherein the cell is actively proliferating and/or producing cytokines in response to a stimulus.

The terms "differentiate" and "differentiated", as used herein, refer to changes in the phenotype of a cell from one cell type or state to another cell type or state. For example, a memory B cell that transitions to a plasma cell is differentiated.

The term "subject" is intended to include living organisms in which an adaptive immune response can be elicited (*e.g.*, mammals). Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof. In one embodiment, the subject is human. B cells can be obtained from a number of sources, including peripheral blood mononuclear cells (PBMCs), bone marrow, lymph node tissue, cord blood, tissue from a site of infection, spleen tissue, and tumors. In a preferred embodiment, the source of B cells is PBMCs. In certain embodiments of the present disclosure, any number of B cell lines available in the art, may be used.

In certain embodiments of the methods described herein, B cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as FICOLL™ (copolymers of sucrose and epichlorohydrin that may be used to prepare high density solutions) separation. In one preferred embodiment, cells from the circulating blood of an individual are obtained by apheresis or leukapheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment of the methods described herein, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. As those of ordinary skill in the art would readily appreciate a

washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, PBS. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

B cells may be isolated from peripheral blood or leukapheresis using techniques known in the art. For example, PBMCs may be isolated using FICOLL™ (Sigma-Aldrich, St Louis, MO) and CD19+ B cells purified by negative or positive selection using any of a variety of antibodies known in the art, such as the Rosette tetrameric complex system (StemCell Technologies, Vancouver, Canada) or MACS™ MicroBead Technology (Miltenyi Biotec, San Diego, CA). In certain embodiments, memory B cells are isolated as described by Jourdan et al., (Blood. 2009 Dec 10; 114(25):5173-81). For example, after removal of CD2+ cells using anti-CD2 magnetic beads, CD19+ CD27+ memory B cells can be sorted by FACS. Bone marrow plasma cells (BMPCs) can be purified using anti-CD138 magnetic microbeads sorting or other similar methods and reagents. Human B cells may be isolated, e.g., using CD19 MicroBeads, human (Miltenyi Biotec, San Diego, CA). Human Memory B cell may be isolated, e.g., using the Memory B Cell Isolation Kit, human (Miltenyi Biotec, San Diego, CA).

Other isolation kits are commercially available, such as R&D Systems' MagCellect Human B Cell Isolation Kit (Minneapolis, MN). In certain embodiments, resting B cells may be prepared by sedimentation on discontinuous Percoll gradients, as described in (Defranco et al., (1982) J. Exp. Med. 155:1523).

In one embodiment, PBMCs are obtained from a blood sample using a gradient based purification (*e.g.*, FICOLL™). In another embodiment, PBMCs are obtained from apheresis based collection. In one embodiment, B cells are isolated from PBMCs by isolating pan B cells. The isolating step may utilize positive and/or negative selection. In one embodiment, the negative selection comprises depleting T cells using anti-CD3 conjugated microbeads, thereby providing a T cell depleted fraction. In a further embodiment, memory B cells are isolated from the pan B cells or the T cell depleted fraction by positive selection for CD27.

In one particular embodiment, memory B cells are isolated by depletion of unwanted cells and subsequent positive selection with CD27 MicroBeads. Unwanted cells, for example, T cells, NK cells, monocytes, dendritic cells, granulocytes, platelets, and erythroid cells may be depleted using a cocktail of biotinylated antibodies against CD2, CD14, CD16, CD36, CD43, and CD235a (glycophorin A), and Anti-Biotin MicroBeads.

In one embodiment, switched memory B cells are obtained. "Switched memory B cell" or "switched B cell," as used herein, refers to a B cell that has undergone isotype class switching. In one embodiment, switched memory B cells are positively selected for IgG. In another embodiment, switched memory B cells are obtained by depleting IgD and IgM expressing cells. Switched memory B cells may be isolated, e.g., using the Switched Memory B Cell Kit, human (Miltenyi Biotec, San Diego, CA).

For example, in one particular embodiment, non-target cells may be labeled with a cocktail of biotinylated CD2, CD14, CD16, CD36, CD43, CD235a (glycophorin A), Anti-IgM, and Anti-IgD antibodies. These cells may be subsequently magnetically labeled with Anti-Biotin MicroBeads. Highly pure switched memory B cells may be obtained by depletion of the magnetically labeled cells.

In a further embodiment the promoter sequence from a gene unique to memory B cells, such as, e.g., the CD27 gene (or other gene specific to memory B cells and not expressed in naive B cells) is used to drive expression of a selectable marker such as, e.g., mutated dihydrofolate reductase allowing for positive selection of the memory B cells in the presence of methotrexate. In another embodiment, the promoter sequence from a pan B cell gene such as, e.g., the CD19 gene is used to drive expression of a selectable marker such as, e.g., mutated dihydrofolate reductase allowing for positive selection of the memory B cells in the presence of methotrexate. In another embodiment T cells are depleted using CD3 or by addition of cyclosporin. In another embodiment, CD138+ cells are isolated from the pan B cells by positive selection. In yet another embodiment, CD138+ cells are isolated from PBMCs by positive selection. In another embodiment, CD38+ cells are isolated from the pan B cells by positive selection. In yet another embodiment, CD38+ cells are isolated from PBMCs by positive selection. In one embodiment, CD27+ cells are isolated from PBMCs by positive selection. In another embodiment, memory B cells and/or plasma cells are selectively expanded from PBMCs using *in vitro* culture methods available in the art.

**Culturing B Cells *In Vitro***

B cells, such as memory B cells, can be cultured using *in vitro* methods to activate and differentiate the B cells into plasma cells or plasmablasts or both. As would be recognized by the skilled person, plasma cells may be identified by cell surface protein expression patterns using standard flow cytometry methods. For example, terminally differentiated plasma cells express relatively few surface antigens, and do not express common pan-B cell markers, such as CD19 and CD20. Instead, plasma cells may be identified by expression of CD38, CD78, CD138, and IL-6R and lack of expression of CD45. CD27 may also be used to identify plasma cells as naïve B cells are CD27-, memory B cells are CD27+ and plasma cells are CD27++. Plasma cells express high levels of CD38 and CD138.

In one embodiment, the B cells are CD138- memory B cells. In one embodiment, the B cells are CD138+ plasma cells. In one embodiment, the B cells are activated and have a cell surface phenotype of CD138-, CD27+.

In one embodiment, the B cells are CD20-, CD138- memory B cells. In one embodiment, the B cells are CD20-, CD138+ plasma cells. In one embodiment, the B cells are activated and have a cell surface phenotype of CD20-, CD138-, CD27+.

In one embodiment, the B cells are CD20-, CD38-, CD138- memory B cells. In one embodiment, the B cells are CD20-, CD38+, CD138+ plasma cells. In one embodiment, the B cells are activated and have a cell surface phenotype of CD20- CD38- CD138- CD27+.

In one embodiment, the B cells are contacted with one or more B cell activating factors, *e.g.*, any of a variety of cytokines, growth factors or cell lines known to activate and/or differentiate B cells (see *e.g.*, Fluckiger, et al. Blood 1998 92: 4509-4520; Luo, et al., Blood 2009 1 13: 1422-1431 ). Such factors may be selected from the group consisting of, but not limited to, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-1 1 , IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, IL-33, IL-34, and IL-35, IFN-γ, IFN-α, IFN-β, IFN-δ, C type chemokines XCL1 and XCL2, C-C type chemokines (to date including CCL1 -CCL28) and CXC type chemokines (to date including CXCL1 -CXCL17), and members of the TNF superfamily (*e.g.*, TNF-α, 4-1 BB ligand, B cell activating factor (BLyS), FAS ligand, sCD40L (including multimeric versions of sCD40L; *e.g.*, histidine-tagged soluble recombinant CD40L in combination

with anti-poly-histidine mAb to group multiple sCD40L molecules together), Lymphotoxin, OX40L, RANKL, TRAIL), CpG, and other toll like receptor agonists (*e.g.*, CpG).

B cell activating factors may be added to *in vitro* cell cultures at various concentrations to achieve the desired outcome (*e.g.*, expansion or differentiation). In one embodiment, a B cell activating factor is utilized in expanding the B cells in culture. In one embodiment, a B cell activating factor is utilized in differentiating the B cells in culture. In another embodiment, the B cell activating factor is utilized in both expanding and differentiating the B cells in culture. In one embodiment, the B cell activating factor is provided at the same concentration for expanding and differentiating. In another embodiment, the B cell activating factor is provided at a first concentration for expanding and at a second concentration for differentiating. It is contemplated that a B cell activating factor may be 1) utilized in expanding the B cells and not in differentiating the B cells, 2) utilized in differentiating the B cells and not in expanding the B cells, or 3) utilized in expanding and differentiating the B cells.

For example, B cells are cultured with one or more B cell activating factors selected from CD40L, IL-2, IL-4, and IL-10 for expansion of the B cells. In one embodiment, the B cells are cultured with 0.25-5.0 μg/ml CD40L. In one embodiment, the concentration of CD40L is 0.5 μg/ml. In one embodiment a crosslinking agent (such as an anti-HIS antibody in combination with HIS-tagged CD40L) is used to create multimers of CD40L. In one embodiment molecules of CD40L are covalently linked or are held together using protein multimerization domains (e.g., the Fc region of an IgG or a leucine zipper domain). In one embodiment CD40L is conjugated to beads. In one embodiment CD40L is expressed from feeder cells. In one embodiment, the B cells are cultured with 1-10 ng/ml IL-2. In one embodiment, the concentration of IL-2 is 5 ng/ml. In one embodiment, the B cells are cultured with 1-10 ng/ml IL-4. In one embodiment, the concentration of IL-4 is 2 ng/ml. In one embodiment, the B cells are cultured with 10-100 ng/ml IL-10. In one embodiment, the concentration of IL-10 is 40 ng/ml.

In one embodiment, B cells are cultured with one or more B cell activating factors selected from CD40L, IL-2, IL-4, IL-10, IL-15 and IL-21 for expansion of the B cells. In one embodiment, the B cells are cultured with 0.25-5.0 μg/ml CD40L. In one embodiment, the concentration of CD40L is 0.5 μg/ml. In one embodiment a crosslinking agent (such as an anti-HIS antibody in combination with HIS-tagged

CD40L) is used to create multimers of CD40L. In one embodiment molecules of CD40L are covalently linked or are held together using protein multimerization domains (e.g., the Fc region of an IgG or a leucine zipper domain). In one embodiment CD40L is conjugated to beads. In one embodiment CD40L is expressed from feeder cells. In one embodiment, the B cells are cultured with 1-10 ng/ml IL-2. In one embodiment, the concentration of IL-2 is 5 ng/ml. In one embodiment, the B cells are cultured with 1-10 ng/ml IL-4. In one embodiment, the concentration of IL-4 is 2 ng/ml. In one embodiment, the B cells are cultured with 10-100 ng/ml IL-10. In one embodiment, the concentration of IL-10 is 40 ng/ml. In one embodiment, the B cells are cultured with 50-150 ng/ml IL-15. In one embodiment, the concentration of IL-15 is 100 ng/ml. In one embodiment, the B cells are cultured with 50-150 ng/ml IL-21. In one embodiment, the concentration of IL-21 is 100 ng/ml. In a particular embodiment, the B cells are cultured with CD40L, IL-2, IL-4, IL-10, IL-15 and IL-21 for expansion of the B cells.

For example, in one embodiment, B cells are cultured with the B cell activating factors CD40L, IL-2, IL-4, IL-10, IL-15 and IL-21 for expansion of the B cells, wherein the CD40L is crosslinked with a crosslinking agent to create multimers of CD40L. Such a culture system may be maintained throughout an entire culture period (e.g., a 7 day culture period), in which the B cells are transfected or otherwise engineered to express a transgene of interest (e.g., an exogenous polypeptide such as, e.g., IDUA). The transgene may be integrated into the B cell (e.g., via a viral or non-viral vector). The transgene may be expressed in the B cell via use of a transposon. The transgene may be expressed in the B cell due to the targeted integration of the transgene into the B cell's genome. The targeted integration may be via homologous recombination. The homologous recombination may occur at a double strand break induced by a nuclease. The nuclease may be, e.g., a zinc finger nuclease, a TALE-nuclease (TALEN), a meganuclease (e.g., a homing endonuclease), or via a CRISPR / CAS9-nulease system.

In another example, B cells are cultured with one or more B cell activating factors selected from CD40L, IFN-α, IL-2, IL-6, IL-10, IL-15, IL-21, and P-class CpG oligodeoxynucleotides (p-ODN) for differentiation of the B cells. In one embodiment, the B cells are cultured with 25-75 ng/ml CD40L. In one embodiment, the concentration of CD40L is 50 ng/ml. In one embodiment, the B cells are cultured with 250-750 U/ml IFN-α. In one embodiment the concentration of the IFN-α is 500 U/ml. In one embodiment, the B cells are cultured with 5-50 U/ml IL-2. In one embodiment the

concentration of IL-2 is 20 U/ml. In one embodiment, the B cells are cultured with 25-75 ng/ml IL-6. In one embodiment, the concentration of IL-6 is 50 ng/ml. In one embodiment, the B cells are cultured with 10-100 ng/ml IL-10. In one embodiment, the concentration of IL-10 is 50 ng/ml. In one embodiment, the B cells are cultured with 1-20 ng/ml IL-15. In one embodiment, the concentration of IL-15 is 10 ng/ml. In one embodiment, the B cells are cultured with 10-100 ng/ml IL-21. In one embodiment, the concentration of IL-21 is 50 ng/ml. In one embodiment, the B cells are cultured with 1-50 μg/ml p-ODN. In one embodiment, the concentration of p-ODN is 10 μg/ml.

In one embodiment, B cells are contacted or cultured on feeder cells. In one embodiment, the feeder cells are a stromal cell line, *e.g.*, murine stromal cell line S17 or MS5. In another embodiment, isolated CD19+ cells are cultured with one or more B cell activating factor cytokines, such as IL-10 and IL-4, in the presence of fibroblasts expressing CD40-ligand (CD40L, CD154). In one embodiment, CD40L is provided bound to a surface such as tissue culture plate or a bead. In another embodiment, purified B cells are cultured, in the presence or absence of feeder cells, with CD40L and one or more cytokines or factors selected from IL-10, IL-4, IL-7, p-ODN, CpG DNA, IL-2, IL-15, IL6, and IFN-α.

In another embodiment, B cell activating factors are provided by transfection into the B cell or other feeder cell. In this context, one or more factors that promote differentiation of the B cell into an antibody secreting cell and/or one or more factors that promote the longevity of the antibody producing cell may be used. Such factors include, for example, Blimp-1, TRF4, anti-apoptotic factors like Bcl-xl or Bcl5, or constitutively active mutants of the CD40 receptor. Further, factors which promote the expression of downstream signaling molecules such as TNF receptor-associated factors (TRAFs) may also be used in the activation/differentiation of the B cells. In this regard, cell activation, cell survival, and antiapoptotic functions of the TNF receptor superfamily are mostly mediated by TRAF1-6 (see *e.g.*, R.H. Arch, et al., Genes Dev. 12 (1998), pp. 2821-2830). Downstream effectors of TRAF signaling include transcription factors in the NF- κB and AP-1 family which can turn on genes involved in various aspects of cellular and immune functions. Further, the activation of NF-κB and AP-1 has been shown to provide cells protection from apoptosis via the transcription of antiapoptotic genes.

In another embodiment, Epstein Barr virus (EBV)-derived proteins are used for the activation and/or differentiation of B cells or to promote the longevity of the

antibody producing cell. EBV-derived proteins include but are not limited to, EBNA-1, EBNA-2, EBNA-3, LMP-1, LMP-2, EBER, miRNAs, EBV-EA, EBV-MA, EBV-VCA and EBV-AN.

In certain embodiments, contacting the B cells with B cell activation factors using the methods provided herein leads to, among other things, cell proliferation (*i.e.*, expansion), modulation of the lgM+ cell surface phenotype to one consistent with an activated mature B cell, secretion of Ig, and isotype switching. CD19+ B cells may be isolated using known and commercially available cell separation kits, such as the MiniMACS™ cell separation system (Miltenyi Biotech, Bergisch Gladbach, Germany). In certain embodiments, CD40L fibroblasts are irradiated before use in the methods described herein. In one embodiment, B cells are cultured in the presence of one or more of IL-3, IL-7, Flt3 ligand, thrombopoietin, SCF, IL-2, IL-10, G-CSF and CpG. In certain embodiments, the methods include culturing the B cells in the presence of one or more of the aforementioned factors in conjunction with transformed stromal cells (*e.g.*, MS5) providing a low level of anchored CD40L and/or CD40L bound to a plate or a bead.

As discussed above, B cell activating factors induce expansion, proliferation, or differentiation of B cells. Accordingly, B cells are contacted with one or more B cell activating factors listed above to obtain an expanded cell population. A cell population may be expanded prior to transfection. Alternatively, or additionally, a cell population may be expanded following transfection. In one embodiment, expanding a B cell population comprises culturing cells with IL-2, IL-4, IL-10 and CD40L (see *e.g.*, Neron et al. PLoS ONE, 2012 7(12):e51946). In one embodiment, expanding a B cell population comprises culturing cells with IL-2, IL-10, CpG, and CD40L. In one embodiment, expanding a B cell population comprises culturing cells with IL-2, IL-4, IL-10, IL-15, IL-21, and CD40L. In one embodiment, expanding a B cell population comprises culturing cells with IL-2, IL-4, IL-10, IL-15, IL-21, and multimerized CD40L.

In another embodiment, expansion of a B cell population is induced and/or enhanced by a transgene introduced into the B cells. For example, a B cell that contains a recombinant receptor or an engineered receptor that induces a cell signaling pathway (*e.g.*, signaling downstream of CD40) upon binding its ligand (*e.g.*, a soluble ligand or a cell surface expressed ligand). In one embodiment, a B cell overexpresses CD40 due to expression of a CD40 transgene. In another embodiment, a B cell expresses an engineered receptor, including, *e.g.*, a recombinantly engineered antibody. In one

embodiment, an engineered receptor is similar to a chimeric antigen receptor (CAR) and comprises a fusion protein of an scFv and an intracellular signaling portion of a B cell receptor (*e.g.*, CD40).

In one embodiment, expansion of a B cell population is induced and/or enhanced by a small molecule compound added to the cell culture. For example, a compound that binds to and dimerizes CD40 can be used to trigger the CD40 signaling pathway.

Any of a variety of culture media may be used in the present methods as would be known to the skilled person (see *e.g.*, Current Protocols in Cell Culture, 2000-2009 by John Wiley & Sons, Inc.). In one embodiment, media for use in the methods described herein includes, but is not limited to Iscove modified Dulbecco medium (with or without fetal bovine or other appropriate serum). Illustrative media also includes, but is not limited to, IMDM, RPMI 1640, AIM-V, DMEM, MEM, a-MEM, F-12, X-Vivo 15, and X-Vivo 20. In further embodiments, the medium may comprise a surfactant, an antibody, plasmanate or a reducing agent (*e.g.* N-acetyl-cysteine, 2-mercaptoethanol), one or more antibiotics, and/or additives such as insulin, transferrin, sodium selenite and cyclosporin. In some embodiments, IL-6, soluble CD40L, and a cross-linking enhancer may also be used.

B cells are cultured under conditions and for sufficient time periods to achieve differentiation and/or activation desired. In certain embodiments, the B cells are cultured under conditions and for sufficient time periods such that 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or even 100% of the B cells are differentiated and/or activated as desired. In one embodiment, the B cells are activated and differentiated into a mixed population of plasmablasts and plasma cells. As would be recognized by the skilled person, plasmablasts and plasma cells may be identified by cell surface protein expression patterns using standard flow cytometry methods as described elsewhere herein, such as expression of one or more of CD38, CD78, IL-6R, $CD27^{high}$, and CD138 and/or lack of, or reduction of, expression of one or more of CD19, CD20 and CD45. As would be understood by the skilled person, memory B cells are generally CD20+ CD19+ CD27+ CD38− while early plasmablasts are CD20− CD19+ CD27++ CD38++. In one embodiment, the cells cultured using the methods described herein are CD20-, CD38+, CD138-. In another embodiment, the cells have a phenotype of CD20-, CD38+, CD138+. In certain embodiments, cells are cultured

for 1-7 days. In further embodiments, cells are cultured 7, 14, 21 days or longer. Thus, cells may be cultured under appropriate conditions for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or more days. Cells are re-plated, and media and supplements may be added or changed as needed using techniques known in the art.

In certain embodiments, the B cells are cultured under conditions and for sufficient time periods such that at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% of the cells are differentiated and activated to produce Ig and/or to express the transgene.

The induction of B cell activation may be measured by techniques such as $^{3}$H-uridine incorporation into RNA (as B cells differentiate, RNA synthesis increases), or by $^{3}$H-thymidine incorporation, which measures DNA synthesis associated with cell proliferation. In one embodiment, interleukin-4 (IL-4) may be added to the culture medium at an appropriate concentration (*e.g.*, about 10 ng/ml) for enhancement of B cell proliferation.

Alternatively, B cell activation is measured as a function of immunoglobulin secretion. For example, CD40L is added to resting B cells together with IL-4 (*e.g.*, 10 ng/ml) and IL-5 (*e.g.*, 5 ng/ml) or other cytokines that activate B cells. Flow cytometry may also be used for measuring cell surface markers typical of activated B cells. See *e.g.*, Civin CI, Loken MR, Int'l J. Cell Cloning 987; 5:1 -16; Loken, MR, et al, Flow Cytometry Characterization of Erythroid, Lymphoid and Monomyeloid Lineages in Normal Human Bone Marrow, in Flow Cytometry in Hematology, Laerum OD, Bjerksnes R. eds., Academic Press, New York 1992; pp. 31 -42; and LeBein TW, et ai, Leukemia 1990; 4:354-358.

After culture for an appropriate period of time, such as from 2, 3, 4, 5, 6, 7, 8, 9, or more days, generally around 3 days, an additional volume of culture medium may be added. Supernatant from individual cultures may be harvested at various times during culture and quantitated for IgM and IgG1 as described in Noelle et al., (1991) J. Immunol. 146:1118-1124. In one embodiment, the culture is harvested and measured for expression of the transgene of interest using flow cytometry, enzyme-linked immunosorbent assay (ELISA), ELISPOT or other assay known in the art.

In another embodiment, ELISA is used to measure antibody isotype production, *e.g.*, IgM, or a product of the transgene of interest. In certain embodiments, IgG determinations are made using commercially available antibodies, such as goat anti-human IgG, as capture antibody followed by detection using any of a variety of appropriate detection reagents such as biotinylated goat antihuman Ig, streptavidin alkaline phosphatase and substrate.

In certain embodiments, the B cells are cultured under conditions and for sufficient time periods such that the number of cells is 1, 10, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 fold or more greater than the number of B cells at the start of culture. In one embodiment, the number of cells is 10-1000 fold greater, including consecutive integers therein, than the number of B cells at the start of culture. For example, an expanded B cell population is at least 10 fold greater than the initial isolated B cell population. In another embodiment, the expanded B cell population is at least 100 fold greater than the initial isolated B cell population. In one embodiment, the expanded B cell population is at least 500 fold greater than the initial isolated B cell population.

**Engineering of B cells**

In one embodiment, the genetically modified B cells are transfected with a transgene. Exemplary methods for transfecting B cells are provided in WO 2014/152832 and WO 2016/100932, both of which are incorporated herein by reference in their entireties. Transfection of B cells may be accomplished using any of a variety of methods available in the art to introduce DNA or RNA into a B cell. Suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, pressure-mediated transfection or "cell squeezing" (*e.g.*, CellSqueeze microfluidic system, SQZ Biotechnologies), nano-particle-mediated or liposome-mediated transfection and transduction using retrovirus or other virus, *e.g.*, vaccinia. See, *e.g.*, Graham et al., 1973, Virology 52:456; Sambrook et al., 2001, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratories; Davis et al., 1986, Basic Methods in Molecular Biology, Elsevier; Chu et al., 1981, Gene 13:197; US 5,124,259; US 5,297,983; US 5,283,185; US 5,661,018; US 6,878,548; US 7,799,555; US 8,551,780; and US 8,633,029. One example of a commercially available electroporation technique suitable for B cells is the Nucleofector™ transfection technology.

Transfection may take place prior to or during *in vitro* culture of the isolated B cells in the presence of one or more activating and/or differentiating factors described above. For example, cells are transfected on day 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, or 39 of *in vitro* culture. In one embodiment, cells are transfected on day 1, 2, or 3 of *in vitro* culture. In a particular embodiment, cells are transfected on day 2. For example, cells are electroporated on day 2 of *in vitro* culture for delivery of, *e.g.*, a plasmid, a transposon, a minicircle, or a self-replicating RNA. In another embodiment, cells are transfected on day 4, 5, 6, or 7 of *in vitro* culture. In a particular embodiment, cells are transfected on day 6 of *in vitro* culture. In another embodiment, cells are transfected on day 5 of *in vitro* culture.

In one embodiment, cells are transfected or otherwise engineered (e.g., via a targeted integration of a transgene) prior to activation. In another embodiment, cells are transfected or otherwise engineered (e.g., via a targeted integration of a transgene) during activation. In one embodiment, cells are transfected or otherwise engineered (e.g., via a targeted integration of a transgene) after activation. In one embodiment, cells are transfected or otherwise engineered (e.g., via a targeted integration of a transgene) prior to differentiation. In another embodiment, cells are transfected or otherwise engineered (e.g., via a targeted integration of a transgene) during differentiation. In one embodiment, cells are transfected or otherwise engineered (e.g., via a targeted integration of a transgene) after differentiation.

In one embodiment, a non-viral vector is used to deliver DNA or RNA to memory B cells and/or plasma cells. For example, systems that may facilitate transfection of memory B cells and/or plasma cells without the need of a viral integration system include, without limitation, transposons (*e.g.*, Sleeping Beauty transposon system), zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPRs), meganucleases, minicircles, replicons, artificial chromosomes (*e.g.*, bacterial artificial chromosomes, mammalian artificial chromosomes, and yeast artificial chromosomes), plasmids, cosmids, and bacteriophage.

In some embodiments, such non-viral-dependent vector systems may also be delivered via a viral vector known in the art or described below. For example, in some embodiments, a viral vector (e.g., a retrovirus, lentivirus, adenovirus, adeno-associated

virus), is utilized to deliver one or more non-viral vector (such as, e.g., one or more of the above-mentioned zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), clustered regularly interspaced short palindromic repeats (CRISPRs) meganucleases, or any other enzyme / complementary vectors, polynucleotides, and/or polypeptides capable of facilitating the targeted integration. Accordingly, in some embodiments, a cell (e.g., B cells such as a memory B cells and/or plasma cells) may be engineered to express an exogenous sequence (e.g., a sequence encoding a therapeutic polypeptide such as IDUA) via a targeted integration method. Such methods are known in the art and may comprise cleaving an endogenous locus in the cell using one or more nucleases (e.g., ZFNs, TALENs, CRISPR/Cas, meganuclease) and administering the transgene to the cell such that it is integrated into the endogenous locus and expressed in the cell. The transgene may be comprised in a donor sequence that is integrated into the host cell's DNA at or near the point of a cleavage by the nuclease.

The integration of the exogenous sequence (e.g., a sequence encoding a therapeutic polypeptide such as IDUA) may occur via recombination. As would be clear to one of skill in the art, "Recombination" refers to a process of exchange of genetic information between two polynucleotides, including but not limited to, donor capture by non-homologous end joining (NHEJ) and homologous recombination. The recombination may be homologous recombination. For the purposes of this disclosure, "homologous recombination (HR)" refers to the specialized form of such exchange that takes place, for example, during repair of double-strand breaks in cells via homology-directed repair mechanisms. This process utilizes nucleotide sequence homology, whereby a "donor" molecule (e.g., donor polynucleotide sequence or donor vector comprising such a sequence) is utilized by a cell's DNA-repair machinery as a template to repair of a "target" molecule (i.e., the one that experienced the double-strand break), and by these means causes the transfer of genetic information from the donor to the target. In some embodiments of HR-directed integration, the donor molecule may contain at least 2 regions of homology to the genome ("homology arms"). In some embodiments, the homology arms may be, e.g., of least 50-100 base pairs in length. The homology arms may have substantial DNA homology to a region of genomic DNA flanking the cleavage site wherein the targeted integration is to occur. The homology arms of the donor molecule may flank the DNA that is to be integrated into the target genome or target DNA locus. Breakage of the chromosome followed by repair using the homologous

region of the plasmid DNA as a template may results in the transfer of the intervening transgene flanked by the homology arms into the genome. See, e.g., Koller et al. (1989) *Proc. Nat'l. Acad. Sci. USA* 86(22):8927-8931; Thomas et al. (1986) *Cell* 44(3):419-428. The frequency of this type of homology-directed targeted integration can be increased by up to a factor of $10^5$ by deliberate creation of a double-strand break in the vicinity of the target region (Hockemeyer et al. (2009) *Nature Biotech.* 27(9):851-857; Lombardo et al. (2007) *Nature Biotech.* 25(11):1298-1306; Moehle et al. (2007) *Proc. Nat'l Acad. Sci. USA* 104(9):3055-3060; Rouet et al. (1994) *Proc. Nat'l Acad. Sci. USA* 91(13):6064-6068.

Any nuclease capable of mediating the targeted cleavage of a genomic locus such that a transgene may be integrated into the genome of a target cell (e.g., by recombination such as HR) may be utilized in engineering a cell (e.g., a memory B cell or plasmablast) according to the present disclosure.

A double-strand break (DSB) or nick can be created by a site-specific nuclease such as a zinc-finger nuclease (ZFN), a TAL effector domain nuclease (TALEN), a meganuclease, or using the CRISPR/Cas9 system with an engineered crRNA/tract RNA (single guide RNA) to guide specific cleavage. See, for example, Burgess (2013) Nature Reviews Genetics 14:80-81, Umov et al. (2010) *Nature* 435(7042):646-51; United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060188987; 20090263900; 20090117617; 20100047805; 20110207221; 20110301073 and International Publication WO 2007/014275, the disclosures of which are incorporated by reference in their entireties for all purposes.

In some embodiments, the cell (e.g., a memory B cell or a plasmablast) is engineered via Zinc Finger Nuclease-mediated targeted integration of a donor construct. A zinc finger nuclease (ZFN) is an enzyme that is able to recognize and cleave a target nucleotide sequence with specificity due to the coupling of a "zinc finger DNA binding protein" (ZFP) (or binding domain), which binds DNA in a sequence-specific manner through one or more zinc fingers, and a nuclease enzyme. ZFNs may comprise any suitable cleavage domains (e.g., a nuclease enzyme) operatively linked to a ZFP DNA-binding domain to form a engineered ZFN that can facilitate site-specific cleavage of a target DNA sequence (see, e.g., Kim et al. (1996) Proc Natl Acad Sci USA 93(3):1156-1160). For example, ZFNs may comprise a target-specific ZFP linked to a FOK1 enzyme

or a portion of a FOK1 enzyme. In some embodiments, ZFN used in a ZFN-mediated targeted integration approach utilize two separate molecules, each comprising a subunit of a FOK1 enzyme each bound to a ZFP, each ZFP with specificity for a DNA sequence flanking a target cleavage site, and when the two ZFPs bind to their respective target DNA sites the FOK1 enzyme subunits are brought into proximity with one another and they bind together activating the nuclease activity which cleaves the target cleavage site. ZFNs have been used for genome modification in a variety of organisms (e.g., United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060188987; 20060063231; and International Publication WO 07/014,275, incorporated herein by reference in their entirety) Custom ZFPs and ZFNs are commercially available from, e.g., Sigma Aldrich (St. Louis, MO), and any location of DNA may be routinely targeted and cleaved using such custom ZFNs.

In some embodiments, the cell (e.g., a memory B cell or a plasmablast) is engineered via CRISPR/Cas (e.g., CRISPR Cas9) Nuclease-mediated integration of a donor construct. A CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR Associated) nuclease system is an engineered nuclease system based on a bacterial system that may be used for genome engineering. It is based on part of the adaptive immune response of many bacteria and archea. When a virus or plasmid invades a bacterium, segments of the invader's DNA are converted into CRISPR RNAs (crRNA) by the 'immune' response. This crRNA then associates, through a region of partial complementarity, with another type of RNA called tracrRNA to guide the Cas9 nuclease to a region homologous to the crRNA in the target DNA called a "protospacer". Cas9 cleaves the DNA to generate blunt ends at the DSB at sites specified by a 20-nucleotide guide sequence contained within the crRNA transcript. Cas9 requires both the crRNA and the tracrRNA for site specific DNA recognition and cleavage. This system has now been engineered such that the crRNA and tracrRNA can be combined into one molecule (the "single guide RNA"), and the crRNA equivalent portion of the single guide RNA can be engineered to guide the Cas9 nuclease to target any desired sequence (see Jinek et al (2012) Science 337, p. 816-821, Jinek et al, (2013), eLife 2:e00471, and David Segal, (2013) eLife 2:e00563). Thus, the CRISPR/Cas system can be engineered to create a DSB at a desired target in a genome, and repair of the DSB can be influenced by the use of repair inhibitors to cause an increase in error prone repair.

In some embodiments, the CRISPR/Cas nuclease-mediated integration utilizes a Type II CRISPR. The Type II CRISPR is one of the most well characterized systems and carries out targeted DNA double-strand break in four sequential steps. First, two non-coding RNA, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the repeat regions of the pre-crRNA and mediates the processing of pre-crRNA into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the target DNA via Wastson-Crick base-pairing between the spacer on the crRNA and the protospacer on the target DNA next to a protospacer adjacent motif (PAM), an additional requirement for target recognition. Forth, Cas9 mediates cleavage of target DNA to create a double-stranded break within the protospacer.

The Cas9 related CRISPR/Cas system comprises two RNA non-coding components: tracrRNA and a pre-crRNA array containing nuclease guide sequences (spacers) interspaced by identical direct repeats (DRs). To use a CRISPR/Cas system to accomplish genome engineering, both functions of these RNAs must be present (see Cong et al, (2013) Sciencexpress 1/10.1126/science 1231143). In some embodiments, the tracrRNA and pre-crRNAs are supplied via separate expression constructs or as separate RNAs. In other embodiments, a chimeric RNA is constructed where an engineered mature crRNA (conferring target specificity) is fused to a tracrRNA (supplying interaction with the Cas9) to create a chimeric cr-RNA-tracrRNA hybrid (also termed a single guide RNA). (see Jinek ibid and Cong, ibid).

In some embodiments, a single guide RNA containing both the crRNA and tracrRNA may be engineered to guide the Cas9 nuclease to target any desired sequence (e.g., Jinek et al (2012) Science 337, p. 816-821, Jinek et al, (2013), eLife 2:e00471, David Segal, (2013) eLife 2:e00563). Thus, the CRISPR/Cas system may be engineered to create a DSB at a desired target in a genome.

Custom CRISPR/Cas systems are commercially available from, e.g., Dharmacon (Lafayette, CO), and any location of DNA may be routinely targeted and cleaved using such custom single guide RNA sequences. Single stranded DNA templates for recombination may be synthesized (e.g., via oligonucleotide synthesis methods known in the art and commercially available) or provided in a vector, e.g., a viral vector such as an AAV.

In some embodiments, the cell (e.g., a memory B cell or a plasmablast) is engineered via TALE-Nuclease (TALEN) mediated targeted integration of a donor construct. A "TALE DNA binding domain" or "TALE" is a polypeptide comprising one or more TALE repeat domains/units. The repeat domains are involved in binding of the TALE to its cognate target DNA sequence. A single "repeat unit" (also referred to as a "repeat") is typically 33-35 amino acids in length and exhibits at least some sequence homology with other TALE repeat sequences within a naturally occurring TALE protein. TAL-effectors may contain a nuclear localization sequence, an acidic transcriptional activation domain and a centralized domain of tandem repeats where each repeat contains approximately 34 amino acids that are key to the DNA binding specificity of these proteins. (e.g., Schornack S, et al (2006) J Plant Physiol 163(3): 256-272). TAL effectors depend on the sequences found in the tandem repeats which comprises approximately 102 bp and the repeats are typically 91-100% homologous with each other (e.g., Bonas et al (1989) MoI Gen Genet 218: 127-136). These DNA binding repeats may be engineered into proteins with new combinations and numbers of repeats, to make artificial transcription factors that are able to interact with new sequences and activate the expression of a non-endogenous reporter gene (e.g., Bonas et al (1989) MoI Gen Genet 218: 127-136). Engineered TAL proteins may be linked to a FokI cleavage half domain to yield a TAL effector domain nuclease fusion (TALEN) to cleave target specific DNA sequence (e.g., Christian et al (2010) Genetics epub 10.1534/genetics.110.120717).

Custom TALEN are commercially available from, e.g., Thermo Fisher Scientific (Waltham, MA), and any location of DNA may be routinely targeted and cleaved.

In some embodiments, the cell (e.g., a memory B cell or a plasmablast) is engineered via Meganuclease-mediated targeted integration of a donor construct. A Meganuclease (or "homing endonuclease") is an endonuclease that binds and cleaves double-stranded DNA at a recognition sequence that is greater than 12 base pairs. Naturally occurring meganucleases may be monomeric (e.g., I-SceI) or dimeric (e.g., I-CreI). Naturally occurring meganucleases recognize 15-40 base-pair cleavage sites and are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cyst box family and the HNH family. Exemplary homing endonucleases include I-SceI, I-CeuI, PI-PspI, PI-Sce, I-SceIV, I-CsmI, I-PanI, I-SceII, I-PpoI, I-SceIII, I-CreI, I-TevI, I-TevII and I-TevIII. Their recognition sequences are known. See also

U.S. Pat. No. 5,420,032; U.S. Pat. No. 6,833,252; Belfort et al. (1997) Nucleic Acids Res. 25:3379-3388; Dujon et al. (1989) Gene 82:115-118; Perler et al. (1994) Nucleic Acids Res. 22, 1125-1127; Jasin (1996) Trends Genet. 12:224-228; Gimble et al. (1996) J. Mol. Biol. 263:163-180; Argast et al. (1998) J. Mol. Biol. 280:345-353 and the New England Biolabs catalogue.. The term "Meganuclease" includes monomeric meganucleases, dimeric meganucleases and monomers that associate to form a dimeric meganucleases.

In certain embodiments, the methods and compositions described herein make use of a nuclease that comprises an engineered (non-naturally occurring) homing endonuclease (meganuclease). The recognition sequences of homing endonucleases and meganucleases such as I-SceI, I-CeuI, PI-PspI, PI-Sce, I-SceIV, I-CsmI, I-PanI, I-SceII, I-PpoI, I-SceIII, I-CreI, I-TevI, I-TevII and I-TevIII are known. See also U.S. Pat. No. 5,420,032; U.S. Pat. No. 6,833,252; Belfort et al. (1997) Nucleic Acids Res. 25:3379-3388; Dujon et al. (1989) Gene 82:115-118; Perler et al. (1994) Nucleic Acids Res. 22, 1125-1127; Jasin (1996) Trends Genet. 12:224-228; Gimble et al. (1996) J. Mol. Biol. 263:163-180; Argast et al. (1998) J. Mol. Biol. 280:345-353 and the New England Biolabs catalogue. In addition, the DNA-binding specificity of homing endonucleases and meganucleases can be engineered to bind non-natural target sites. See, for example, Chevalier et al. (2002) Molec. Cell 10:895-905; Epinat et al. (2003) Nucleic Acids Res. 31:2952-2962; Ashworth et al. (2006) Nature 441:656-659; Paques et al. (2007) Current Gene Therapy 7:49-66; U.S. Patent Publication No. 20070117128. The DNA-binding domains of the homing endonucleases and meganucleases may be altered in the context of the nuclease as a whole (i.e., such that the nuclease includes the cognate cleavage domain) or may be fused to a heterologous cleavage domain. Custom Meganuclease are commercially available from, e.g., New England Biolabs (Ipswich, MA), and any location of DNA may be routinely targeted and cleaved.

The engineering of the B cell may comprise administering one or more nucleases (e.g., ZFNs, TALENs, CRISPR/Cas, meganuclease) to a B cell, e.g., via one or more vectors encoding the nucleases, such that the vectors comprising the encoded nucleases are taken up by the B cell. The vectors may be viral vectors.

In some embodiments, the nucleases cleave a specific endogenous locus (e.g. safe harbor gene or locus of interest) in the cell (e.g., memory B cell or plasma cell) and one or more exogenous (donor) sequences (e.g., transgenes) are administered (e.g. one or more vectors comprising these exogenous sequences). The nuclease may induce a

double-stranded (DSB) or single-stranded break (nick) in the target DNA. In some embodiments, targeted insertion of a donor transgene may be performed via homology directed repair (HDR), non-homology repair mechanisms (e.g., NHEJ- mediated end capture), or insertions and/or deletion of nucleotides (e.g. endogenous sequence) at the site of integration of a transgene into the cell's genome.

In one embodiment, a method of transfecting a B cell comprises electroporating the B cell prior to contacting the B cell with a vector. In one embodiment, cells are electroporated on day 1, 2, 3, 4, 5, 6, 7, 8, or 9 of *in vitro* culture. In one embodiment, cells are electroporated on day 2 of *in vitro* culture for delivery of a plasmid. In one embodiment, cells are transfected using a transposon on day 1, 2, 3, 4, 5, 6, 7, 8, or 9 of *in vitro* culture. In another embodiment, cells are transfected using a minicircle on day 1, 2, 3, 4, 5, 6, 7, 8, or 9 of *in vitro* culture. In one embodiment, electroporation of a Sleeping Beauty transposon takes place on day 2 of *in vitro* culture.

In one embodiment, the B cells are contacted with a vector comprising a nucleic acid of interest operably linked to a promoter, under conditions sufficient to transfect at least a portion of the B cells. In one embodiment the B cells are contacted with a vector comprising a nucleic acid of interest operably linked to a promoter, under conditions sufficient to transfect at least 5% of the B cells. In a further embodiment, the B cells are contacted with a vector under conditions sufficient to transfect at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or even 100% of the B cells. In one particular embodiment, the B cells, cultured *in vitro* as described herein, are transfected, in which case the cultured B cells are contacted with a vector as described herein under conditions sufficient to transfect at least 5%, 10% 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or even 100% of the B cells.

Viral vectors may be employed to transduce memory B cells and/or plasma cells. Examples of viral vectors include, without limitation, adenovirus-based vectors, adeno-associated virus (AAV)-based vectors, retroviral vectors, retroviral-adenoviral vectors, and vectors derived from herpes simplex viruses (HSVs), including amplicon vectors, replication-defective HSV and attenuated HSV (see, *e.g.*, Krisky, Gene Ther. 5: 1517-30, 1998; Pfeifer, Annu. Rev. Genomics Hum. Genet. 2:177-211, 2001, each of which is incorporated by reference in its entirety).

In one embodiment, cells are transduced with a viral vector (*e.g.*, a lentiviral vector) on day 1, 2, 3, 4, 5, 6, 7, 8, or 9 of *in vitro* culture. In a particular embodiment, cells are transduced with a viral vector on day 5 of *in vitro* culture. In one embodiment, the viral vector is a lentivirus. In one embodiment, cells are transduced with a measles virus pseudotyped lentivirus on day 1 of *in vitro* culture.

In one embodiment, B cells are transduced with retroviral vectors using any of a variety of known techniques in the art (see, *e.g.*, Science 12 April 1996 272: 263-267; Blood 2007, 99:2342- 2350; Blood 2009, 1 13:1422-1431 ; Blood 2009 Oct 8; 1 14(15):3173-80; Blood. 2003;101 (6):2167-2174; Current Protocols in Molecular Biology or Current Protocols in Immunology, John Wiley & Sons, New York, N.Y.(2009)). Additional description of viral transduction of B cells may be found in WO 2011/085247 and WO 2014/152832, each of which is herein incorporated by reference in its entirety.

For example, PBMCs, B- or T-lymphocytes from donors, and other B cell cancer cells such as B-CLLs may be isolated and cultured in IMDM medium or RPMI 1640 (GibcoBRL Invitrogen, Auckland, New Zealand) or other suitable medium as described herein, either serum-free or supplemented with serum (*e.g.*, 5-10% FCS, human AB serum, and serum substitutes) and penicillin/streptomycin and/or other suitable supplements such as transferrin and/or insulin. In one embodiment, cells are seeded at 1 x $10^5$ cells in 48-well plates and concentrated vector added at various doses that may be routinely optimized by the skilled person using routine methodologies. In one embodiment, B cells are transferred to an MS5 cell monolayer in RPMI supplemented with 10% AB serum, 5% FCS, 50ng/ml rhSCF, 10ng/ml rhlL-15 and 5ng/ml rhlL-2 and medium refreshed periodically as needed. As would be recognized by the skilled person, other suitable media and supplements may be used as desired.

Certain embodiments relate to the use of retroviral vectors, or vectors derived from retroviruses. "Retroviruses" are enveloped RNA viruses that are capable of infecting animal cells, and that utilize the enzyme reverse transcriptase in the early stages of infection to generate a DNA copy from their RNA genome, which is then typically integrated into the host genome. Examples of retroviral vectors Moloney murine leukemia virus (MLV)-derived vectors, retroviral vectors based on a Murine Stem Cell Virus, which provides long-term stable expression in target cells such as hematopoietic precursor cells and their differentiated progeny (see, *e.g.*, Hawley et al., PNAS USA

93:10297-10302, 1996; Keller et al., Blood 92:877-887, 1998), hybrid vectors (see, *e.g.*, Choi, et al., Stem Cells 19:236-246, 2001), and complex retrovirus-derived vectors, such as lentiviral vectors.

In one embodiment, the B cells are contacted with a retroviral vector comprising a nucleic acid of interest operably linked to a promoter, under conditions sufficient to transduce at least a portion of the B cells. In one embodiment the B cells are contacted with a retroviral vector comprising a nucleic acid of interest operably linked to a promoter, under conditions sufficient to transduce at least 2% of the B cells. In a further embodiment, the B cells are contacted with a vector under conditions sufficient to transduce at least 2%, 3%, 4%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or even 100% of the resting B cells. In one particular embodiment, the differentiated and activated B cells, cultured *in vitro* as described herein, are transduced, in which case the cultured differentiated/activated B cells are contacted with a vector as described herein under conditions sufficient to transduce at least 2%, 3%, 4%, 5%, 10% 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or even 100% of the differentiated and activated B cells.

In certain embodiments, prior to transduction, the cells are prestimulated with Staphylococcus Aureus Cowan (SAC; Calbiochem, San Diego, CA) and/or IL-2 at appropriate concentrations known to the skilled person and routinely optimized. Other B cell activating factors (*e.g.*, PMA), as are known to the skilled artisan and described herein may be used.

As noted above, certain embodiments employ lentiviral vectors. The term "lentivirus" refers to a genus of complex retroviruses that are capable of infecting both dividing and non-dividing cells. Examples of lentiviruses include HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2), visna-maedi, the caprine arthritis-encephalitis virus, equine infectious anemia virus, feline immunodeficiency virus (FIV), bovine immune deficiency virus (BIV), and simian immunodeficiency virus (SIV). Lentiviral vectors can be derived from any one or more of these lentiviruses (see, *e.g.*, Evans et al., Hum Gene Ther. 10:1479-1489, 1999; Case et al., PNAS USA 96:2988-2993, 1999; Uchida et al., PNAS USA 95:1 1939-1 1944, 1998; Miyoshi et al., Science 283:682-686, 1999; Sutton et al., J Virol 72:5781 -5788,

1998; and Frecha et al., Blood. 1 12:4843-52, 2008, each of which is incorporated by reference in its entirety).

It has been documented that resting T and B cells can be transduced by a VSVG-coated LV carrying most of the HIV accessory proteins (vif, vpr, vpu, and nef) (see *e.g.*, Frecha et al., 2010 Mol. Therapy 18:1748). In certain embodiments the retroviral vector comprises certain minimal sequences from a lentivirus genome, such as the HIV genome or the SIV genome. The genome of a lentivirus is typically organized into a 5' long terminal repeat (LTR) region, the gag gene, the pol gene, the env gene, the accessory genes (*e.g.*, nef, vif, vpr, vpu, tat, rev) and a 3' LTR region. The viral LTR is divided into three regions referred to as U3, R (repeat) and U5. The U3 region contains the enhancer and promoter elements, the U5 region contains the polyadenylation signals, and the R region separates the U3 and U5 regions. The transcribed sequences of the R region appear at both the 5' and 3' ends of the viral RNA (see, *e.g.*, "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, 2000); O Narayan, J. Gen. Virology. 70:1617-1639, 1989; Fields et al., Fundamental Virology Raven Press., 1990; Miyoshi et al., J Virol. 72:8150-7,1998; and U.S. Pat. No. 6,013,516, each of which is incorporated by reference in its entirety). Lentiviral vectors may comprise any one or more of these elements of the lentiviral genome, to regulate the activity of the vector as desired, or, they may contain deletions, insertions, substitutions, or mutations in one or more of these elements, such as to reduce the pathological effects of lentiviral replication, or to limit the lentiviral vector to a single round of infection.

Typically, a minimal retroviral vector comprises certain 5'LTR and 3'LTR sequences, one or more genes of interest (to be expressed in the target cell), one or more promoters, and a cis-acting sequence for packaging of the RNA. Other regulatory sequences can be included, as described herein and known in the art. The viral vector is typically cloned into a plasmid that may be transfected into a packaging cell line, such as a eukaryotic cell (*e.g.*, 293-HEK), and also typically comprises sequences useful for replication of the plasmid in bacteria.

In certain embodiments, the viral vector comprises sequences from the 5' and/or the 3' LTRs of a retrovirus such as a lentivirus. The LTR sequences may be LTR sequences from any lentivirus from any species. For example, they may be LTR sequences from HIV, SIV, FIV or BIV. Preferably the LTR sequences are HIV LTR sequences.

In certain embodiments, the viral vector comprises the R and U5 sequences from the 5' LTR of a lentivirus and an inactivated or "self-inactivating" 3' LTR from a lentivirus. A "self-inactivating 3' LTR" is a 3' long terminal repeat (LTR) that contains a mutation, substitution or deletion that prevents the LTR sequences from driving expression of a downstream gene. A copy of the U3 region from the 3' LTR acts as a template for the generation of both LTR's in the integrated provirus. Thus, when the 3' LTR with an inactivating deletion or mutation integrates as the 5' LTR of the provirus, no transcription from the 5' LTR is possible. This eliminates competition between the viral enhancer/promoter and any internal enhancer/promoter. Self-inactivating 3' LTRs are described, for example, in Zufferey et al., J Virol. 72:9873-9880, 1998; Miyoshi et al., J Virol. 72:8150-8157, 1998; and Iwakuma et al., J Virology 261: 120-132, 1999, each of which is incorporated by reference in its entirety. Self-inactivating 3' LTRs may be generated by any method known in the art. In certain embodiments, the U3 element of the 3' LTR contains a deletion of its enhancer sequence, preferably the TATA box, Spl and/or NF-kappa B sites. As a result of the self-inactivating 3' LTR, the provirus that is integrated into the host cell genome will comprise an inactivated 5' LTR.

The vectors provided herein typically comprise a gene that encodes a protein (or other molecule, such as siRNA) that is desirably expressed in one or more target cells. In a viral vector, the gene of interest is preferably located between the 5' LTR and 3' LTR sequences. Further, the gene of interest is preferably in a functional relationship with other genetic elements, for example, transcription regulatory sequences such as promoters and/or enhancers, to regulate expression of the gene of interest in a particular manner once the gene is incorporated into the target cell. In certain embodiments, the useful transcriptional regulatory sequences are those that are highly regulated with respect to activity, both temporally and spatially.

In certain embodiments, one or more additional genes may be incorporated as a safety measure, mainly to allow for the selective killing of transfected target cells within a heterogeneous population, such as within a human patient. In one exemplary embodiment, the selected gene is a thymidine kinase gene (TK), the expression of which renders a target cell susceptible to the action of the drug gancyclovir. In a further embodiment, the suicide gene is a caspase 9 suicide gene activated by a dimerizing drug (see, *e.g.*, Tey et al., Biology of Blood and Marrow Transplantation 13:913-924, 2007).

In certain embodiments, a gene encoding a marker protein may be placed before or after the primary gene in a viral or non-viral vector to allow for identification and/or selection of cells that are expressing the desired protein. Certain embodiments incorporate a fluorescent marker protein, such as green fluorescent protein (GFP) or red fluorescent protein (RFP), along with the primary gene of interest. If one or more additional reporter genes are included, IRES sequences or 2A elements may also be included, separating the primary gene of interest from a reporter gene and/or any other gene of interest.

Certain embodiments may employ genes that encode one or more selectable markers. Examples include selectable markers that are effective in a eukaryotic cell or a prokaryotic cell, such as a gene for a drug resistance that encodes a factor necessary for the survival or growth of transformed host cells grown in a selective culture medium. Exemplary selection genes encode proteins that confer resistance to antibiotics or other toxins, *e.g.*, G418, hygromycin B, puromycin, zeocin, ouabain, blasticidin, ampicillin, neomycin, methotrexate, or tetracycline, complement auxotrophic deficiencies, or supply may be present on a separate plasmid and introduced by co-transfection with the viral vector. In one embodiment, the gene encodes for a mutant dihydrofolate reductase (DHFR) that confers methotrexate resistance. Certain other embodiments may employ genes that encode one or cell surface receptors that can be used for tagging and detection or purification of transfected cells (*e.g.*, low-affinity nerve growth factor receptor (LNGFR) or other such receptors useful as transduction tag systems. See *e.g.*, Lauer et al., Cancer Gene Ther. 2000 Mar;7(3):430-7.

Certain viral vectors such as retroviral vectors employ one or more heterologous promoters, enhancers, or both. In certain embodiments, the U3 sequence from a retroviral or lentiviral 5' LTR may be replaced with a promoter or enhancer sequence in the viral construct. Certain embodiments employ an "internal" promoter/enhancer that is located between the 5' LTR and 3' LTR sequences of the viral vector, and is operably linked to the gene of interest.

A "functional relationship" and "operably linked" mean, without limitation, that the gene is in the correct location and orientation with respect to the promoter and/or enhancer, such that expression of the gene will be affected when the promoter and/or enhancer is contacted with the appropriate regulatory molecules. Any enhancer/promoter combination may be used that either regulates (*e.g.*, increases,

decreases) expression of the viral RNA genome in the packaging cell line, regulates expression of the selected gene of interest in an infected target cell, or both.

A promoter is an expression control element formed by a DNA sequence that permits polymerase binding and transcription to occur. Promoters are untranslated sequences that are located upstream (5') of the start codon of a selected gene of interest (typically within about 100 to 1000 bp) and control the transcription and translation of the coding polynucleotide sequence to which they are operably linked. Promoters may be inducible or constitutive. Inducible promoters initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, such as a change in temperature. Promoters may be unidirectional or bidirectional. Bidirectional promoters can be used to co-express two genes, *e.g.*, a gene of interest and a selection marker. Alternatively, a bidirectional promoter configuration comprising two promoters, each controlling expression of a different gene, in opposite orientation in the same vector may be utilized.

A variety of promoters are known in the art, as are methods for operably linking the promoter to the polynucleotide coding sequence. Both native promoter sequences and many heterologous promoters may be used to direct expression of the selected gene of interest. Certain embodiments employ heterologous promoters, because they generally permit greater transcription and higher yields of the desired protein as compared to the native promoter.

Certain embodiments may employ heterologous viral promoters. Examples of such promoters include those obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus, bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40). Certain embodiments may employ heterologous mammalian promoter, such as the actin promoter, an immunoglobulin promoter, a heat-shock promoter, or a promoter that is associated with the native sequence of the gene of interest. Typically, the promoter is compatible with the target cell, such as an activated B-lymphocyte, a plasma B cell, a memory B cell or other lymphocyte target cell.

Certain embodiments may employ one or more of the RNA polymerase II and III promoters. A suitable selection of RNA polymerase III promoters can be found, for example, in Paule and White. Nucleic Acids Research., Vol. 28, pp 1283-1298, 2000, which is incorporated by reference in its entirety. RNA polymerase II and III promoters

also include any synthetic or engineered DNA fragments that can direct RNA polymerase II or III, respectively, to transcribe its downstream RNA coding sequences. Further, the RNA polymerase II or III (Pol II or III) promoter or promoters used as part of the viral vector can be inducible. Any suitable inducible Pol II or III promoter can be used with the methods described herein. Exemplary Pol II or III promoters include the tetracycline responsive promoters provided in Ohkawa and Taira, Human Gene Therapy, Vol. 11, pp 577-585, 2000; and Meissner et al., Nucleic Acids Research, Vol. 29, pp 1672-1682, 2001, each of which is incorporated by reference in its entirety.

Non-limiting examples of constitutive promoters that may be used include the promoter for ubiquitin, the CMV promoter (see, *e.g.*, Karasuyama et al., J. Exp. Med. 169:13, 1989), the β-actin (see, *e.g.*, Gunning et al., PNAS USA 84:4831 -4835, 1987), the elongation factor-1 alpha (EF-1 alpha) promoter, the CAG promoter, and the pgk promoter (see, *e.g.*, Adra et al., Gene 60:65-74, 1987); Singer-Sam et al., Gene 32:409-417, 1984; and Dobson et al., Nucleic Acids Res. 10:2635-2637, 1982, each of which is incorporated by reference). Non-limiting examples of tissue specific promoters include the lck promoter (see, *e.g.*, Garvin et al., Mol. Cell Biol. 8:3058-3064, 1988; and Takadera et al., Mol. Cell Biol. 9:2173-2180, 1989), the myogenin promoter (Yee et al., Genes and Development 7:1277-1289. 1993), and the thyl promoter (see, *e.g.*, Gundersen et al., Gene 1 13:207-214, 1992).

Additional examples of promoters include the ubiquitin-C promoter, the human μ heavy chain promoter or the Ig heavy chain promoter (*e.g.*, MH), and the human κ light chain promoter or the Ig light chain promoter (*e.g.*, EEK), which are functional in B-lymphocytes. The MH promoter contains the human μ heavy chain promoter preceded by the iEμ enhancer flanked by matrix association regions, and the EEK promoter contains the κ light chain promoter preceded an intronic enhancer (iEκ), a matrix associated region, and a 3' enhancer (3Eκ) (see, *e.g.*, Luo et al., Blood. 1 13:1422-1431, 2009, and U.S. Patent Application Publication No. 2010/0203630). Accordingly, certain embodiments may employ one or more of these promoter or enhancer elements.

In one embodiment, one promoter drives expression of a selectable marker and a second promoter drives expression of the gene of interest. For example, in one embodiment, the EF-1 alpha promoter drives the production of a selection marker (*e.g.*, DHFR) and a miniature CAG promoter (see, *e.g.*, Fan et al. Human Gene Therapy 10:2273–2285, 1999) drives expression of the gene of interest (*e.g.*, IDUA).

As noted above, certain embodiments employ enhancer elements, such as an internal enhancer, to increase expression of the gene of interest. Enhancers are cis-acting elements of DNA, usually about 10 to 300 bp in length, that act on a promoter to increase its transcription. Enhancer sequences may be derived from mammalian genes (*e.g.*, globin, elastase, albumin, α-fetoprotein, insulin), such as the □□□ enhancer, the □□□ intronic enhancer, and the 3' □□ enhancer. Also included are enhancers from a eukaryotic virus, including the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. Enhancers may be spliced into the vector at a position 5' or 3' to the antigen- specific polynucleotide sequence, but are preferably located at a site 5' from the promoter. Persons of skill in the art will select the appropriate enhancer based on the desired expression pattern.

In certain embodiments, promoters are selected to allow for inducible expression of the gene. A number of systems for inducible expression are known in the art, including the tetracycline responsive system and the lac operator-repressor system. It is also contemplated that a combination of promoters may be used to obtain the desired expression of the gene of interest. The skilled artisan will be able to select a promoter based on the desired expression pattern of the gene in the organism and/or the target cell of interest.

Certain viral vectors contain cis-acting packaging sequences to promote incorporation of the genomic viral RNA into the viral particle. Examples include psi-sequences. Such cis-acting sequences are known in the art. In certain embodiments, the viral vectors described herein may express two or more genes, which may be accomplished, for example, by incorporating an internal promoter that is operably linked to each separate gene beyond the first gene, by incorporating an element that facilitates co-expression such as an internal ribosomal entry sequence (IRES) element (U.S. Pat. No. 4,937,190, incorporated by reference) or a 2A element, or both. Merely by way of illustration, IRES or 2A elements may be used when a single vector comprises sequences encoding each chain of an immunoglobulin molecule with a desired specificity. For instance, the first coding region (encoding either the heavy or light chain) may be located immediately downstream from the promoter, and the second coding region (encoding the other chain) may be located downstream from the first coding region, with an IRES or 2A element located between the first and second coding regions, preferably immediately

preceding the second coding region. In other embodiments, an IRES or 2A element is used to co-express an unrelated gene, such as a reporter gene, a selectable marker, or a gene that enhances immune function. Examples of IRES sequences that can be used include, without limitation, the IRES elements of encephalomyelitis virus (EMCV), foot-and- mouth disease virus (FMDV), Theiler's murine encephalomyelitis virus (TMEV), human rhinovirus (HRV), coxsackievirus (CSV), poliovirus (POLIO), Hepatitis A virus (HAV), Hepatitis C virus (HCV), and Pestiviruses (*e.g.*, hog cholera virus (HOCV) and bovine viral diarrhea virus (BVDV)) (see, *e.g.*, Le et al., Virus Genes 12:135-147, 1996; and Le et al., Nuc. Acids Res. 25:362-369, 1997, each of which is incorporated by reference in their entirety). One example of a 2A element includes the F2A sequence from foot-and-mouth disease virus.

In certain embodiments, the vectors provided herein also contain additional genetic elements to achieve a desired result. For example, certain viral vectors may include a signal that facilitates nuclear entry of the viral genome in the target cell, such as an HIV-1 flap signal. As a further example, certain viral vectors may include elements that facilitate the characterization of the provirus integration site in the target cell, such as a tRNA amber suppressor sequence. Certain viral vectors may contain one or more genetic elements designed to enhance expression of the gene of interest. For example, a woodchuck hepatitis virus responsive element (WRE) may be placed into the construct (see, *e.g.*, Zufferey et al., J. Virol. 74:3668-3681, 1999; and Deglon et al., Hum. Gene Ther. 11:179-190, 2000, each of which is incorporated by reference in its entirety). As another example, a chicken β-globin insulator may also be included in the construct. This element has been shown to reduce the chance of silencing the integrated DNA in the target cell due to methylation and heterochromatinization effects. In addition, the insulator may shield the internal enhancer, promoter and exogenous gene from positive or negative positional effects from surrounding DNA at the integration site on the chromosome. Certain embodiments employ each of these genetic elements. In another embodiment, the viral vectors provided herein may also contain a Ubiquitous Chromatin Opening Element (UCOE) to increase expression (see *e.g.*, Zhang F, et al., Molecular Therapy: The journal of the American Society of Gene Therapy 2010 Sep;18(9):1640–9.)

In certain embodiments, the viral vectors (*e.g.*, retroviral, lentiviral) provided herein are “pseudo-typed” with one or more selected viral glycoproteins or

envelope proteins, mainly to target selected cell types. Pseudo-typing refers to generally to the incorporation of one or more heterologous viral glycoproteins onto the cell-surface virus particle, often allowing the virus particle to infect a selected cell that differs from its normal target cells. A "heterologous" element is derived from a virus other than the virus from which the RNA genome of the viral vector is derived. Typically, the glycoprotein-coding regions of the viral vector have been genetically altered such as by deletion to prevent expression of its own glycoprotein. Merely by way of illustration, the envelope glycoproteins gp41 and/or gp120 from an HIV-derived lentiviral vector are typically deleted prior to pseudo-typing with a heterologous viral glycoprotein.

In certain embodiments, the viral vector is pseudo-typed with a heterologous viral glycoprotein that targets B lymphocytes. In certain embodiments, the viral glycoprotein allows selective infection or transduction of resting or quiescent B lymphocytes. In certain embodiments, the viral glycoprotein allows selective infection of B lymphocyte plasma cells, plasmablasts, and activated B cells. In certain embodiments, the viral glycoprotein allows infection or transduction of quiescent B lymphocytes, plasmablasts, plasma cells, and activated B cells. In certain embodiments, viral glycoprotein allows infection of B cell chronic lymphocyte leukemia cells. In one embodiment, the viral vector is pseudo-typed with VSV-G. In another embodiment, the heterologous viral glycoprotein is derived from the glycoprotein of the measles virus, such as the Edmonton measles virus. Certain embodiments pseudo-type the measles virus glycoproteins hemagglutinin (H), fusion protein (F), or both (see, *e.g.*, Frecha et al., Blood. 1 12:4843-52, 2008; and Frecha et al., Blood. 1 14:3173-80, 2009, each of which is incorporated by reference in its entirety). In one embodiment, the viral vector is pseudo-typed with gibbon ape leukemia virus (GALV). In one embodiment, the viral vector is pseudo-typed with cat endogenous retrovirus (RD114). In one embodiment, the viral vector is pseudo-typed with baboon endogenous retrovirus (BaEV). In one embodiment, the viral vector is pseudo-typed with murine leukemia virus (MLV). In one embodiment, the viral vector is pseudo-typed with gibbon ape leukemia virus (GALV). In further embodiments, the viral vector comprises an embedded antibody binding domain, such as one or more variable regions (*e.g.*, heavy and light chain variable regions) which serves to target the vector to a particular cell type.

Generation of viral vectors can be accomplished using any suitable genetic engineering techniques known in the art, including, without limitation, the standard

techniques of restriction endonuclease digestion, ligation, transformation, plasmid purification, PCR amplification, and DNA sequencing, for example as described in Sambrook et al. (Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, N.Y. (1989)), Coffin et al. (Retroviruses. Cold Spring Harbor Laboratory Press, N.Y. (1997)) and "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, (2000)).

Any variety of methods known in the art may be used to produce suitable retroviral particles whose genome comprises an RNA copy of the viral vector. As one method, the viral vector may be introduced into a packaging cell line that packages the viral genomic RNA based on the viral vector into viral particles with a desired target cell specificity. The packaging cell line typically provides in trans the viral proteins that are required for packaging the viral genomic RNA into viral particles and infecting the target cell, including the structural gag proteins, the enzymatic pol proteins, and the envelope glycoproteins.

In certain embodiments, the packaging cell line stably expresses certain necessary or desired viral proteins (*e.g.*, gag, pol) (see, *e.g.*, U.S. Pat. No. 6,218,181, herein incorporated by reference). In certain embodiments, the packaging cell line is transiently transfected with plasmids that encode certain of the necessary or desired viral proteins (*e.g.*, gag, pol, glycoprotein), including the measles virus glycoprotein sequences described herein. In one exemplary embodiment, the packaging cell line stably expresses the gag and pol sequences, and the cell line is then transfected with a plasmid encoding the viral vector and a plasmid encoding the glycoprotein. Following introduction of the desired plasmids, viral particles are collected and processed accordingly, such as by ultracentrifugation to achieve a concentrated stock of viral particles. Exemplary packaging cell lines include 293 (ATCC CCL X), HeLa (ATCC CCL 2), D17 (ATCC CCL 183), MDCK (ATCC CCL 34), BHK (ATCC CCL-10) and Cf2Th (ATCC CRL 1430) cell lines.

**Therapeutic Agent**

As used herein "gene of interest" or "gene" or "nucleic acid of interest" refers to a transgene to be expressed in the target transfected cell. While the term "gene" may be used, this is not to imply that this is a gene as found in genomic DNA and is used interchangeably with the term "nucleic acid". Generally, the nucleic acid of interest

provides suitable nucleic acid for encoding a therapeutic agent and may comprise cDNA or DNA and may or may not include introns, but generally does not include introns. As noted elsewhere, the nucleic acid of interest is operably linked to expression control sequences to effectively express the protein of interest in the target cell. In certain embodiments, the vectors described herein may comprise one or more genes of interest, and may include 2, 3, 4, or 5 or more genes of interest, such as for example, the heavy and light chains of an immunoglobulin that may be organized using an internal promoter as described herein.

The recitation "polynucleotide" or "nucleic acid" as used herein designates mRNA, RNA, cRNA, cDNA or DNA. The term typically refers to polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA and RNA. The nucleic acid or gene of interest may be any nucleic acid encoding a protein of interest.

A therapeutic agent to be delivered by a genetically modified B cell as described herein may be a protein. A protein of interest for use as described herein comprises any protein providing an activity desired. In this regard, a protein of interest includes, but is not limited to, an antibody or antigen-binding fragment thereof, a cell surface receptor, a secreted protein such as a cytokine (lymphokines, interleukins, interferons, or chemokines), other secreted signaling molecules such as TGF-beta and fibroblast growth factor, an antigenic fragment of a protein, a DNA-encoded small molecule (see *e.g.*, Nature Chemical Biology 5, 647 - 654 (2009)), an enzyme, a clotting factor, and an adhesion molecule. In one embodiment, the nucleic acid encodes an antibody or antigen-binding fragment thereof. Exemplary antigen binding fragments include domain antibodies, sFv, scFv, Fab, Fab', F(ab')2, and Fv. In one embodiment, the nucleic acid encodes the protein of interest as a fusion protein comprising a cleavable linker. For example, an antibody heavy chain and a light chain can be expressed with a self-cleavable linker peptide, *e.g.*, F2A.

In one embodiment, the antibody encoded by the nucleic acid comprises at least the antigen binding domain of the HIV neutralizing antibody, b12 (see, *e.g.*, J Virol 2003, 77:5863- 5876; J Virol. 1994 Aug; 68(8):4821 -8; Proc Natl Acad Sci U S A. 1992, 89:9339-9343; exemplary sequences are provided in GenBank Accession Nos. for the b12 light chain (AAB26306.1 Gl 299737) and heavy chain (AAB26315.1 Gl

299746)). In a further embodiment, the antibody encoded by the nucleic acid of interest comprises Fuzeon(TM) (T-20 / enfuvirtide / pentafuside / DP-178). DP-178 is an amino acid sequence from gp41 on HIV and interferes with HIV's ability to fuse with its target cell. Fuzeon may be produced synthetically using methods known to the skilled person (see *e.g.*, 2001 J. Virol. 75:3038-3042; It should be noted that it is highly unlikely that the methods described in this paper resulted in secretion of a therapeutic dose of the DP-178 peptide).

In one particular embodiment, the nucleic acid of interest encodes an immunologically active protein. In certain embodiments, a nucleic acid of interest encodes a protein, or a biologically active fragment thereof (*e.g.*, an antigenic fragment), that induces an immune vaccine-like reaction through the presentation of the protein on the surface of a B cell, T cell or other immune cell. In certain embodiments, the protein of interest influences the regulation of B cells, for example but not limited to promoting cell division, promoting differentiation into different B lineages, inactivating or killing cells, or regulates production or activity of other introduced DNA elements. Interleukins are known to the skilled person and to date include IL-1 , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21 , IL-22, IL-23, IL-24, IL- 25, IL-26, IL-27, secreted form of the p28 subunit of IL27, IL-28, IL-29, IL-30, IL-31, IL-32, IL-33, IL-34, and IL-35. Interferons include IFN-γ, IFN-α, IFN-β and IFN-o. The chemokines contemplated for use herein include the C type chemokines XCL1 and XCL2, C-C type chemokines (to date including CCL1 - CCL28) and CXC type chemokines (to date including CXCL1 -CXCL17). Also contemplated as a gene of interest are members of the TNF superfamily (*e.g.*, TNF-a, 4-1 BB ligand, B cell activating factor, FAS ligand, Lymphotoxin, OX40L RANKL, and TRAIL).

In certain embodiments, the protein of interest induces immunological tolerance. In this regard, the protein of interest may comprise an IgG-antigen fusion protein (see *e.g.*, Cellular Immunology 235(1), 2005, 12-20). In certain embodiments, expression of a protein of interest may be accompanied by stimulation of the cells with factors such as TGF-β, IL-10 and LPS. In certain embodiments, factors such as IL-10 or transcription factors that induce tolerance are expressed with the cultured B cells.

In a further embodiment, the gene(s) of interest encodes one or more factors that promote differentiation of the B cell into an antibody secreting cell and/or

one or more factors that promote the longevity of the antibody producing cell. Such factors include, for example, Blimp-1, Xbp1, IRF4, Zbtb20, TRF4, anti-apoptotic factors like Bcl-xl, Bcl-2, Mcl-1, or Bcl5, and constitutively active mutants of the CD40 receptor. Further genes of interest encode factors which promote the expression of downstream signaling molecules such as TNF receptor- associated factors (TRAFs). In this regard, cell activation, cell survival, and anti-apoptotic functions of the TNF receptor superfamily are mostly mediated by TRAF 1-6 (see *e.g.*, R.H. Arch, et al., Genes Dev. 12 (1998), pp. 2821-2830). Downstream effectors of TRAF signaling include transcription factors in the NF-KB and AP-1 family which can turn on genes involved in various aspects of cellular and immune functions. Further, the activation of NF-κβ and AP-1 has been shown to provide cells protection from apoptosis via the transcription of anti-apoptotic genes. In an additional embodiment the encoded factor, such as IL-10, IL-35, TGF-beta or an Fc-fusion protein, is associated with induction of immune tolerance.

In an additional embodiment, the nucleic acid(s) of interest encodes one or more Epstein Barr virus (EBV)-derived proteins. EBV-derived proteins include but are not limited to, EBNA-1, EBNA-2, EBNA-3, LMP-1, LMP-2, EBER, EBV-EA, EBV-MA, EBV-VCA and EBV-AN. In one particular embodiment, the nucleic acid of interest encodes an antibody or an antigen-binding fragment thereof. In this regard, the antibody may be a natural antibody or a custom, recombinantly engineered antibody. Fusion proteins comprising an antibody or portion thereof are specifically contemplated to be encoded by the vectors described herein.

In one embodiment, an antibody or fragment thereof according to the present disclosure has an amino acid sequence of an anti-HIV antibody, such as the m36 anti-HIV antibody (see *e.g.*, Proc Natl Acad Sci U S A. 2008 Nov 4;105(44):17121 -6), or an amino acid molecule having at least 60%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity with an amino acid sequence of an anti-HIV antibody, such as m36. In particular, fusion proteins comprising m36, or derivatives thereof, are specifically contemplated, such as m36L2CD4Fc (see *e.g.*, Antiviral Research volume 88, Issue 1, October 2010, Pages 107-1 15). In one embodiment, the anti-HIV antibody is the broadly neutralizing monoclonal antibody VRC01 (see, *e.g.*, Wu et al., Science, 2010, 329(5993):856861 and Li et al., J Virol, 2011, 85(17):8954-8967).

In a further embodiment, the antibody encoded by the transgene of the disclosure binds to an autoantigen. In certain embodiments, the autoantigen in this regard

is associated with the development of multiple sclerosis or Type 1 diabetes, including but not limited to MBP, alphaB-crystallin, S100beta, proteolipid protein (PLP), HSP105, epithelial isoform of bullous pemphigoid (BP) antigen 1 (BPAG1-e), lipids, and myelin oligodendrocyte glycoprotein (MOG)-alpha and MOG-beta isoforms or any of a variety of islet cell autoantigens (*e.g.*, sialoglycolipid, glutamate decarboxylase, insulin, insulin receptor, 38 kD, bovine serum albumin, glucose transporter, hsp 65, carboxypeptidase H, 52 kD, ICA 12/ICA512, 150 kD, and RIN polar). Antibodies to these autoantigens are known in the art and may be sequenced and made recombinantly using routine techniques (see *e.g.*, J. Clin. Invest. 107(5): 555-564 (2001)).

In a further embodiment, the antibody binds to a cancer- associated antigen. Cancer-associated antigens may be derived from a variety of tumor proteins. Illustrative tumor proteins useful in the present disclosure include, but are not limited to any one or more of, p53, MAGE-A1 , MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A6, MAGE-A10, MAGE-A12, BAGE, DAM-6, -10, GAGE-1 , -2, -8, GAGE-3, -4, -5, -6, -7B, NA88-A, NY-ESO-1 , MART-1 , MC1 R, Gp100, PSA, PSM, Tyrosinase, TRP-1 , TRP-2, ART-4, CAMEL, CEA, Cyp-B, Her2/neu (*e.g.*, the antibody may be derived from the Her2-specific mAb, Herceptin(R)), hTERT, hTRT, iCE, MUC1 , MUC2, PRAME, P15, RU1 , RU2, SART-1 , SART-3, WT1 , AFP, □-catenin/m, Caspase-8/m, CEA, CDK-4/m, ELF2M, GnT-V, G250, HSP70-2M, HST-2, KIAA0205, MUM-1 , MUM-2, MUM- 3, Myosin/m, RAGE, SART-2, TRP-2/INT2, 707-AP, Annexin II, CDC27/m, TPI/mbcr-abl, ETV6/AML, LDLR/FUT, Pml/RARa, and TEL/AML1 . These and other tumor proteins are known to the skilled artisan.

In further embodiments, the nucleic acid of interest encodes a peptide or other binding domain with a particular functional attribute, such as, but not limited to, an inhibitory activity, ability to induce cell death in cancer cells, or ability to slow or inhibit cancer cell proliferation. In this regard, in one embodiment, a peptide or binding domain encoded by the nucleic acid of interest may bind any of the target proteins described herein, such as a cancer- associated antigen as described above, CD4, HIV gp120 or other viral protein, ICAM-3, DC-SIGN (see *e.g.*, U.S. patent 7,301,010). In certain embodiments, the peptides may be derived from pathogenic and nonpathogenic bacteria and green plants. Illustrative peptides are disclosed in U.S. patents 7084105, 7301010, 7338766, 7381701, 7491394, 7511117, 7556810. In one embodiment, the nucleic acid of interest encodes azurin-p28 (NSC745104) a peptide inhibitor of p53 ubiquitination (see

*e.g.*, Cancer Chemother Pharmacol 2010, DOI 10.1007/S00280-010-1518-3; U.S. Patent 7,084,105). In a further embodiment, the nucleic acid of interest encodes a factor known as Ghrelin, which induces appetite and can be used to treat cancer patients (see *e.g.*, Obes Facts. 2010 3:285-92; FASEB J. 18 (3): 439-56). In another embodiment, the nucleic acid of interest encodes a binding peptide that binds to and inhibits angiopoietin 1 and 2 (see, *e.g.*, AMG386, an Fc fragment of an antibody (peptibody) used to treat cancer; In certain embodiments, tumor antigens may be identified directly from an individual with cancer. In this regard, screens can be carried out using a variety of known technologies. For example, in one embodiment, a tumor biopsy is taken from a patient, RNA is isolated from the tumor cells and screened using a gene chip (for example, from Affymetrix, Santa Clara, CA) and a tumor antigen is identified. Once the tumor target antigen is identified, it may then be cloned, expressed and purified using techniques known in the art.

In one particular embodiment, the nucleic acid of interest encodes an enzyme. In one embodiment, the nucleic acid of interest encodes an enzyme to treat a lysosomal storage disorder. In one embodiment, the nucleic acid of interest encodes iduronidase (IDUA) for treatment or prevention of mucopolysaccharidosis type I (MPS I). In one embodiment, the nucleic acid of interest encodes idursulfase for treatment or prevention of mucopolysaccharidosis type II (MPS II). In one embodiment, the nucleic acid of interest encodes galsulfase for treatment or prevention of mucopolysaccharidosis type VI (MPS VI). In one embodiment, the nucleic acid of interest encodes elosulfase alfa for treatment or prevention of mucopolysaccharidosis type IVA (MPS IVA). In one embodiment, the nucleic acid of interest encodes agalsidase beta for treatment or prevention of Fabry's disease. In one embodiment, the nucleic acid of interest encodes agalsidase alpha for treatment or prevention of Fabry's disease. In one embodiment, the nucleic acid of interest encodes alpha-1-anti-trypsin for treatment or prevention of Alpha-1-anti-trypsin deficiency. In one embodiment, the nucleic acid of interest encodes alpha-N-acetylglucosaminidase for treatment or prevention of mucopolysaccharidosis type IIIB (MPS IIIB). In another embodiment, the nucleic acid of interest encodes factor VII for treatment or prevention of hemophilia. In one embodiment, the nucleic acid of interest encodes lecithin-cholesterol acyltransferase (LCAT) useful for treatment or prevention of, *e.g.*, LCAT deficiency and atherosclerosis. In another embodiment, the nucleic acid of interest encodes Apolipoprotein A-1 Milano (ApoA-1 Milano) for treatment or prevention of cardiovascular diseases and disorders, such as, *e.g.*, atherosclerosis. In one

embodiment, the nucleic acid of interest encodes lipoprotein lipase (LPL) for treatment or prevention of LPL deficiency. In another embodiment, the nucleic acid of interest encodes a broadly neutralizing antibody (bNAb), or a fusion protein thereof, that binds to and neutralizes multiple HIV-1 strains (*e.g.*, b12). In yet another embodiment, the nucleic acid of interest encodes phenylalanine hydroxylase for treatment or prevention of phenyketonuria (PKU).

An "antibody", as used herein, includes both polyclonal and monoclonal antibodies; primatized (*e.g.*, humanized); murine; mouse-human; mouse-primate; and chimeric; and may be an intact molecule, a fragment thereof (such as scFv, Fv, Fd, Fab, Fab' and F(ab)'2 fragments), or multimers or aggregates of intact molecules and/or fragments; and may occur in nature or be produced, *e.g.*, by immunization, synthesis or genetic engineering; an "antibody fragment," as used herein, refers to fragments, derived from or related to an antibody, which bind antigen and which in some embodiments may be derivatized to exhibit structural features that facilitate clearance and uptake, *e.g.*, by the incorporation of galactose residues. This includes, *e.g.*, F(ab), F(ab)'2, scFv, light chain variable region (VL), heavy chain variable region (VH), and combinations thereof. Sources include antibody gene sequences from various species (which can be formatted as antibodies, sFvs, scFvs or Fabs, such as in a phage library), including human, camelid (from camels, dromedaries, or llamas; Hamers-Casterman et al. (1993) Nature, 363:446 and Nguyen et al. (1998) J. Mol. Biol., 275:413), shark (Roux et al. (1998) Proc. Nat'l. Acad. Sci. (USA) 95:1 1804), fish (Nguyen et al. (2002) Immunogenetics, 54:39), rodent, avian, ovine, sequences that encode random peptide libraries or sequences that encode an engineered diversity of amino acids in loop regions of alternative non-antibody scaffolds, such as fibrinogen domains (see, *e.g.*, Weisel et al. (1985) Science 230:1388), Kunitz domains (see, *e.g.*, US Patent No. 6,423,498), lipocalin domains (see, *e.g.*, WO 2006/095164), V-like domains (see, *e.g.*, US Patent Application Publication No. 2007/0065431 ), C-type lectin domains (Zelensky and Gready (2005) FEBS J. 272:6179), etc. (see, *e.g.*, PCT Patent Application Publication Nos. WO 2007/098934; WO 2006/072620), or the like.

Terms understood by those in the art as referring to antibody technology are each given the meaning acquired in the art, unless expressly defined herein. For example, the terms "VL" and "VH" refer to the variable binding region derived from an antibody light and heavy chain, respectively. The variable binding regions are made up

of discrete, well-defined sub-regions known as "complementarity determining regions" (CDRs) and "framework regions" (FRs). The terms "CL" and "CH" refer to an "immunoglobulin constant region," *i.e.*, a constant region derived from an antibody light or heavy chain, respectively, with the latter region understood to be further divisible into Cm, CH2, CH3 and CH4 constant region domains, depending on the antibody isotype (IgA, IgD, IgE, IgG, IgM) from which the region was derived. A portion of the constant region domains makes up the Fc region (the "fragment crystallizable" region), which contains domains responsible for the effector functions of an immunoglobulin, such as ADCC (antibody-dependent cell-mediated cytotoxicity), CDC (complement-dependent cytotoxicity) and complement fixation, binding to Fc receptors, greater half-life *in vivo* relative to a polypeptide lacking an Fc region, protein A binding, and perhaps even placental transfer (see Capon et al. (1989) Nature, 337:525). Further, a polypeptide containing an Fc region allows for dimerization or multimerization of the polypeptide.

The domain structure of immunoglobulins is amenable to engineering, in that the antigen binding domains and the domains conferring effector functions may be exchanged between immunoglobulin classes and subclasses. For example, amino acid changes (*e.g.*, deletions, insertions, substitutions) may alter post-translational processes of the immunoglobulin, such as changing the number or position of glycosylation and/or fucosylation sites. Methods for enhancing ADCC via glycosylation are known in the art and contemplated for use herein. For example, enzymes that enhance glycosylation may be co-expressed with the antibody. In one embodiment, MGAT3 is overexpressed in cells producing the antibody to enhance glycosylation of the antibody and its ADCC function. In one embodiment, inhibition of Fut8 via, *e.g.*, siRNA, enhances glycosylation of the antibody and ADCC.

Immunoglobulin structure and function are reviewed, for example, in Harlow et al., Eds., Antibodies: A Laboratory Manual, Chapter 14 (Cold Spring Harbor Laboratory, Cold Spring Harbor, 1988). An extensive introduction as well as detailed information about all aspects of recombinant antibody technology can be found in the textbook Recombinant Antibodies (John Wiley & Sons, NY, 1999). A comprehensive collection of detailed antibody engineering lab Protocols can be found in R. Kontermann and S. Dubel, Eds., The Antibody Engineering Lab Manual (Springer Verlag, Heidelberg/New York, 2000). Further related protocols are also available in Current Protocols in Immunology (August 2009,) published by John Wiley & Sons, Inc., Boston,

MA. Methods for production of enzymes and protein engineering (*e.g.*, IDUA) are also known in the art and contemplated for use herein.

Thus, this disclosure provides polynucleotides (isolated or purified or pure polynucleotides) encoding therapeutic agents (e.g., proteins of interest) of this disclosure for genetically modifying B cells, vectors (including cloning vectors and expression vectors) comprising such polynucleotides, and cells (*e.g.*, host cells) transformed or transfected with a polynucleotide or vector according to this disclosure. In certain embodiments, a polynucleotide (DNA or RNA) encoding a protein of interest of this disclosure is contemplated. Expression cassettes encoding proteins of interest are also contemplated herein.

The present disclosure also relates to vectors that include a polynucleotide of this disclosure and, in particular, to recombinant expression constructs. In one embodiment, this disclosure contemplates a vector comprising a polynucleotide encoding a protein of this disclosure, along with other polynucleotide sequences that cause or facilitate transcription, translation, and processing of such a protein-encoding sequences. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described, for example, in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, (1989). Exemplary cloning/expression vectors include cloning vectors, shuttle vectors, and expression constructs, that may be based on plasmids, phagemids, phasmids, cosmids, viruses, artificial chromosomes, or any nucleic acid vehicle known in the art suitable for amplification, transfer, and/or expression of a polynucleotide contained therein.

As used herein, unless as otherwise described with regard to viral vectors, "vector" means a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Exemplary vectors include plasmids, minicircles, transposons (e.g., Sleeping Beauty transposon), yeast artificial chromosomes, self-replicating RNAs, and viral genomes. Certain vectors can autonomously replicate in a host cell, while other vectors can be integrated into the genome of a host cell and thereby are replicated with the host genome. In addition, certain vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"), which contain nucleic acid sequences that are operatively linked to an expression control sequence and, therefore, are capable of directing the expression of those sequences. In certain embodiments, expression constructs are derived from plasmid vectors. Illustrative constructs include

modified pNASS vector (Clontech, Palo Alto, CA), which has nucleic acid sequences encoding an ampicillin resistance gene, a polyadenylation signal and a T7 promoter site; pDEF38 and pNEF38 (CMC ICOS Biologies, Inc.), which have a CHEF1 promoter; and pD18 (Lonza), which has a CMV promoter. Other suitable mammalian expression vectors are well known (see, *e.g.*, Ausubel et al., 1995; Sambrook et al., supra; see also, *e.g.*, catalogs from Invitrogen, San Diego, CA; Novagen, Madison, Wl; Pharmacia, Piscataway, NJ).

Useful constructs may be prepared that include a dihydrofolate reductase (DHFR)-encoding sequence under suitable regulatory control, for promoting enhanced production levels of the fusion proteins, which levels result from gene amplification following application of an appropriate selection agent (*e.g.*, methotrexate). In one embodiment, use of a bifunctional transposon encoding a therapeutic gene (e.g., IDUA) along with drug-resistant DHFR in combination with incubation in methotrexate (MTX) to enrich for successfully transposed B cells, generates a more potent product.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence, as described above. A vector in operable linkage with a polynucleotide according to this disclosure yields a cloning or expression construct. Exemplary cloning/expression constructs contain at least one expression control element, *e.g.*, a promoter, operably linked to a polynucleotide of this disclosure. Additional expression control elements, such as enhancers, factor-specific binding sites, terminators, and ribosome binding sites are also contemplated in the vectors and cloning/expression constructs according to this disclosure. The heterologous structural sequence of the polynucleotide according to this disclosure is assembled in appropriate phase with translation initiation and termination sequences. Thus, for example, encoding nucleic acids as provided herein may be included in any one of a variety of expression vector constructs (*e.g.*, minicircles) as a recombinant expression construct for expressing such a protein in a host cell.

The appropriate DNA sequence(s) may be inserted into a vector, for example, by a variety of procedures. In general, a DNA sequence is inserted into an appropriate restriction endonuclease cleavage site(s) by procedures known in the art. Standard techniques for cloning, DNA isolation, amplification and purification, for

enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are contemplated. A number of standard techniques are described, for example, in Ausubel et al. (Current Protocols in Molecular Biology, Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., Boston, MA, 1993); Sambrook et al. (Molecular Cloning, Second Ed., Cold Spring Harbor Laboratory, Plainview, NY, 1989); Maniatis et al. (Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, NY, 1982); Glover (Ed.) (DNA Cloning Vol. I and II, IRL Press, Oxford, UK, 1985); Hames and Higgins (Eds.) (Nucleic Acid Hybridization, IRL Press, Oxford, UK, 1985); and elsewhere.

The DNA sequence in the expression vector is operatively linked to at least one appropriate expression control sequence (*e.g.*, a constitutive promoter or a regulated promoter) to direct mRNA synthesis. Representative examples of such expression control sequences include promoters of eukaryotic cells or their viruses, as described above. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors, kanamycin vectors, or other vectors with selectable markers. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-l. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art, and preparation of certain particularly preferred recombinant expression constructs comprising at least one promoter or regulated promoter operably linked to a nucleic acid encoding a protein or polypeptide according to this disclosure is described herein.

For example, in one embodiment, the vector may be a plasmid having the structure shown in FIG. 1b. In one embodiment, the plasmid may comprise a sequence of SEQ ID NO: 1. In one embodiment, the plasmid may consist of a sequence of SEQ ID NO: 1. In one embodiment, the plasmid may comprise or consist of a sequence that is at least about 60% identical to SEQ ID NO: 1. In one embodiment, the plasmid may comprise or consist of a sequence that is at least about 85% identical to SEQ ID NO: 1, or at least about 90%, 95%, 96%, 97%, 98%, 99%, or greater than 99% identical to SEQ ID NO: 1.

Variants of the polynucleotides of this disclosure are also contemplated. Variant polynucleotides are at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, and preferably 95%, 96%, 97%, 98%, 99%, or 99.9% identical to one of the polynucleotides

of defined sequence as described herein, or that hybridizes to one of those polynucleotides of defined sequence under stringent hybridization conditions of 0.015M sodium chloride, 0.0015M sodium citrate at about 65-68°C or 0.015M sodium chloride, 0.0015M sodium citrate, and 50% formamide at about 42°C. The polynucleotide variants retain the capacity to encode a binding domain or fusion protein thereof having the functionality described herein.

The term "stringent" is used to refer to conditions that are commonly understood in the art as stringent. Hybridization stringency is principally determined by temperature, ionic strength, and the concentration of denaturing agents such as formamide. Examples of stringent conditions for hybridization and washing are 0.015M sodium chloride, 0.0015M sodium citrate at about 65-68°C or 0.015M sodium chloride, 0.0015M sodium citrate, and 50% formamide at about 42°C (see Sambrook et ai, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989). More stringent conditions (such as higher temperature, lower ionic strength, higher formamide, or other denaturing agent) may also be used; however, the rate of hybridization will be affected. In instances wherein hybridization of deoxyoligonucleotides is concerned, additional exemplary stringent hybridization conditions include washing in 6x SSC, 0.05% sodium pyrophosphate at 37°C (for 14-base oligonucleotides), 48°C (for 17-base oligonucleotides), 55°C (for 20-base oligonucleotides), and 60°C (for 23-base oligonucleotides).

A further aspect of this disclosure provides a host cell transformed or transfected with, or otherwise containing, any of the polynucleotides or vector/expression constructs of this disclosure. The polynucleotides or cloning/expression constructs of this disclosure are introduced into suitable cells using any method known in the art, including transformation, transfection and transduction. Host cells include the cells of a subject undergoing *ex vivo* cell therapy including, for example, *ex vivo* gene therapy. Eukaryotic host cells contemplated as an aspect of this disclosure when harboring a polynucleotide, vector, or protein according to this disclosure include, in addition to a subject's own cells (*e.g.*, a human patient's own cells), VERO cells, HeLa cells, Chinese hamster ovary (CHO) cell lines (including modified CHO cells capable of modifying the glycosylation pattern of expressed multivalent binding molecules, see US Patent Application Publication No. 2003/01 15614), COS cells (such as COS-7), W138, BHK, HepG2, 3T3, RIN, MDCK, A549, PC12, K562, HEK293 cells, HepG2 cells, N cells, 3T3 cells,

Spodoptera frugiperda cells (*e.g.*, Sf9 cells), Saccharomyces cerevisiae cells, and any other eukaryotic cell known in the art to be useful in expressing, and optionally isolating, a protein or peptide according to this disclosure. Also contemplated are prokaryotic cells, including Escherichia coli, Bacillus subtilis, Salmonella typhimurium, a Streptomycete, or any prokaryotic cell known in the art to be suitable for expressing, and optionally isolating, a protein or peptide according to this disclosure. In isolating protein or peptide from prokaryotic cells, in particular, it is contemplated that techniques known in the art for extracting protein from inclusion bodies may be used. The selection of an appropriate host is within the scope of those skilled in the art from the teachings herein. Host cells that glycosylate the fusion proteins of this disclosure are contemplated.

The term "recombinant host cell" (or simply "host cell") refers to a cell containing a recombinant expression vector. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term "host cell" as used herein. Recombinant host cells can be cultured in a conventional nutrient medium modified as appropriate for activating promoters, selecting transformants, or amplifying particular genes. The culture conditions for particular host cells selected for expression, such as temperature, pH and the like, will be readily apparent to the ordinarily skilled artisan. Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman (1981) Cell 23:175, and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and, optionally, enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5'-flanking nontranscribed sequences, for example, as described herein regarding the preparation of multivalent binding protein expression constructs. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Introduction of the construct into the host cell can be effected by a variety of methods with which those skilled in the art will be familiar, including calcium phosphate transfection, DEAE-Dextran-mediated

transfection, or electroporation (Davis et al. (1986) Basic Methods in Molecular Biology).

**Cells and Compositions**

In one embodiment, the modified B cells described herein have been activated/differentiated *in vitro* and transfected to express a therapeutic agent as described herein. In one embodiment, the modified B cells described herein have been activated/differentiated *in vitro* and engineered (e.g., using a targeted transgene integration approach such as a zinc finger nuclease, TALEN, meganuclease, or CRISPR/CAS9-mediated transgene integration) to express a therapeutic agent as described herein. In one embodiment, the compositions comprise B cells that have differentiated into plasma B cells, have been transfected or otherwise engineered and express one or more proteins of interest. Target cell populations, such as the transfected or otherwise engineered and activated B cell populations of the present disclosure may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as cytokines or cell populations.

In one embodiment, the modified B cells that have been engineered to express one or more proteins of interest are harvested from culture after activation/differentiation *in vitro* at a time-point at which the modified B cells have optimal migratory capacity for a particular chemoattractant. In some embodiments, the optimal migratory capacity may be on day 7, day 8, or day 9 of the B cell culture. In some embodiments, the optimal migratory capacity may be on day 5, day 6, or day 7 of the B cell culture after transfection or engineering. In some embodiments, the optimal migratory capacity may be on day 8 of the B cell culture after transfection or engineering or later in culture than day 8 after transfection or engineering (e.g., day 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or later than day 20). In some embodiments, the optimal migratory capacity may be prior to day 10 of the B cell culture. In some embodiments, the optimal migratory capacity may be prior to day 8 of the B cell culture after transfection or engineering. In some embodiments, the optimal migratory capacity may be on day 6 or day 7 of the B cell culture. In some embodiments, the optimal migratory capacity may be on day 4 or day 5 of the B cell culture after transfection or engineering. In some embodiments, the optimal migratory capacity may be prior to day 9 of the B cell culture. In some embodiments, the optimal migratory capacity may be prior to day 7 of

the B cell culture after transfection or engineering. In some embodiments, the optimal migratory capacity is optimal for modified B cell homing to CXCL12. In some embodiments, the optimal migratory capacity is optimal for modified B cell homing to the bone marrow of a subject receiving one or more administration of the modified B cells. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL12 and/or to the bone marrow of a subject on from about day 7 to about day 9 in culture. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL12 and/or to the bone marrow of a subject on from about day 5 to about day 7 in culture after transfection or engineering. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL12 and/or to the bone marrow of a subject prior to about day 10 in culture. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL12 and/or to the bone marrow of a subject prior to about day 8 in culture after transfection or engineering. In some embodiments, the optimal migratory capacity is optimal for modified B cell homing to CXCL13. In some embodiments, the optimal migratory capacity is optimal for modified B cell homing to a site of inflammation in a subject receiving one or more administration of the modified B cells. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL13 and/or to a site of inflammation in the subject on about day 6 or about day 7 in culture. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL13 and/or to a site of inflammation in the subject on about day 4 or about day 5 in culture after transfection or engineering. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL13 and/or to a site of inflammation prior to about day 10 in culture. In some embodiments, the B cells are harvested for administration to a subject at optimal migratory capacity to CXCL13 and/or to a site of inflammation prior to about day 8 in culture after transfection or engineering.

In some embodiments, the optimal migratory capacity is optimal for modified B cell homing to both CXCL12 and CXCL13. In some embodiments, the B cells are harvested at optimal migratory capacity for homing to both CXCL12 and CXCL13 on day 7 of the B cell culture. In some embodiments, the B cells are harvested

at optimal migratory capacity for homing to both CXCL12 and CXCL13 on day 5 of the B cell culture after transfection or engineering.

In some embodiments, the engineered B cells are harvested when at least about 20%, of the B cells migrate in a chemotaxis assay to a particular chemoattractant. For example, but not to be limited by example, the engineered B cells (e.g., that produce IDUA) may be harvested when at least about 20% of the B cells migrate in a chemotaxis assay to CXCL12. Or, in another non-limiting example, the engineered B cells (e.g., that produce IDUA) may be harvested when at least about 20% of the B cells migrate in a chemotaxis assay to CXCL13. Furthermore, the engineered B cells (e.g., that produce IDUA) may be harvested when at least about 30% of the B cells migrate in a chemotaxis assay to a particular chemoattractant (e.g., CXCL12 or CXCL13), or when at least about 40%, 45%, 50%, 55%, 60%, 65%, or at least about 70% of the B cells migrate in a chemotaxis assay to a particular chemoattractant (e.g., CXCL12 or CXCL13). Furthermore, the engineered B cells (e.g., that produce IDUA) may be harvested when more than 70% of the B cells migrate in a chemotaxis assay. Such chemotaxis assays are known in the art and are described herein (see, e.g., Example 6 herein).

Briefly, cell compositions of the present disclosure may comprise a differentiated and activated B cell population that has been transfected and is expressing a therapeutic agent as described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline, Lactated Ringer's solution and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (*e.g.*, aluminum hydroxide); and preservatives. Compositions of the present disclosure are preferably formulated for intravenous or subcutaneous administration.

In one embodiment, a cell composition is assessed for purity prior to administration. In another embodiment, a cell composition is tested for robustness of therapeutic agent production. In one embodiment, a cell composition is tested for sterility. In another embodiment, a cell composition is screened to confirm it matches the recipient subject.

In one embodiment, an engineered B cell population is assessed for polyclonality prior to administration to a subject. Ensuring polyclonality of the final cell

product is an important safety parameter. Specifically, the emergence of a dominant clone is viewed as potentially contributing to *in vivo* tumorigenesis or auto-immune disease. Polyclonality may be assessed by any means known in the art or described herein. For example, in some embodiments, polyclonality is assessed by sequencing (e.g., by deep sequencing) the B cell receptors expressed in an engineered B cell population. Since the B cell receptor undergoes changes during B cell development that makes it unique between B cells, this method allows for quantifying how many cells share the same B cell receptor sequence (meaning they are clonal). Thus, in some embodiments, the more B cells in an engineered B cell population that express the same B cell receptor sequence, the more clonal the population and, therefore, the less safe the population is for administration to a subject. Conversely, in some embodiments, the less B cells in an engineered B cell population that express the same B cell receptor sequence, the less clonal the population (*i.e.*, more polyclonal) and, thus, the more safe the population is for administration to a subject.

In some embodiments, the engineered B cells are administered to a subject after they have been determined to be sufficiently polyclonal. For example, the engineered B cells may be administered to a subject after it has been determined that no particular B cell clone in the final population comprises more than about 0.2% of the total B cell population. The engineered B cells may be administered to a subject after it has been determined that no particular B cell clone in the final population comprises more than about 0.1% of the total B cell population, or more than about 0.09%, 0.08%, 0.07%, 0.06%, 0.05%, or about 0.04%, of the total B cell population. In particular embodiments, the engineered B cells (e.g., which produce IDUA) are administered to a subject after it has been determined that no particular B cell clone in the final population comprises more than about 0.03% of the total B cell population.

In one embodiment, a cell composition is stored and/or shipped at 4°C. In another embodiment, a cell composition is frozen for storage and/or shipment. A cell composition may be frozen at, *e.g.*, -20°C or -80°C. In one embodiment, a step of freezing a cell composition comprises liquid nitrogen. In one embodiment, a cell composition is frozen using a controlled rate freezer. Accordingly, methods described herein may further include a thawing step.

**Methods of Use**

One aspect of the present invention is directed to the long term *in vivo* delivery of a therapeutic agent. In particular embodiments, the modified B cells are used in methods of treating and/or preventing chronic diseases and disorders.

Modified B cells described herein may be administered in a manner appropriate to the disease or disorder to be treated or prevented. The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease, although appropriate dosages may be determined by clinical trials.

In one embodiment, a single dose of modified B cells is administered to a subject. In one embodiment, two or more doses of modified B cells are administered sequentially to a subject. In one embodiment, three doses of modified B cells are administered sequentially to a subject. In one embodiment, a dose of modified B cells is administered weekly, biweekly, monthly, bimonthly, quarterly, semiannually, annually, or biannually to a subject. In one embodiment, a second or subsequent dose of modified B cells is administered to a subject when an amount of a therapeutic agent produced by the modified B cells decreases.

In one embodiment, a dose of modified B cells is administered to a subject at a certain frequency (e.g., weekly, biweekly, monthly, biomonthly, or quarterly) until a desired amount (e.g., an effective amount) of a therapeutic agent is detected in the subject. In one embodiment, an amount of the therapeutic agent is monitored in the subject. In one embodiment, a subsequent dose of modified B cells is administered to the subject when the amount of the therapeutic agent produced by the modified B cells decreases below the desired amount. In one embodiment, the desired amount is a range that produces the desired effect. For example, in a method for reducing the amount of glycosaminoglycans (GAGs) in an individual with MPS I, a desired amount of IDUA is an amount that decreases the level of GAGs in a certain tissue in comparison to the level of GAGs in the absence of IDUA.

When “an effective amount”, “an anti-tumor effective amount”, “a tumor-inhibiting effective amount”, or “therapeutic amount” is indicated, the precise amount of the compositions of the present disclosure to be administered can be determined by a physician with consideration of individual differences in age, weight, tumor size, extent of infection or metastasis, and condition of the patient (subject). B cell compositions may also be administered multiple times at an appropriate dosage(s). The cells can be

administered by using infusion techniques that are commonly known in immunotherapy (see, *e.g.*, Rosenberg et al., New Eng. J. of Med. 319:1676, 1988).

The optimal dosage and treatment regime for a particular patient can be determined by one skilled in the art of medicine by monitoring the patient for signs of disease and adjusting the treatment accordingly. The treatment may also be adjusted after measuring the levels of a therapeutic agent (*e.g.*, a gene or protein of interest) in a biological sample (*e.g.*, body fluid or tissue sample) can also be used to assess the treatment efficacy, and the treatment may be adjusted accordingly to increase or decrease. Typically, in related adoptive immunotherapy studies, antigen-specific T cells are administered approximately at 2 x $10^9$ to 2 x $10^{11}$ cells to the patient. (See, *e.g.*, U.S. Pat. No. 5,057,423).

In some aspects of the present disclosure, an optimal dosage of the modified B cells for a multi-dose regime may be determined by first determining an optimal single-dose concentration of the B cells for a subject, decreasing the number of B cells present in the optimal single-dose concentration to provide a sub-optimal single-dose concentration of the modified B cells, and administering two or more dosages of the sub-optimal single-dose concentration of modified B cells to the subject. In some aspects, 2, 3, or more dosages of a sub-optimal single-dose concentration of modified B cells are administered to the subject. In some aspects, the administration of 2, 3, or more dosages of a sub-optimal single-dose concentration of modified B cells to a subject results in synergistic *in vivo* production of a therapeutic polypeptide that the modified B cells are engineered to express. In some aspects, the sub-optimal single-dose concentration comprises 1/2 or 3, 4, 5, 6, 7, 8, 9, 10 fold, or less than the optimal single-dose concentration. In some aspects, the therapeutic polypeptide is IDUA. In some aspects, the therapeutic polypeptide is human coagulation factor X (FIX). In some aspects, the therapeutic polypeptide is human Lecithin–cholesterol acyltransferase (LCAT). In some aspects, the therapeutic polypeptide is human lipoprotein lipase (LPL).

In some aspects of the present disclosure, lower numbers of the transfected B cells of the present disclosure, in the range of $10^6$/kilogram ($10^6$-$10^{11}$ per patient) may be administered. In certain embodiments, the B cells are administered at 1 x $10^4$, 5 x $10^4$, 1 x $10^5$, 5 x $10^5$, 1 x $10^6$, 5 x $10^6$, 1 x $10^7$, 5 x $10^7$, 1 x $10^8$, 5 x $10^8$, 5 x $10^9$, 1 x $10^{10}$, 5 x $10^{10}$, 1 x $10^{11}$, 5 x $10^{11}$, or 1 x $10^{12}$ cells to the subject. B cell compositions may be administered multiple times at dosages within these ranges. The cells may be autologous

or heterologous (e.g., allogeneic) to the patient undergoing therapy. If desired, the treatment may also include administration of mitogens (*e.g.*, PHA) or lymphokines, cytokines, and/or chemokines (*e.g.*, GM-CSF, IL-4, IL-6, IL-13, IL-21, Flt3-L, RANTES, MIP1α, BAFF, etc.) as described herein to enhance induction of an immune response and engraftment of the infused B cells.

The administration of the subject compositions may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The compositions described herein may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intrathecally, intramuscularly, by intravenous (i.v.) injection, or intraperitoneally. The compositions described herein may be administered to a patient directly into the nervous system. In one embodiment, the B cell compositions of the present disclosure are administered to a patient by intradermal or subcutaneous injection. In another embodiment, the B cell compositions as described herein are preferably administered by i.v. injection. The compositions of B cells may be injected directly into a tumor, lymph node, bone marrow or site of infection.

In yet another embodiment, the pharmaceutical composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, 1990, Science 249:1527-1533; Sefton 1987, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald et al., 1980; Surgery 88:507; Saudek et al., 1989, N. Engl. J. Med. 321 :574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, 1974, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla.; Controlled Drug Bioavailability, Drug Product Design and Performance, 1984, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983; J. Macromol. Sci. Rev. Macromol. Chem. 23:61 ; see also Levy et al., 1985, Science 228:190; During et al., 1989, Ann. Neurol. 25:351 ; Howard et al., 1989, J. Neurosurg. 71 :105). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, thus requiring only a fraction of the systemic dose (see, *e.g.*, Medical Applications of Controlled Release, 1984, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla., vol. 2, pp. 1 15-138).

The B cell compositions of the present disclosure may also be administered using any number of matrices. Matrices have been utilized for a number of years within the context of tissue engineering (see, *e.g.*, Principles of Tissue Engineering

(Lanza, Langer, and Chick (eds.)), 1997. The present disclosure utilizes such matrices within the novel context of acting as an artificial lymphoid organ to support and maintain the B cells. Accordingly, the present disclosure can utilize those matrix compositions and formulations which have demonstrated utility in tissue engineering. Accordingly, the type of matrix that may be used in the compositions, devices and methods of the disclosure is virtually limitless and may include both biological and synthetic matrices. In one particular example, the compositions and devices set forth by U.S. Patent Nos: 5,980,889; 5,913,998; 5,902,745; 5,843,069; 5,787,900; or 5,626,561 are utilized. Matrices comprise features commonly associated with being biocompatible when administered to a mammalian host. Matrices may be formed from natural and/or synthetic materials. The matrices may be nonbiodegradable in instances where it is desirable to leave permanent structures or removable structures in the body of an animal, such as an implant; or biodegradable. The matrices may take the form of sponges, implants, tubes, telfa pads, fibers, hollow fibers, lyophilized components, gels, powders, porous compositions, or nanoparticles. In addition, matrices can be designed to allow for sustained release seeded cells or produced cytokine or other active agent. In certain embodiments, the matrix of the present disclosure is flexible and elastic, and may be described as a semisolid scaffold that is permeable to substances such as inorganic salts, aqueous fluids and dissolved gaseous agents including oxygen.

A matrix is used herein as an example of a biocompatible substance. However, the current disclosure is not limited to matrices and thus, wherever the term matrix or matrices appears these terms should be read to include devices and other substances which allow for cellular retention or cellular traversal, are biocompatible, and are capable of allowing traversal of macromolecules either directly through the substance such that the substance itself is a semi-permeable membrane or used in conjunction with a particular semi-permeable substance.

In certain embodiments of the present disclosure, B cells transfected and activated using the methods described herein, or other methods known in the art, are administered to a patient in conjunction with (*e.g.* before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, bisulfin, bortezomib, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3

antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506), the proteasome (bortezomib), or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin). (Liu et al., Cell 66:807-815, 1991; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Curr. Opin. Immun. 5:763-773, 1993; Isoniemi (supra)). In a further embodiment, the cell compositions of the present disclosure are administered to a patient in conjunction with (*e.g.* before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH. In one embodiment, the cell compositions of the present disclosure are administered following B-cell ablative therapy such as agents that react with CD20, *e.g.* Rituxan®. In one embodiment, the cell compositions of the present disclosure are administered following B cell ablative therapy using an agent such as bortezomib. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present disclosure. In an additional embodiment, expanded cells are administered before or following surgery.

The dosage of the above treatments to be administered to a patient will vary with the precise nature of the condition being treated and the recipient of the treatment. The scaling of dosages for human administration can be performed according to art-accepted practices.

The modified B cells can be used in the treatment or prevention of various infectious diseases, cancers, degenerative diseases and immunological disorders.

Compositions comprising the modified B cells as described herein may be used in treatment of any of a variety of infectious diseases caused by infectious organisms, such as viruses, bacteria, parasites and fungi. Infectious organisms may comprise viruses, (*e.g.*, RNA viruses, DNA viruses, human immunodeficiency virus (HIV), hepatitis A, B, and C virus, herpes simplex virus (HSV), cytomegalovirus (CMV) Epstein-Barr virus (EBV), human papilloma virus (HPV)), parasites (*e.g.*, protozoan and metazoan pathogens such as Plasmodia species, Leishmania species, Schistosoma species, Trypanosoma species), bacteria (*e.g.*, Mycobacteria, in particular, M.

tuberculosis, Salmonella, Streptococci, E. coli, Staphylococci), fungi (*e.g.*, Candida species, Aspergillus species), Pneumocystis carinii, and prions (known prions infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats, as well as bovine spongiform encephalopathy (BSE), or "mad cow disease", and feline spongiform encephalopathy of cats. Four prion diseases known to affect humans are (1) kuru, (2) Creutzfeldt-Jakob Disease (CJD), (3) Gerstmann-Straussler-Scheinker Disease (GSS), and (4) fatal familial insomnia (FFI)). As used herein "prion" includes all forms of prions causing all or any of these diseases or others in any animals used-and in particular in humans and domesticated farm animals. Illustrative infectious diseases include, but are not limited to, toxoplasmosis, histoplasmosis, CMV, EBV, coccidiomycosis, tuberculosis, HIV, and the like.

In certain embodiments, the modified B cell compositions as described herein may also be used for the prevention or treatment of a variety of cancers. In this regard, in certain embodiments, the compositions comprising transfected B cells are useful for preventing or treating melanoma, non-Hodgkin's lymphoma, Hodgkin's disease, leukemia, plasmocytoma, sarcoma, glioma, thymoma, breast cancer, prostate cancer, colo-rectal cancer, kidney cancer, renal cell carcinoma, uterine cancer, pancreatic cancer, esophageal cancer, brain cancer, lung cancer, ovarian cancer, cervical cancer, testicular cancer, gastric cancer, esophageal cancer, multiple myeloma, hepatoma, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL), or other cancers.

In one embodiment, the modified B cells may also be used in the treatment of immunological disorders such as acquired immune deficiency syndrome (AIDS), agammaglobulinemia, hypogammaglobulinemia, other immunodeficiencies, immunosuppression, and severe combined immunodeficiency disease (SCID).

In one embodiment, the modified B cells as described herein may also be used in the treatment of autoimmune diseases such as, but not limited to, rheumatoid arthritis, multiple sclerosis, insulin dependent diabetes, Addison's disease, celiac disease, chronic fatigue syndrome, inflammatory bowel disease, ulcerative colitis, Crohn's disease, Fibromyalgia, systemic lupus erythematosus, psoriasis, Sjogren's syndrome, hyperthyroid ism/Graves disease, hypothyroidism/Hashimoto's disease, Insulin-dependent diabetes (type 1), Myasthenia Gravis, endometriosis, scleroderma, pernicious

anemia, Goodpasture syndrome, Wegener's disease, glomerulonephritis, aplastic anemia, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome, idiopathic thrombocytopenic purpura, autoimmune hemolytic anemia, Evan's syndrome, Factor VIII inhibitor syndrome, systemic vasculitis, dermatomyositis, polymyositis and rheumatic fever. Thus, in one embodiment, the methods herein include methods for treating a disease comprising administering to a subject or patient in need thereof a therapeutically effective amount of the compositions comprising the modified B cells as described herein, thereby treating the disease.

In one embodiment, the modified B cells as described herein may also be used in the treatment of enzyme deficiency diseases and disorders such as, but not limited to, MPS I, MPS II, MPS III, MP IV, MPS V, MPS VI, MPS VII, lysosomal storage disorders, Nieman-pick disease (types A, B and C), Guacher's disease (types I, II and III), Tay-Sachs disease and Pompe disorder.

One embodiment provides a method for treating MPS I in an individual comprising administering a B cell genetically modified to express IDUA (IDUA+ B cells) to a subject having, or suspected of having, MPS I. In one embodiment, a single, maximally effective dose of IDUA+ B cells is administered to the subject. In another embodiment, two or more doses of IDUA+ B cells are administered to the subject, thereby maximizing the amount of engrafted IDUA+ B cells. In some embodiments, the two or more doses of IDUA+ B cells that are administered to the subject comprise less IDUA+ B cells than the single, maximally effective dose of IDUA+ B cells. In some embodiments, when two or more doses of IDUA+ B cells are administered to a subject at a dosage of IDUA+ B cells that is below the maximally effective single dose of IDUA+ B cells, a resultant synergistic increase in IDUA production occurs. In one embodiment, administering IDUA+ B cells to a subject results in normal levels of IDUA seen in a healthy, control subject. In one embodiment, administering IDUA+ B cells to a subject results in greater than normal levels of IDUA in the subject. In one embodiment, administering IDUA+ B cells to a subject reduces levels of GAGs in the subject to a normal level. In one embodiment, administering IDUA+ B cells to a subject reduces levels of GAGs in the subject to less than a normal level of GAGs in the subject.

## EXAMPLES

### EXAMPLE 1

### PRODUCTION OF IDUA EXPRESSING B CELLS

*Sleeping Beauty* transposon and transposase constructs for transposition and expression of human IDUA were generated. Transposons assembled to achieve IDUA gene integration and expression in B cells are shown in Figure 1. We used the EEK promoter, consisting of promoter and enhancer elements from the human immunoglobulin gene as well as other regulatory elements previously described, to achieve high level expression in B cells. To test for IDUA transposition and expression, human B cells were isolated from two separate donors and expanded in culture, electroporating on day 2 with pKT2/EEK-IDUA plus pCMV-SB100x. Cell lysates prepared 8 days post-electroporation contained about 60 nmol/hr/mg IDUA activity, about 50 times the level of IDUA found in wild-type cells, demonstrating the effectiveness of the SB transposon system to achieve high- level IDUA expression in expanded human B cells (Figure 2).

In order to enrich for IDUA expressing cells, we also generated a bifunctional transposon (pKT2/EEK-IDUA-DHFR, Figure 1) encoding human IDUA along with a human dihydrofolate reductase synthesized to encode a novel variant enzyme (L22Y, F31S) that is resistant to the folate antagonist methotrexate (MTX: (McIvor RS. 1996. Bone Marrow Transplantation 18:S50-54.)). The plasmid map for this construct is shown in FIG. 1B and its sequence is provided as SEQ ID NO: 1.

We also established a technique using a commercially available anti-human IDUA antibody to identify cells expressing high levels of IDUA by permeabilizing and intracellular staining followed by flow cytometry. Using a GFP-DHFR transposon similar to pKT2/EEK-IDUA-DHFR, we first established conditions for selective outgrowth of transposed B cells by incubating at several different concentrations of MTX between days 2 and 4 of the cell expansion. We found that 200 nM provided the most effective conditions for selective outgrowth of B cells expressing the GFP reporter transgene (Table 1). We then applied these conditions to the expansion of pKT2/EEK-IDUA-DHFR + pCMV-SB100x electroporated cells, evaluating for the % IDUA+ cells by intracellular staining on day 7. While a 10-12% frequency of IDUA+

cells was observed in the transposed cell population, this frequency was increased to 25% or greater by applying MTX selection (Figure 3).

Table 1. Methotrexate Selection Conditions*

| [MTX], nM | Control | Donor 1 | Donor 2 |
|---|---|---|---|
| 100 | 0.01 | 8.97 | 13.50 |
| 200 | | 25.20 | 39.00 |
| 300 | | 13.40 | 5.49 |

*% GFP positive cells on day 7 after MTX selection on days 2 to 4

Initial *in vivo* studies were started using an MPS I mouse strain that had been backcrossed onto NOD-SCID, but there was no evidence for B cell maintenance one week after infusion into this strain. This problem was eventually solved by crossing in the IL-2 receptor gamma-C knockout allele generating NSG-MPS I mice as described below in Example 2, but in the meantime we tested for adoptive transfer of MTX-selected IDUA+ B cells (as shown in Figure 3) in IDUA+ NSG mice. Autologous peripheral blood cells were enriched for CD4+ and infused intraperitoneally (i.p.) on days -30 and -4 to provide support for the pKT2/EEK-IDUA-DHFR transposed B cells injected either i.p. or intravenously on day 0. We observed a range of plasma IDUA, from wild-type level to 200 times wild-type, in animals administered IDUA expressing B cells both i.p. and i.v. (Figure 4). We also observed extremely high levels of human immunoglobulin (mean of 1 to 4 mg/mL) in plasma - strong evidence for successful human B cell adoptive transfer. Although not carried out in IDUA deficient animals, the results from this experiment nonetheless provide an example of the levels of human IDUA that can be achieved after introduction of a highly potent IDUA+ B cell population into NSG mice.

## EXAMPLE 2

### *IN VIVO* PRODUCTION OF IDUA IN MPS I MICE

In order to determine if the relationship between the number of modified B cells administered and the amount of the therapeutic agent produced is linear, a mouse model of mucopolysaccharidosis type I (MPS I) was used with allogeneic B cells genetically modified to express iduronidase (IDUA).

NSG (NOD-SCID gamma-C deficient) mice were crossed with NOD-SCID IDUA deficient mice to collect the gamma-C and IDUA deficiency alleles in the

same strain and generate NSG MPS I mice, also referred to herein as "MPS I mice". When sufficient NSG MPS I mice were generated, these animals were infused i.p. with 3 x $10^6$ CD4+ T cells at day -7 and then 3 x $10^6$, 1 x $10^7$, or 3 x $10^7$ pKT2/EEK-IDUA transposed B cells (approximately 10% IDUA+ by intracellular staining) i.v. on day 0. MPS I mice were given a single dose of B cells engineered to produce IDUA (or no cells as a control) in the presence of CD4+ memory T cells (or no cells as a control) and IDUA enzyme activity levels measured in serum through day 38 (Figure 5) using the IDUA enzyme assay protocol previously reported by Hopwood JJ, et al., Clin Chim Acta. 1979 Mar 1;92(2):257-65, the contents of which is incorporated herein by reference in its entirety. Because the mice lack factors required for long term survival of the B cells, CD4+ T memory cells were administered intraperitoneally to the mice also in order to promote B cell survival.

Unexpectedly, these results demonstrated that the relationship between cell number and serum IDUA levels was not linear at certain cell doses. Without wishing to be bound by theory, this finding may be due to the B cells interacting with each other *in vivo* that produce different survival outcomes at different cell concentrations. Thus, these results indicate that there is an ideal dose that achieves sufficient production of the therapeutic agent while administering the fewest number of cells necessary. This concept is illustrated in Figure 5, whereby the 3 x $10^7$ cell dose resulted in a greater than 3-fold increase in IDUA activity levels in plasma compared to the 1 x $10^7$ cell dose.

## EXAMPLE 3

### MULTIPLE DOSES OF IDUA PRODUCING B CELLS IN MPS I MICE

In order to determine if the dosage of the B cells effects the amount of therapeutic agent produced *in vivo*, MPS I mice were given a series of 3 doses of IDUA producing B cells.

MPS I mice were given a series of 3 doses of 1 x $10^7$ B cells engineered to produce IDUA (or no cells as a control) in the presence of CD4+ memory T cells (or no cells as a control) on day 0 and IDUA enzyme activity levels measured in serum through day 56 (Figure 6). Specifically, MPS I mice were infused i.p. with 3 x $10^6$ CD4+ T cells at day -7 and then $10^7$ pKT2/EEK-IDUA transposed B cells (approximately 10% IDUA+ by intracellular staining) either i.p. or i.v. on day 0. The animals were given

additional infusions of $10^7$ pKT2/EEK-IDUA transposed B cells by the same route of administration on days 21 and 42 after the first injection.

Using this procedure, we found that wild type levels of plasma IDUA (about 1 nmol/hr/ml) were achieved in most of the B cell treated animals, and that this required the prior administration of CD4+ T cells (Figure 6). We found human IgG in plasma ranged from 200 μg/mL up to 1 mg/mL as evidence for B cell adoptive transfer (Figure 7).

These results demonstrate that administering the same number of modified B cells over the course of several doses results in greater ultimate levels of a therapeutic agent than is achieved by administering all or the modified B cells in a single dose. As can be seen in Figure 6, initial levels of IDUA after the first dose of 1 x $10^7$ B cells were low compared to those achieved after the third dose, which unexpectedly resulted in serum levels of IDUA well in excess of 3-fold of the serum levels of IDUA observed after the first dose. This phenomena can also be observed by comparing the 1 x $10^7$ cells/mouse data in Figure 5 with the data in Figure 6, as each dose is 1 x $10^7$. Whereas in Figure 5 it is shown that a single dosage of 1 x $10^7$ cells administered intravenously resulted in declining levels of IDUA by D38, it is clear in Figure 6 that the levels of IDUA resulting from 3 doses of 1 x $10^7$ cells administered intravenously resulted in expression levels that continued to both greatly increase and also greatly exceed 3 times the 1 x $10^7$ single dosage level and the 3 x $10^7$ single dosage level at the same time point. Unexpectedly, this synergy across multiple dosages was only observed in these groups of mice that were delivered the engineered B cells intravenously, but not in the mice that received the engineered B cells via intraperitoneal injection.

In addition to showing that multiple doses resulted in levels of therapeutic agent greater than expected from a single dosage of the same number of cells, the mere concept that multiple dosages resulted in greater levels of therapeutic agent is advantageous and unexpected. Mechanistically, it is thought that differentiated B cells occupy a finite level of survival niches, and when new differentiated cells are created, they displace some of the old cells. Therefore, it might be expected that subsequent doses of B cells would not lead to concomitant increases in serum levels of the therapeutic agent. However, we have shown this is not the case and that additional B cell infusions do result in greater steady state plasma levels of the therapeutic agent they are making.

This phenomenon is useful in achieving the proper dosage of the therapeutic drug *in vivo* while minimizing the cell dosage that is required.

Specifically, patients can be administered a dosage, and then plasma levels of the drug measured. In the event the levels are lower than desired an additional dosage of cells can be administered. Given the average half-life of most injected biologics, these findings are certainly not applicable to direct infusion of a biologic. Furthermore, these results are not likely to be achievable using other methods such as viral based drug delivery, which may also elicit an immune response to the vector, thereby thus reducing efficacy of/hinder future attempts to administer a further dose.

Accordingly, the methods for delivering a therapeutic agent disclosed herein provide advantages over methods of the prior art, including the ability to administer more than one dose of modified B cells and the dosage stacking that results therefrom.

## EXAMPLE 4

### B CELL BASED DELIVERY TO MULTIPLE TISSUES

Direct infusion of a therapeutic agent, delivery via viral vectors, and hematopoietic stem cell transfer all fail to successfully deliver a therapeutic agent to multiple tissues. In order to determine if modified B cells can be used to deliver a therapeutic agent *in vivo* to a variety of tissues, MPS I mice were given a series of 3 doses of IDUA producing B cells as described in the previous example.

Following the protocol described in the previous Example, MPS I mice were given a dosage regiment comprising 3 doses of $1 \times 10^7$ B cells engineered to produce IDUA (or no cells as a control) in the presence of CD4+ T cells (or no cells as a control). Animals were euthanized and tissues harvested at 60 days post the first B cell infusion, and IDUA enzyme activity levels measured in the liver, lung, spleen, kidney, intestine, muscle, brain, heart, peritoneal lavage, and bone marrow (Figure 8).

Flow cytometry showed 20% to 35% human CD 45+ and 2% to 10% CD19+ cells in spleen and lymph nodes of animals infused with human T and IDUA expressing B cells. There was substantial metabolic cross-correction observed by the IDUA activities restored in peripheral tissues and even in the brain (Figure 8).

IDUA degrades GAGs, and the amount of GAGs present increases in the absence of IDUA, such as in the tissues of MPS I mice. Accordingly, glycosaminoglycans

(GAGs) were also measured in the brain, lung, liver, heart, kidney, muscle, spleen, and intestine on day 60 in order to determine if the IDUA is degrading GAGs in those tissues (Figure 9). As a result of the metabolic cross-correction due to IDUA, tissue glycosaminoglycans were significantly reduced in B cell treated animals also infused with CD4+ T cells (Figure 9).

These results demonstrated that delivery of IDUA via modified B cells allows for both enhanced delivery of therapeutic agents to tissues as well as activity of therapeutic agents in those tissues. Figure 8 shows the IDUA enzyme activity levels in tissues resulting from infusion of B cells producing IDUA. Figure 9 shows the GAG levels in these same mice. GAGs are toxic cellular products that build up in MPS I mice tissues and that are broken down by the enzymatic activity of IDUA. As can be observed in both figures, IDUA production and GAG reduction took place effectively in multiple tissues including lung, spleen, liver, heart, and intestine. Of note, infusion of IDUA is not thought to adequately address disease manifestations in tissues such as heart, spleen and liver. These results demonstrated the effectiveness of IDUA expressing human B cells for metabolic correction of MPS I for the first time. Therefore, this data supports that *in vivo* delivery of the enzyme via genetically modified B cells posits the propensity to enhance treatment of various organs in the body.

## EXAMPLE 5

### LONG-TERM EFFICACY OF B CELL BASED THERAPEUTIC DELIVERY

To determine whether B cell-based delivery of therapeutics results in long-term protein production, we analyzed long-term IDUA activities in plasma and tissues of MPSI NSG mice that had received IDUA producing B cells.

B cells over-expressing IDUA were prepared as previously described. NSG MPS I animals were infused intraperitoneally with 3e6 CD4+ Memory T cells one week before B cell infusions. The animals were then infused with 2e7 IDUA-expressing B cells on days 0 and 30.

We first analyzed IDUA enzyme activity in plasma from MPSI NSG mice that had received IDUA producing B cells as described in the previous examples. Untreated MPSI NSG mice were also analyzed over this period as a control. Blood was harvested from these animals around every 2 weeks for 6.5 months and assayed for IDUA enzymatic activity as previously described.

As shown in Figure 10, plasma IDUA activity was strongly induced in mice receiving the engineered B cells, with peek plasma responses occurring at around 5 weeks post-infusion, and IDUA activity remained higher in these animals until the time of sacrifice 6.5 months post-infusion.

Moreover, long-term treatment with the B cell product also resulted in prolonged increased levels of IDUA in multiple tissues analyzed at 3, 6, and 6.5 months post-infusion with engineered B cells (Figure 11), showing that the enhanced tissue delivery of IDUA that the we observed in above Example 4 is not merely transient, but persists long-term.

Furthermore, 3 months, 6 months, and 6.5 months after the first infusion of B cells, animals were euthanized and extracts from tissues were assayed for GAG levels, as described in Example 4, and long-term reductions in the levels of GAGs in multiple tissues was observed (FIG. 12). As a positive control for reduction in GAGs, NSG-IDUA$^{+/-}$ mice (which are heterozyogous for IDUA and are phenotypically normal) were assayed. As a negative control for reduction in GAGs, a group of MPSI NSG mice received no cells.

Thus, these data show that the delivery of therapeutic agents via modified B cells allows for prolonged enhancement of enzyme activity levels in vivo, not only in plasma, but also in tissues such as the heart, spleen and liver, which are typically not treatable with infusion of IDUA.

## EXAMPLE 6

### OPTIMIZATION OF THE MIGRATORY CAPACITY OF ENGINEERED B CELLS

In order for plasma cells to survive for the long-term it is generally accepted that the pre-cursor cells (i.e. plasmablasts) must have the ability to migrate to long-term plasma cell survival niches found in locations such as bone marrow. In contrast, it is generally accepted that once plasma cells complete differentiation, they down regulate their ability to migrate. Therefore, if one intends to generate a population of plasma cells from an infused population of plasmablasts, it is deemed important that the cells have robust migratory capacity. Specifically, migration towards CXCL12 is important for migration of plasma cell precursors to the bone marrow. Additionally, chemokines such as CXCL13 may be important for migration to sites of inflammation and tissues such as spleen.

To determine if the culture conditions generate migratory B cells and to determine whether the migratory capacity of B cells engineered to express a therapeutic protein is dependent on the amount of time the engineered B cells remain in culture after engineering, but before harvesting for administration to a subject, we prepared B cells over-expressing IDUA as previously described and we maintained the engineered B cells in culture for four to nine days prior to analyzing their migratory capacity.

The assay was conducted using two-chambered culture vessels, in which the chambers are connected (e.g., a Transwell® plate). Engineered B cells were seeded in one chamber and the other chamber was loaded with 100 ng/mL of CXCL12, which is a chemoattractant that draws B cells to the bone marrow, or CXCL13, which is a chemoattractant that draws B cells to sites of inflammation as well as tissues such as spleen. After allowing the B cells to migrate for 3 hours, B cells were collected from the second well and counted. In each assay, a negative control was used in which no chemoattractant (CXCL12 or CXCL13) was added to the second chamber. A schematic of the assay is presented in Figure 13D with respect to CXCL12. The same assay was used for CXCL13.

The test groups were B cells that were exposed to a culture system that we have previously shown greatly enhances the migratory capacity of engineered B cells (*see* PCT/US2015/066908, incorporated herein by reference in its entirety). This culture system, which was utilized for all of the experiments described herein (unless otherwise stated) comprises CD40L (HIS tagged for multimerization), a CD40L crosslinking agent (anti-HIS antibody that induces multimerization of the CD40L), IL-2, IL-4, IL-10, IL-15, and IL-21. B cells were cultured for the relevant number of days after engineering prior to Transwell analysis.

Surprisingly, we observed that B cells engineered under our culture conditions have optimal migratory potential to CXCL12 that peaks around 7-9 days (in culture, or 5-7 days after engineering) and optimal migratory potential to CXCL13 that peaks around 6-7 days (in culture, or around 4-5 days after engineering) (Figures 13A-C). These data suggest that in some instances it may be beneficial to harvest and administer the engineered B cells to a subject within this window to ensure optimal migratory potential and to induced optimal migration to target tissues.

## EXAMPLE 7

### CLONALITY ASSESSMENT OF FINAL B CELLS ENGINEERED TO EXPRESS IDUA

Ensuring polyclonality of the final cell product is an important safety parameter. Specifically, the emergence of a dominant clone is viewed as potentially contributing to *in vivo* tumorigenesis or auto-immune disease. To assess in the B cell final product, cells were grown in culture as previously described (see culture conditions presented in Example 6), engineered to express IDUA using the method described in Example 1, harvested on day 7 of culture and cryopreserved. DNA was extracted and subjected to high-throughput deep sequencing of the B cell receptor. Since the B cell receptor undergoes changes during B cell development that make it unique between B cells, this method allows for quantifying how many cells share the same B cell receptor sequence (meaning they are clonal).

Results of the deep sequencing are shown in Figure 14 and in Table 2 below. More than 248,000 template molecules were analyzed and around 241,000 unique rearrangements were observed representing a Max frequency of clonality (i.e. the prevalence of any specific B cell clone) of 0.03%.

Table 2: Sequencing Summary of B cell clonality sequencing

| SEQUENCING SUMMARY | | | | |
|---|---|---|---|---|
| | Template molecules | Unique rearrangements | Max Frequency | Max Productive Clonality |
| Cryopreserved cells | 248,503 | 241,586 | 0.03% | 0.03 |

This experiment demonstrates that the final B cell product is highly-polyclonal and that no single clone represents a significant portion of the cell population. Specifically, results from sequencing demonstrate that no single clone represents greater than 0.03% of the final population. Thus, the final engineered B cells are expected to be sufficiently polyclonal for therapeutic purposes and do not appear to contain any particular clone in abundance. Since it is plausible that culture conditions, genome modification, and the presence of foreign factors (e.g. fetal bovine serum present in the culture media) might engender the emergence of clonality, we view this result as unexpected in that no single clone achieved a significant frequency.

## EXAMPLE 8

### INFLAMMATORY POTENTIAL OF ENGINEERED B CELLS.

Like most therapeutics, the instant invention has the potential to stimulate the immune system, potentially leading to deleterious side effects and/or neutralizing antibodies that might reduce or eliminate efficacy. One useful predictor of whether such an adverse immune reaction may be triggered is whether or not the final engineered B cell products produce inflammatory cytokines. Thus, we measured whether the end of culture B cell product (B cells engineered to produce IDUA according to the above examples) produces any of various inflammatory cytokines.

B cells were cultured for 7 days in culture as previously described (see culture conditions presented in Example 6), engineered to express IDUA using the method described in Example 1, harvested on day 7 of culture and cryopreserved. During multiple timepoints during the culture, media samples were taken and assayed for a panel of cytokines using a Luminex device. Specifically, the presence of the following factors was assayed for: IL6, IFN alpha, IFN gamma, sFAS, TNFRp75, BAFF, HGF, IL5, IL2R alpha, TNF alpha, IL1ra, TNFRp55, VEGF, IL1 alpha, sIL6R. Because the media formulation contains FBS, which may contain these factors, media without B cells was used as a negative control. In the case of IL6, recombinant protein (in some cases) was added to the media without B cells to serve as positive controls.

Surprisingly, in most cases, by the end of the B cell culture the interrogated factor was either undetectable or not elevated above media only controls (Figures 15A and B). This result was unexpected given that the engineered cells are immune cells undergoing significant stimulation during the culturing and engineering process. The exceptions to this were IL6 and sFAS (Figure 15A, first group from the left, and Figure 15B, first group from the left). The only factor found to be increasing during culture was sFAS. In the case of IL6, while elevated levels were detected on day 2 of the culture, by day 7 the levels of IL6 where very close to that found in the media alone.

Overall, these results illustrate that the end of culture B cell product is not producing significant levels of the inflammatory cytokines tested. Given that we were providing a number of stimulatory cytokines to the B cells, it was unanticipated the final product would not produce significant levels of these cytokines. Most unexpectedly, we found that over time the B cells reduced their production of IL6 to near background levels. This is very relevant to clinical implementation in that IL6 is known to be a potent

immune-stimulatory signal. Overall, we believe these results reflect that the final B cell product is expected to be safe *in vivo* in terms of potential to avoid significant stimulation of the immune system at large.

## EXAMPLE 9

## PRODUCTION OF B CELLS EXPRESSING HUMAN LCAT OR FIX

*Sleeping Beauty* transposon and transposase constructs for transposition and expression of human LCAT, human LPL, and human FIX were generated as described in Example 1 and primary memory B cells were transfected by electroporation on day 2 in culture. Media was collected 2-days post-electroporation (day 4 in culture, "D4") and also 6 days post-electroporation for LCAT and LPL (day 8 in culture, "D8") and analyzed for expression.

Expression of LCAT in the transfected B cells was confirmed using a fluorescence-based LCAT enzyme activity-assay (see the method below). Media collected from transfected cells had strong LCAT activity on both D4 and D8, whereas significant activity was not observed in the media only control (Figure 16A).

Expression of LPL in the transfected B cells was confirmed using a fluorescence-based LPL enzyme activity-assay (see the method below). Media collected from transfected cells had strong LPL activity on both D4 and D8, whereas significant activity was not observed in the media only control (Figure 16B).

For detection of Lecithin–Cholesterol Acyltransferase (LCAT) and Lipoprotein Lipase (LPL), B cells were cultured (see culture conditions presented in Example 6) and electroporated on day 2 of culture (as in Example 1) with a transposon encoding either LCAT or LPL as well as a construct encoding a source of transposase (SB100x, see Example 1 and Figure 1). Media samples were taken on day 4 and day 8 of culture and assayed for the presence of LCAT and LPL using a flourometric enzyme assay. Specifically, 4-methylumbelliferyl palmitate substrate (4-MUP) was used and cleavage of the substrate by LCAT and LPL detected by measuring the increase in fluorescence at wave lengths of 340, 390 and 460nm. Reaction buffer containing 100 mM Sodium Phosphate Buffer (pH 7.4) was prepared and placed at 37°C. The 4-MUP was diluted by mixing with 2mg of 4-MUP with 16mg of Triton X-100. 4-MUP and reaction buffer where combined and a total volume of 150ul was added to a well on a 96 well plate along with 2ul activator compound (e.g. p-Nitrophenyl Butyrate). Serial

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dilutions of B cell culture media were prepared and 50ul of the dilutions were then added an the mixture and incubated at 37°C. Fluorescence measurements begin immediately after adding the culture media and were collected every minute for a total duration of 30 minutes.

Expression of FIX in the transfected B cells was confirmed by ELISA. More specifically, cell lysates of the B cells were prepared and detected via ELISA using a commercially available ELISA kit (FIX-EIA, Enzyme Research Laboratories, South Bend, IN) according to the manufacturer's recommended protocol. Media was collected 2 days post-electroporation and the media contained FIX protein at a concentration of approximately 15 ng/ml, whereas no FIX protein was detected in the negative control cells (B cells transfected with GPF) (Figure 16C).

Thus, these data demonstrate that B cells can be used in the methods disclosed herein to express and deliver a wide-range of polypeptides to a subject.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent application, foreign patents, foreign patent application and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, application and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

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**THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:**

1. A method of administering genetically modified B cells to a subject to enable synergistic *in vivo* production of a therapeutic protein comprising: determining an optimal single-dose concentration of the modified B cells for inducing the greatest *in vivo* production of the therapeutic protein, wherein the B cells are engineered to express the therapeutic protein and the therapeutic protein is iduronidase (IDUA); decreasing the optimal single-dose concentration of the modified B cells to obtain a sub-optimal single-dose concentration of the modified B cells; and administering two or more doses of the sub-optimal single-dose concentration of the modified B cells to the subject.

2. The method of claim 1, wherein multiple single dosages of the modified B cells are tested such that an optimal single-dose concentration of the modified B cells is determined.

3. The method of claim 2, wherein increasing the dosage of modified B cells present in a single-dose concentration of modified B cells results in a linear increase in the production of IDUA .

4. The method of claim 1, wherein multiple sub-optimal single dose concentrations of the modified B cells are tested such that an optimal dosage is found, wherein the resulting dosage results in a greater than linear increase over lower dosages.

5. The method of claim 1, wherein the sub-optimal single-dose concentration is one half or one third the dose of the optimal single-dose concentration.

6. The method of claim 1, wherein the sub-optimal single-dose concentration is less than one third the dose of the optimal single-dose concentration.

7. The method of any one of claims 1-6, wherein the synergistic *in vivo* production of the therapeutic protein that results from administering two or more doses of the sub-optimal single-dose concentration of the modified B cells to the subject results from intravenous injection of the B cell product.

8. The method of any one of claims 1-7, wherein the modified B cells are genetically engineered to secrete IDUA .

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9. The method of any one of claims 1-8, wherein the genetically modified B cells comprise a polynucleotide having a sequence that is identical to SEQ ID NO: 1.

10. The method of any one of claims 1-8, wherein the genetically modified B cells comprise a polynucleotide having a sequence that is at least about 85% identical to SEQ ID NO: 1, or at least about 90%, 95%, 96%, 97%, 98%, 99%, or greater than 99% identical to SEQ ID NO: 1.

11. The method of any one of claims 1-8, wherein the genetically modified B cells comprise a bifunctional transposon encoding a therapeutic gene and a selectable marker.

Figure 1

FIG. 1A

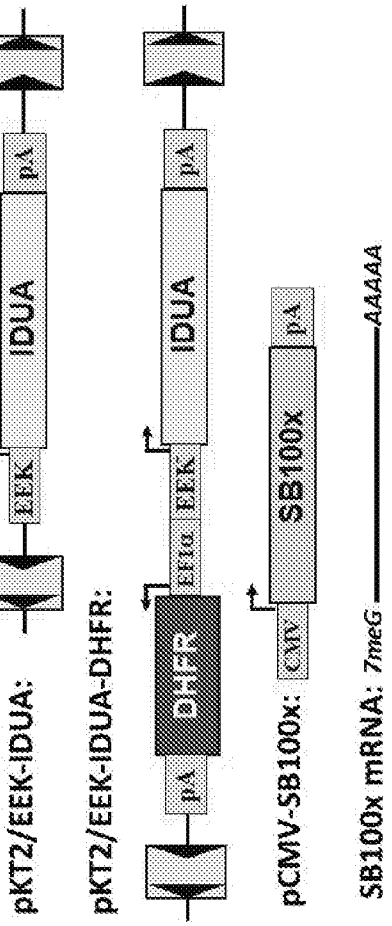



FIG. 1B

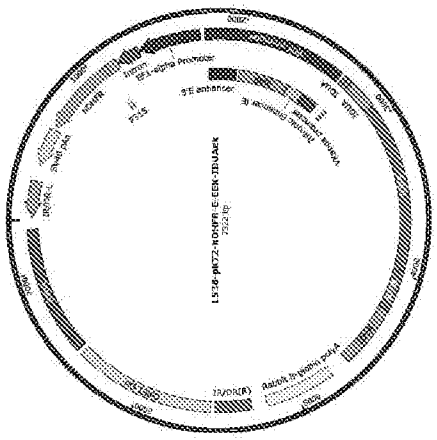


Figure 2

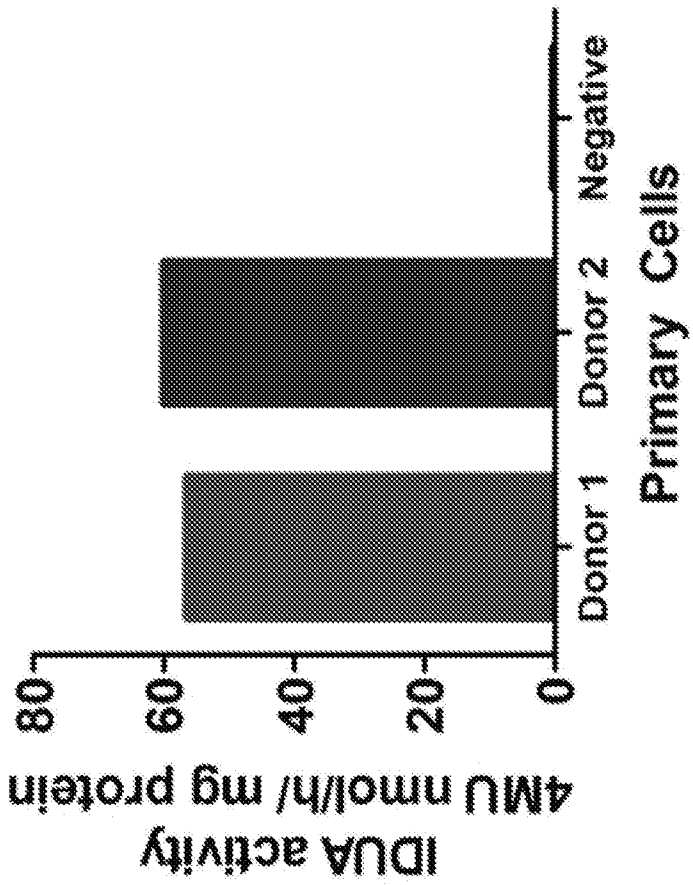

IDUA activity
4MU nmol/h/ mg protein
80
60
40
20
0
Donor 1
Donor 2
Negative
Primary Cells

Figure 3

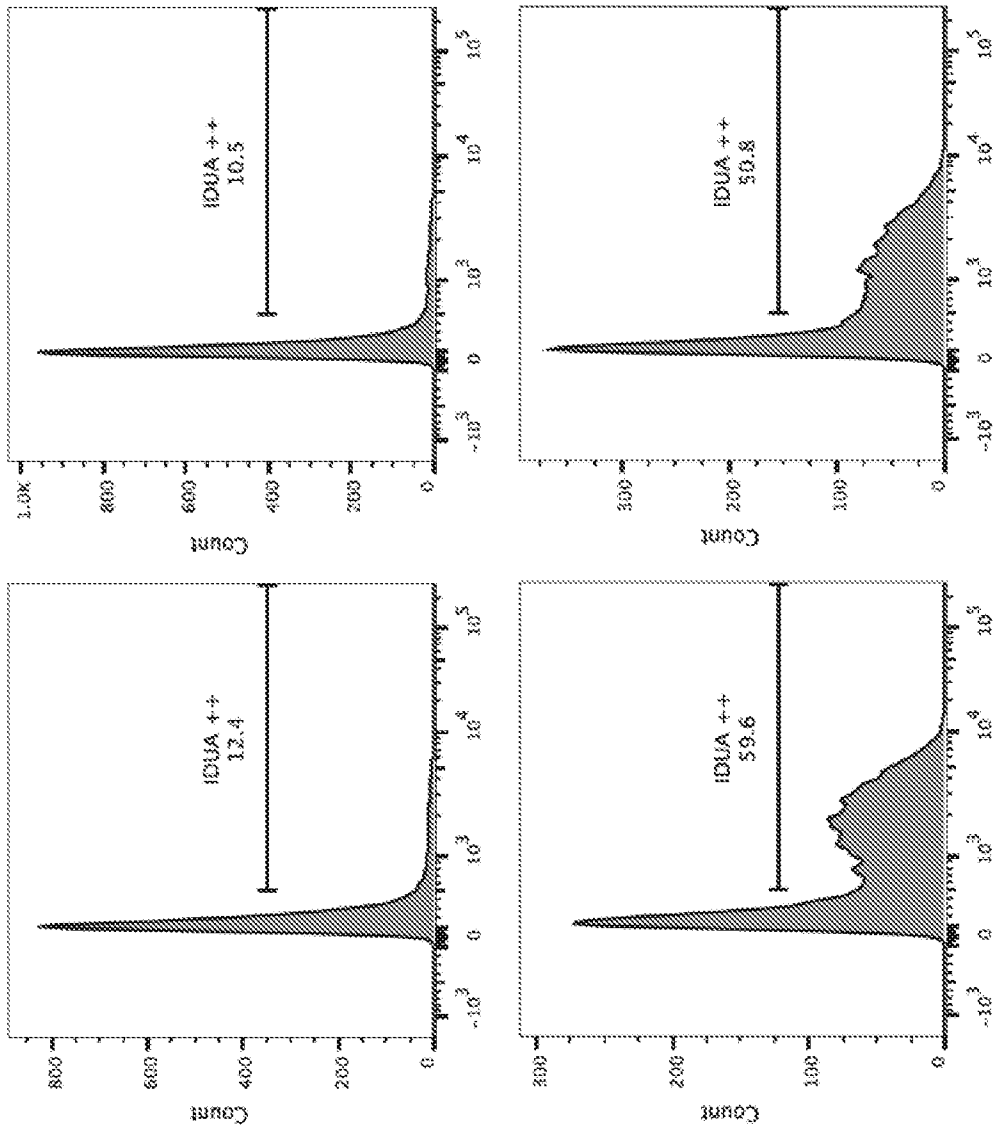


Figure 4

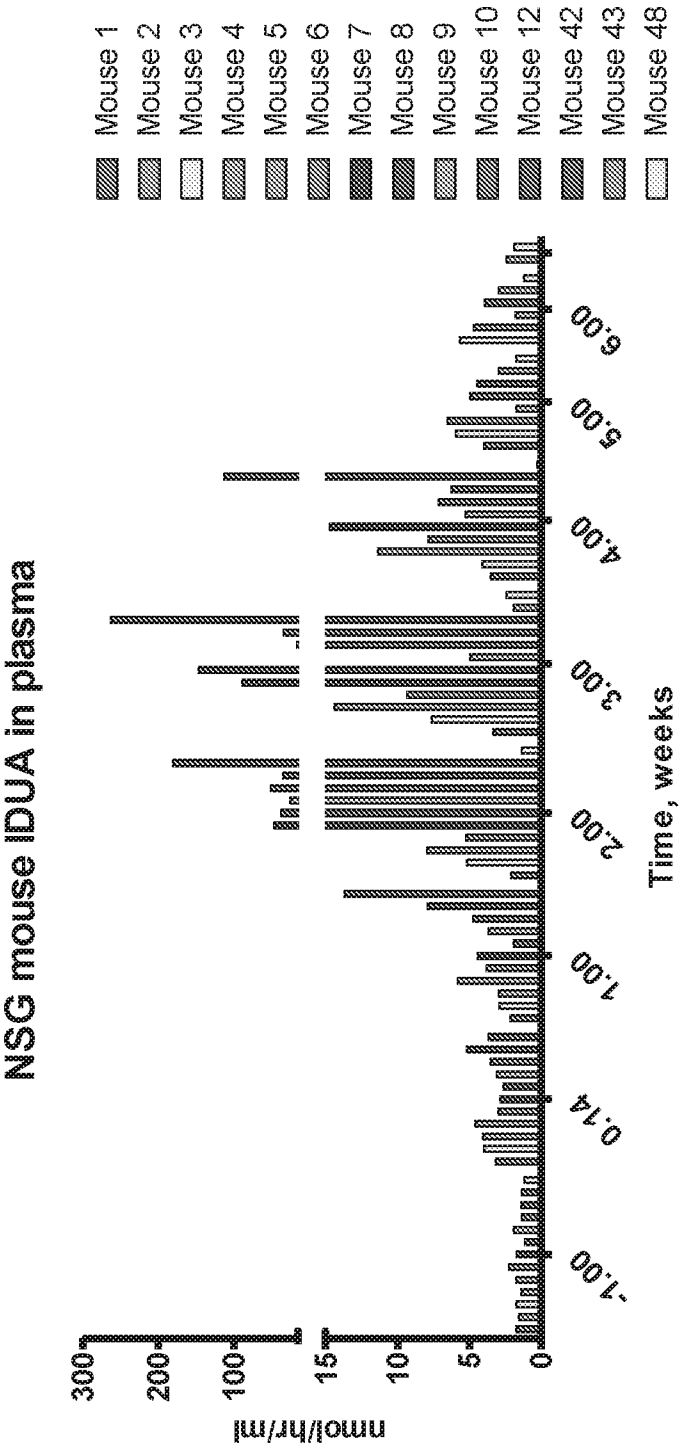

NSG mouse IDUA in plasma
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9
Mouse 10
Mouse 12
Mouse 42
Mouse 43
Mouse 48
nmol/hr/ml
300
200
100
15
10
5
0
-1.00
0.14
1.00
2.00
3.00
4.00
5.00
6.00
Time, weeks

Figure 5

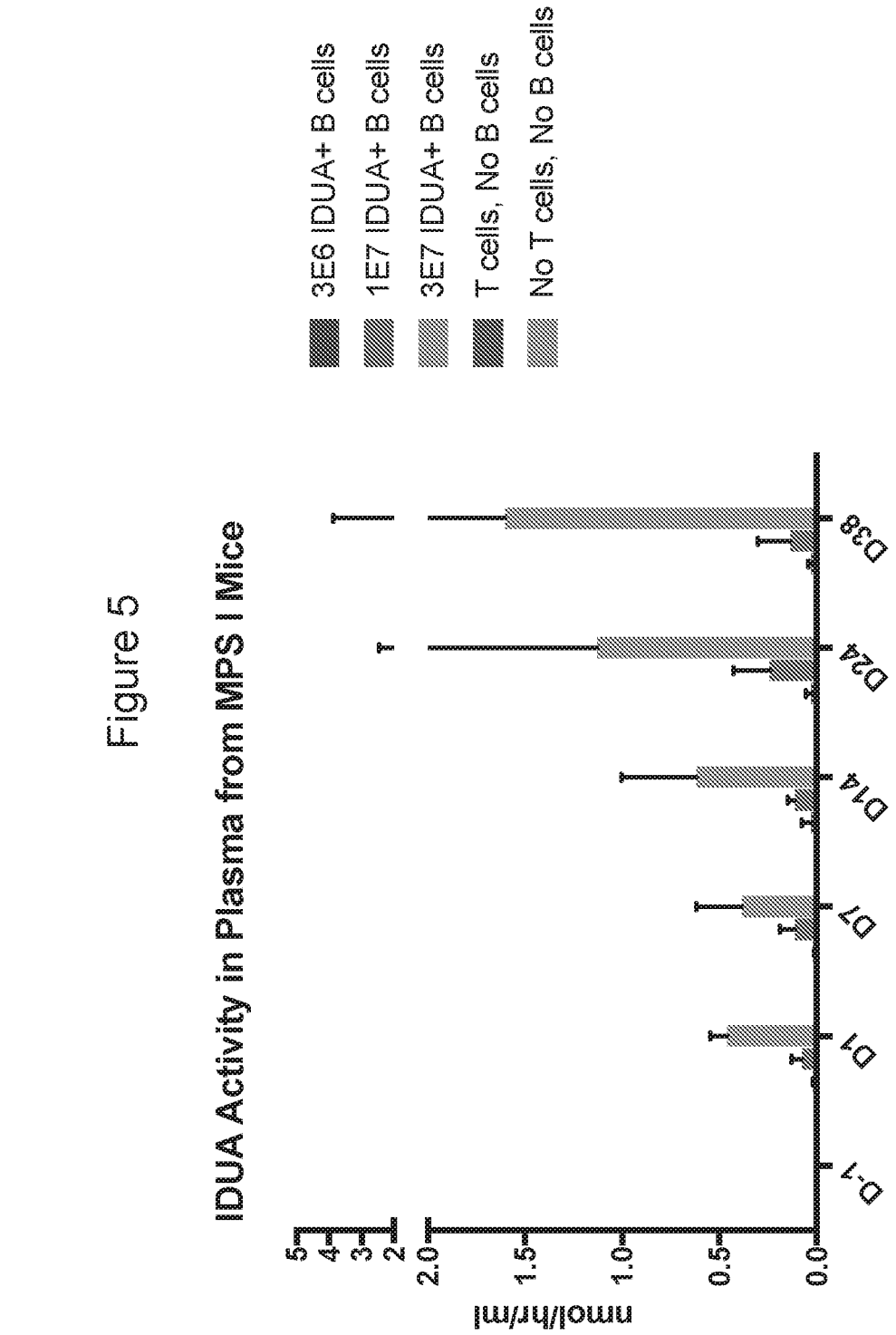

IDUA Activity in Plasma from MPS I Mice
nmol/hr/ml
0.0
0.5
1.0
1.5
2.0
2
3
4
5
D-1
D1
D7
D14
D24
D38
3E6 IDUA+ B cells
1E7 IDUA+ B cells
3E7 IDUA+ B cells
T cells, No B cells
No T cells, No B cells

Figure 6

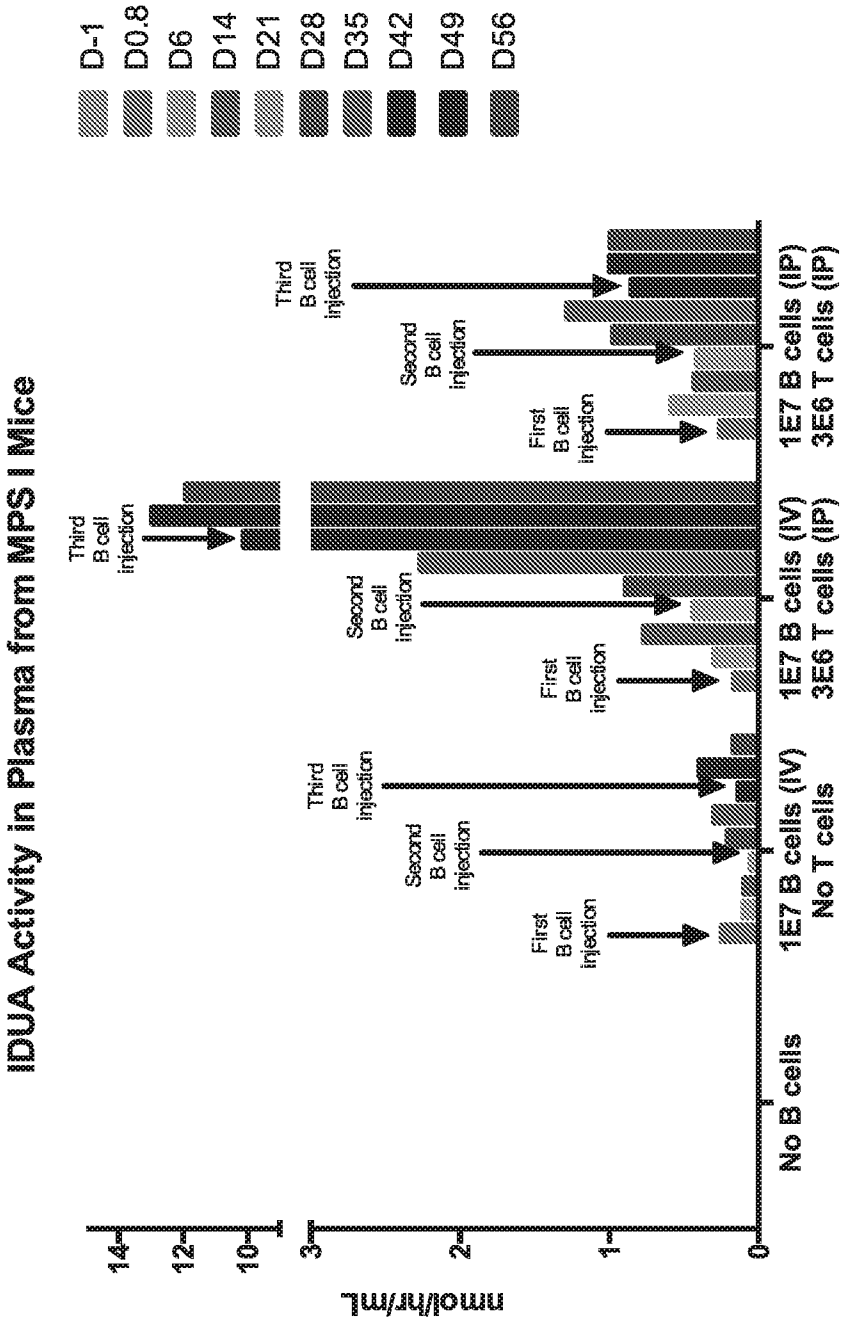

IDUA Activity in Plasma from MPS I Mice
nmol/hr/mL
14
12
10
3
2
1
0
D-1
D0.8
D6
D14
D21
D28
D35
D42
D49
D56
No B cells
1E7 B cells (IV)
No T cells
1E7 B cells (IV)
3E6 T cells (IP)
1E7 B cells (IP)
3E6 T cells (IP)
First B cell injection
Second B cell injection
Third B cell injection

Figure 7

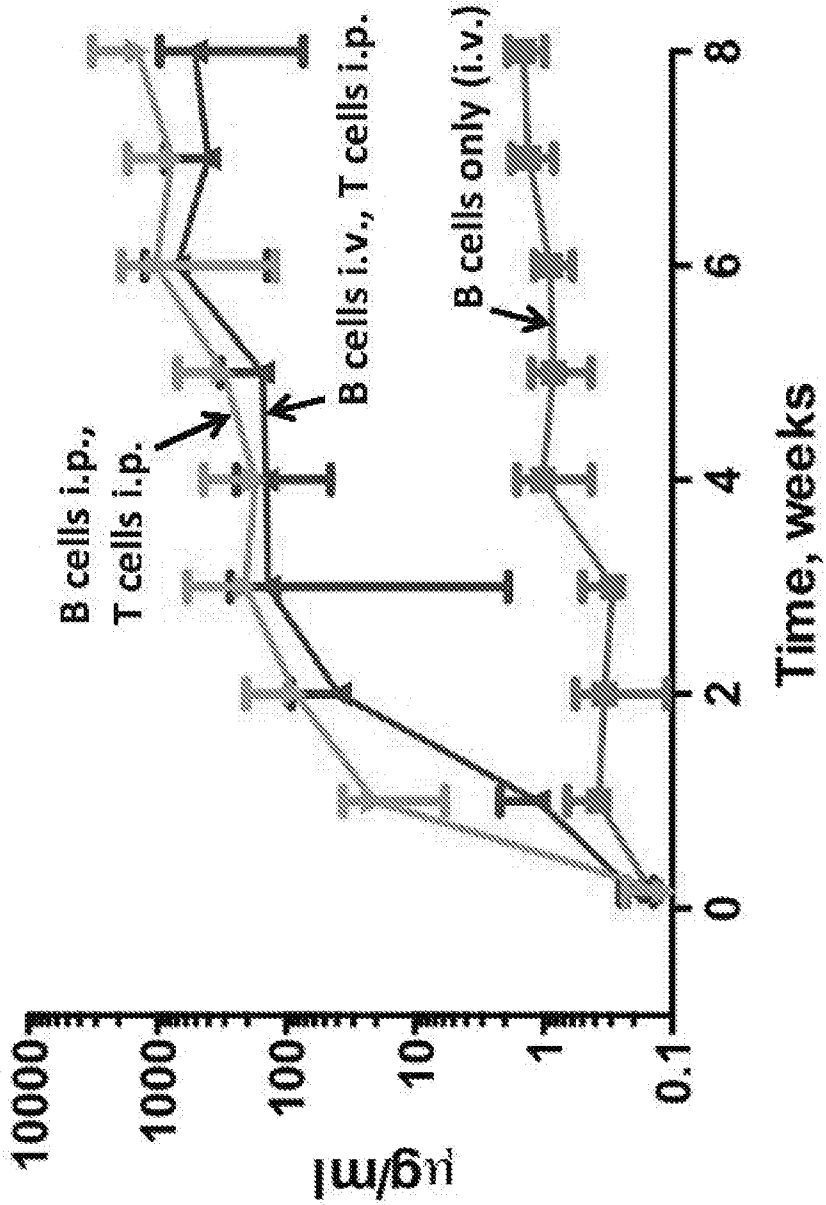

B cells i.p.,
T cells i.p.
B cells i.v., T cells i.p.
B cells only (i.v.)
μg/ml
10000
1000
100
10
1
0.1
0
2
4
6
8
Time, weeks

Figure 8

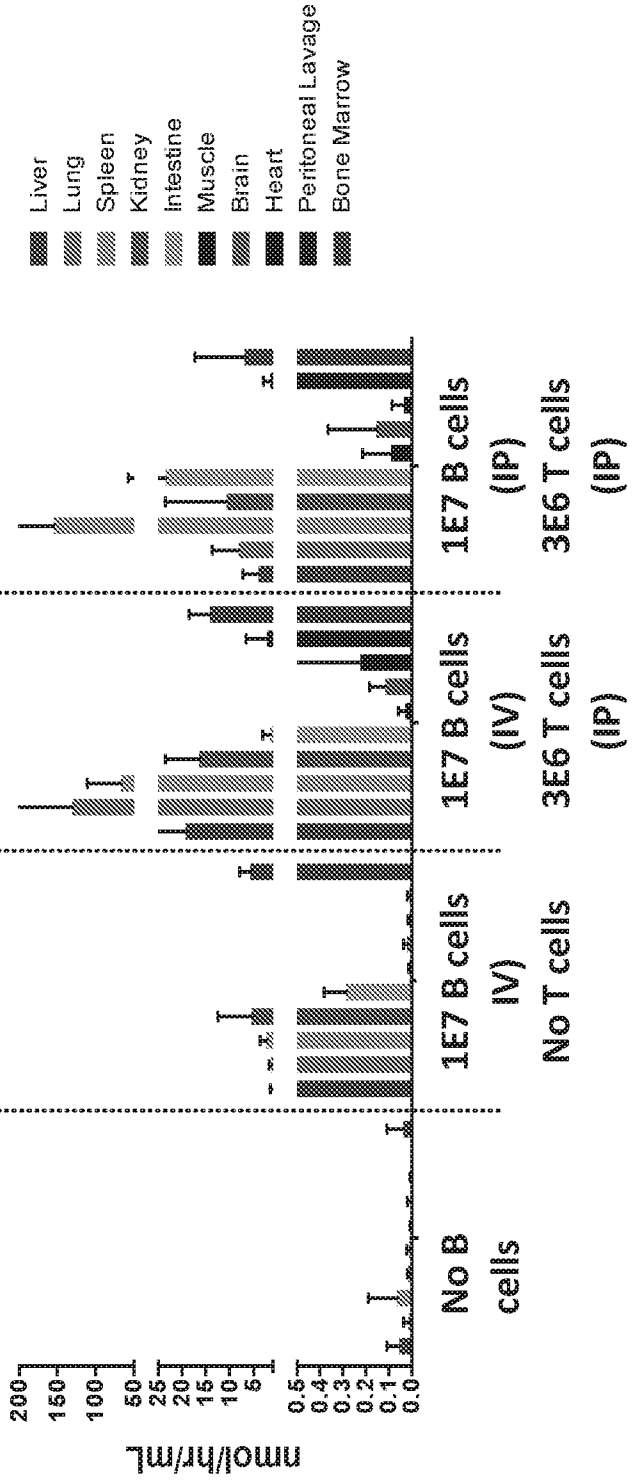

IDUA Activity in Tissues of MPS I Mice
nmol/hr/mL
200
150
100
50
25
20
15
10
5
0.5
0.4
0.3
0.2
0.1
0.0
Liver
Lung
Spleen
Kidney
Intestine
Muscle
Brain
Heart
Peritoneal Lavage
Bone Marrow
No B cells
1E7 B cells (IV)
No T cells
1E7 B cells (IV)
3E6 T cells (IP)
1E7 B cells (IP)
3E6 T cells (IP)

Figure 9

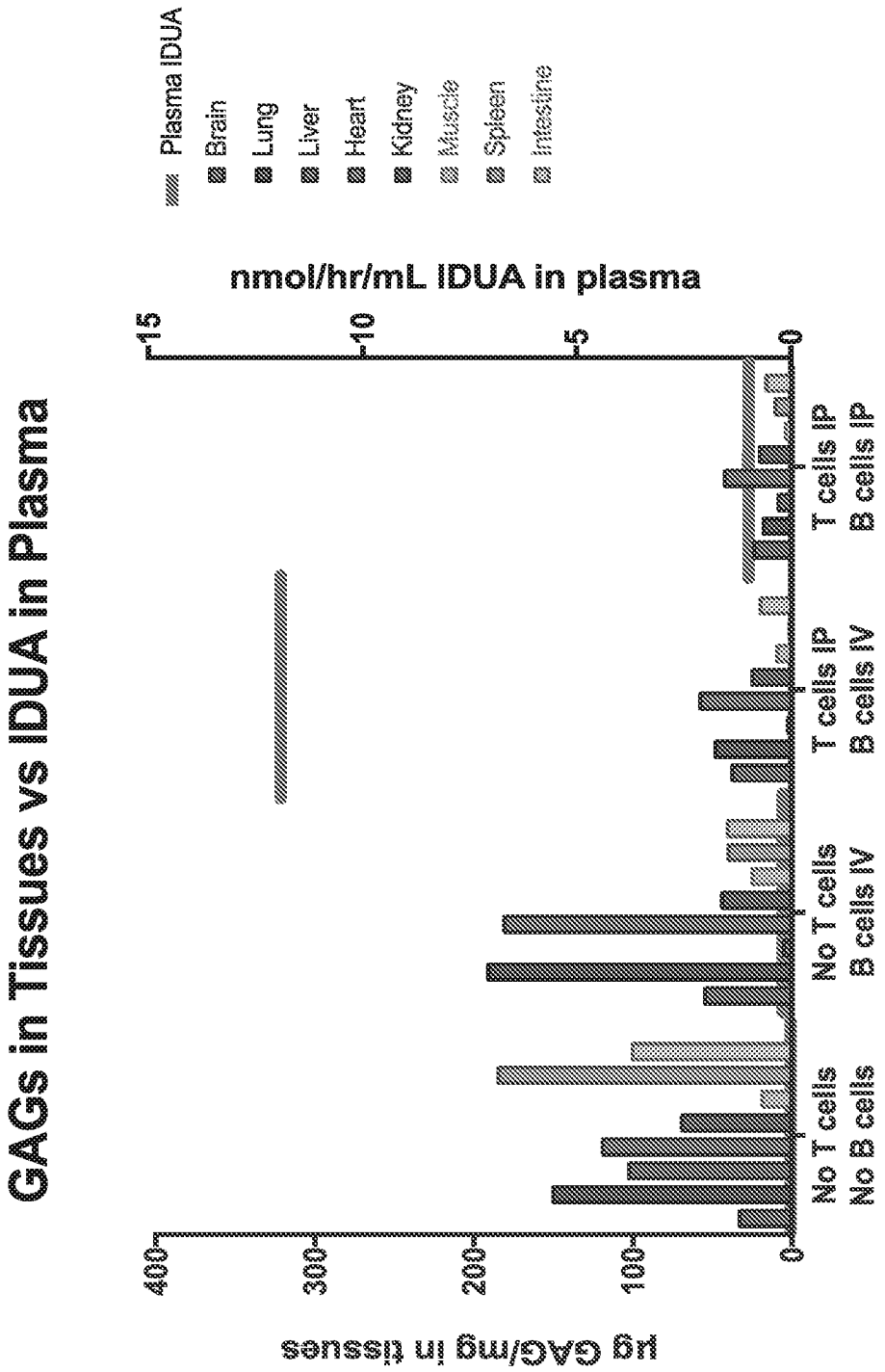

GAGs in Tissues vs IDUA in Plasma
Plasma IDUA
Brain
Lung
Liver
Heart
Kidney
Muscle
Spleen
Intestine
nmol/hr/mL IDUA in plasma
15
10
5
0
T cells IP
B cells IP
T cells IP
B cells IV
No T cells
B cells IV
No T cells
No B cells
400
300
200
100
0
µg GAG/mg in tissues

Figure 10

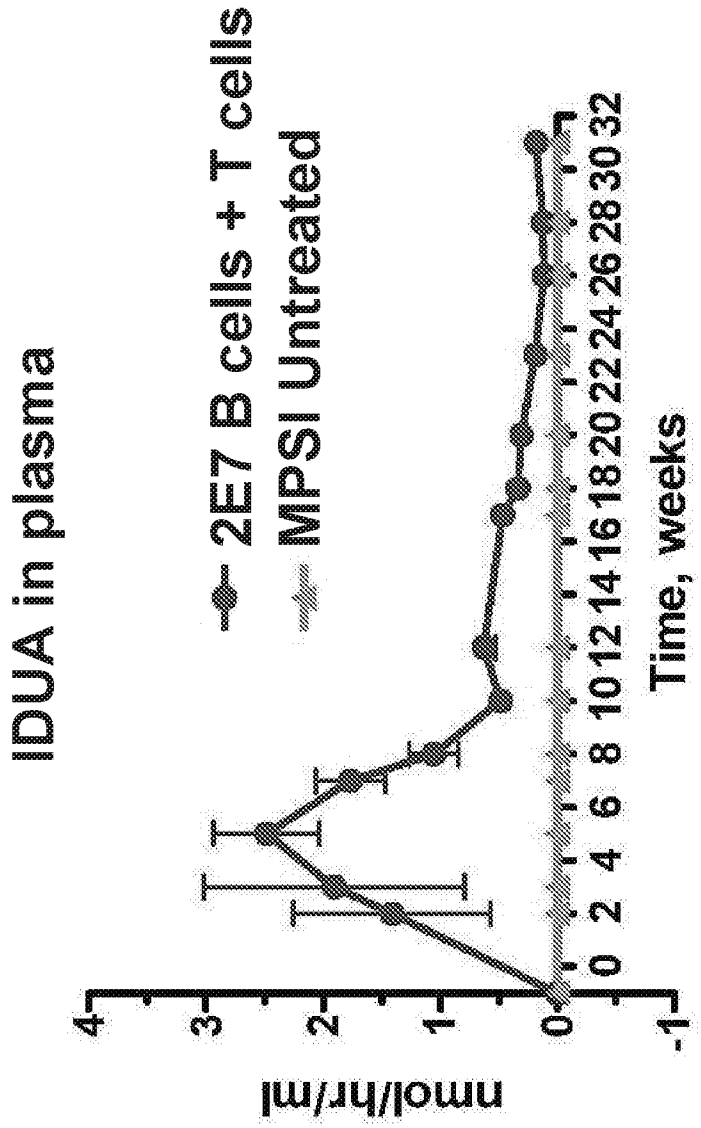

IDUA in plasma
2E7 B cells + T cells
MPSI Untreated
nmol/hr/ml
4
3
2
1
0
-1
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time, weeks

Figure 11

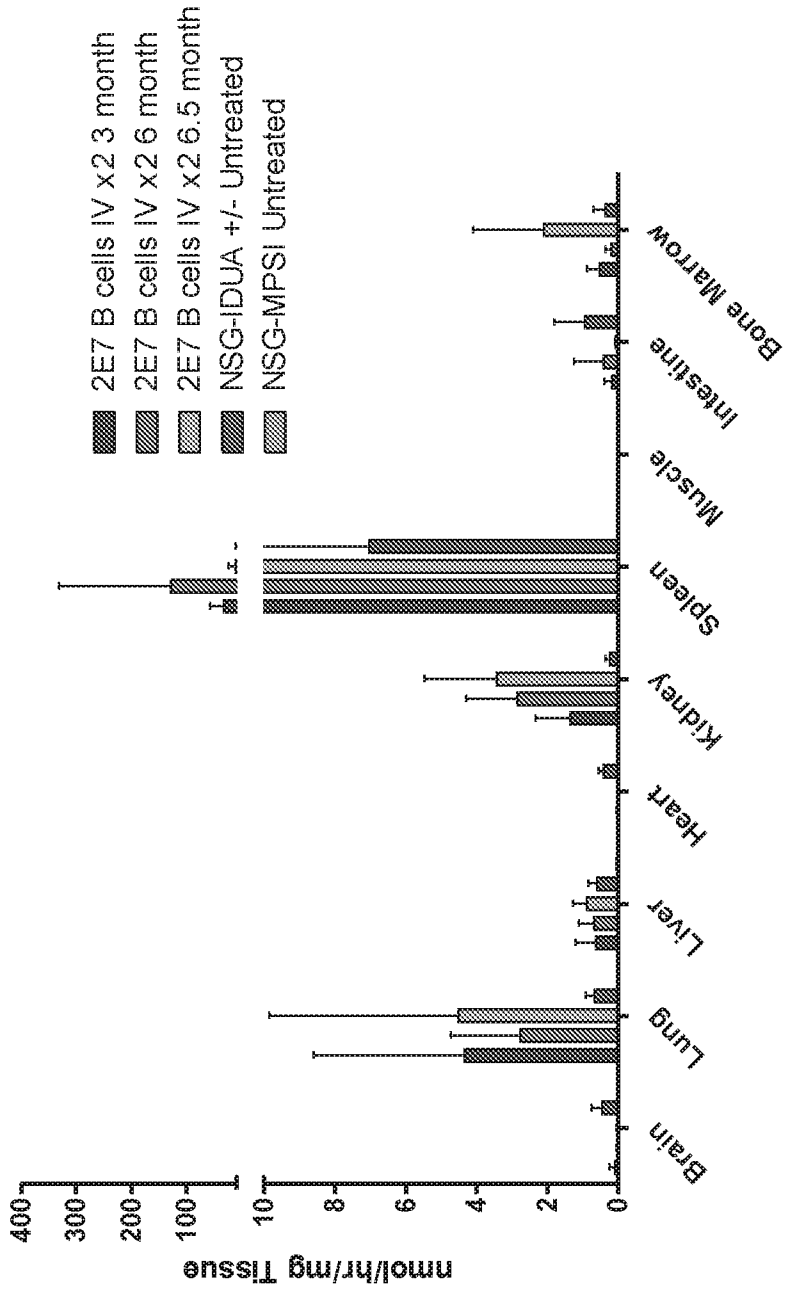

IDUA in tissues
2E7 B cells IV x2 3 month
2E7 B cells IV x2 6 month
2E7 B cells IV x2 6.5 month
NSG-IDUA +/- Untreated
NSG-MPSI Untreated
nmol/hr/mg Tissue
400
300
200
100
10
8
6
4
2
0
Brain
Lung
Liver
Heart
Kidney
Spleen
Muscle
Intestine
Bone Marrow

Figure 12

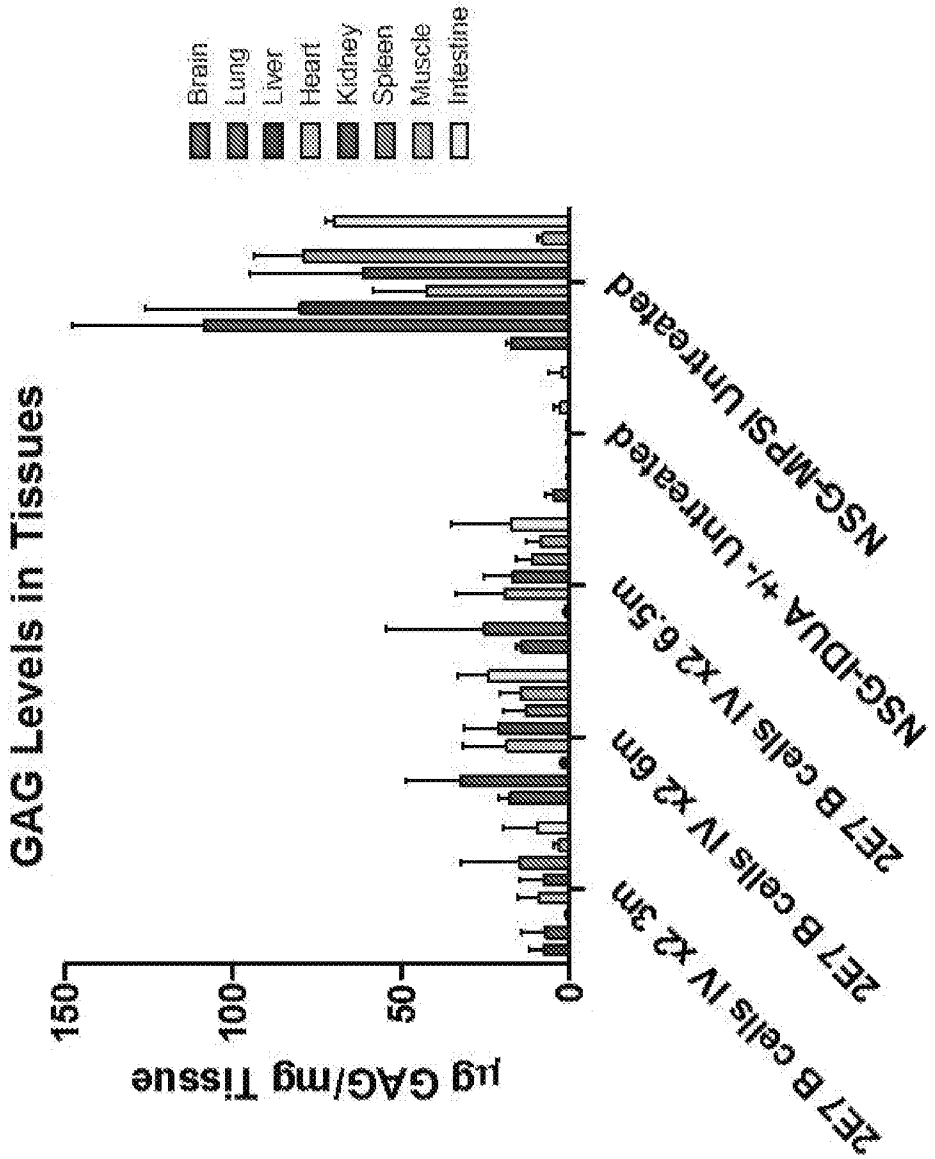

GAG Levels in Tissues
μg GAG/mg Tissue
150
100
50
0
Brain
Lung
Liver
Heart
Kidney
Spleen
Muscle
Intestine
2E7 B cells IV x2 3m
2E7 B cells IV x2 6m
2E7 B cells IV x2 6.5m
NSG-IDUA +/- Untreated
NSG-MPSI Untreated

Figure 13

FIG. 13A

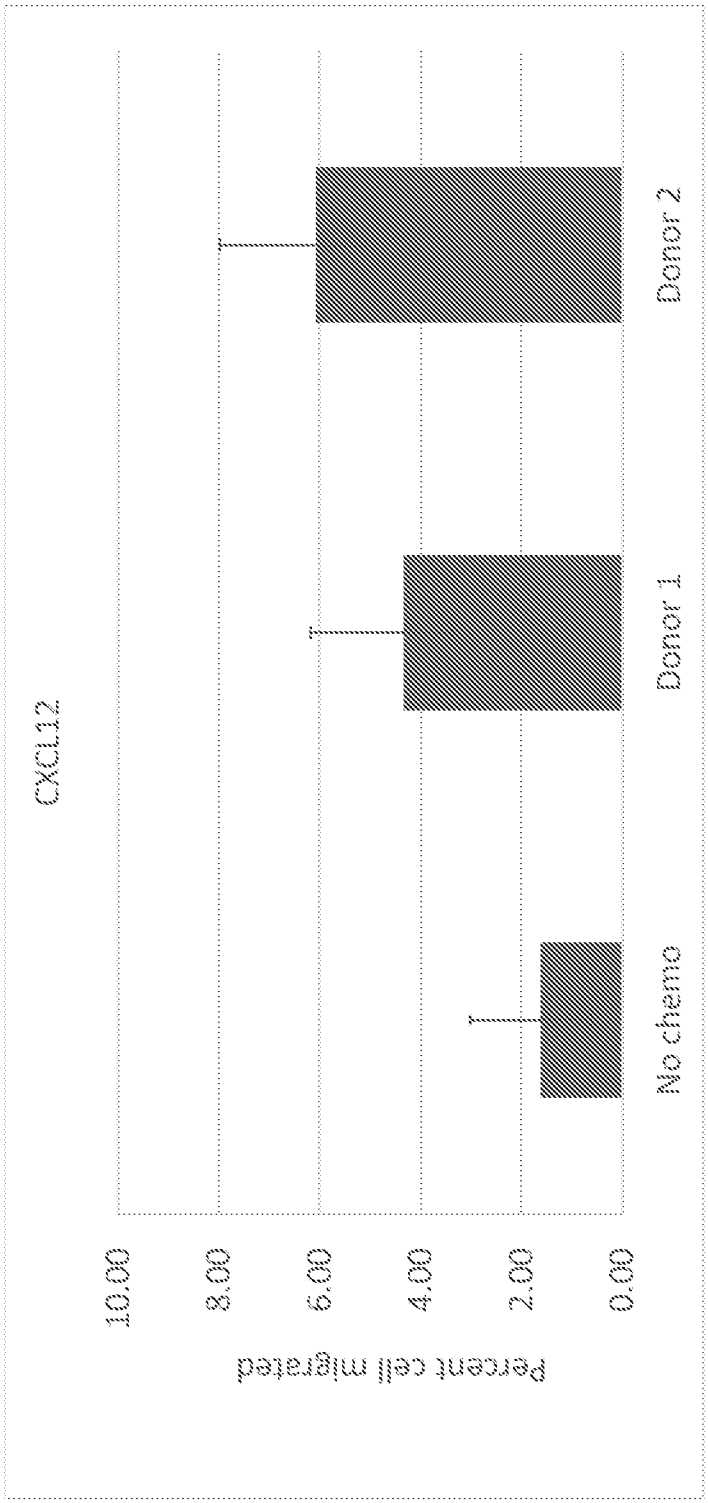


Figure 13 (Continued)

FIG. 13B

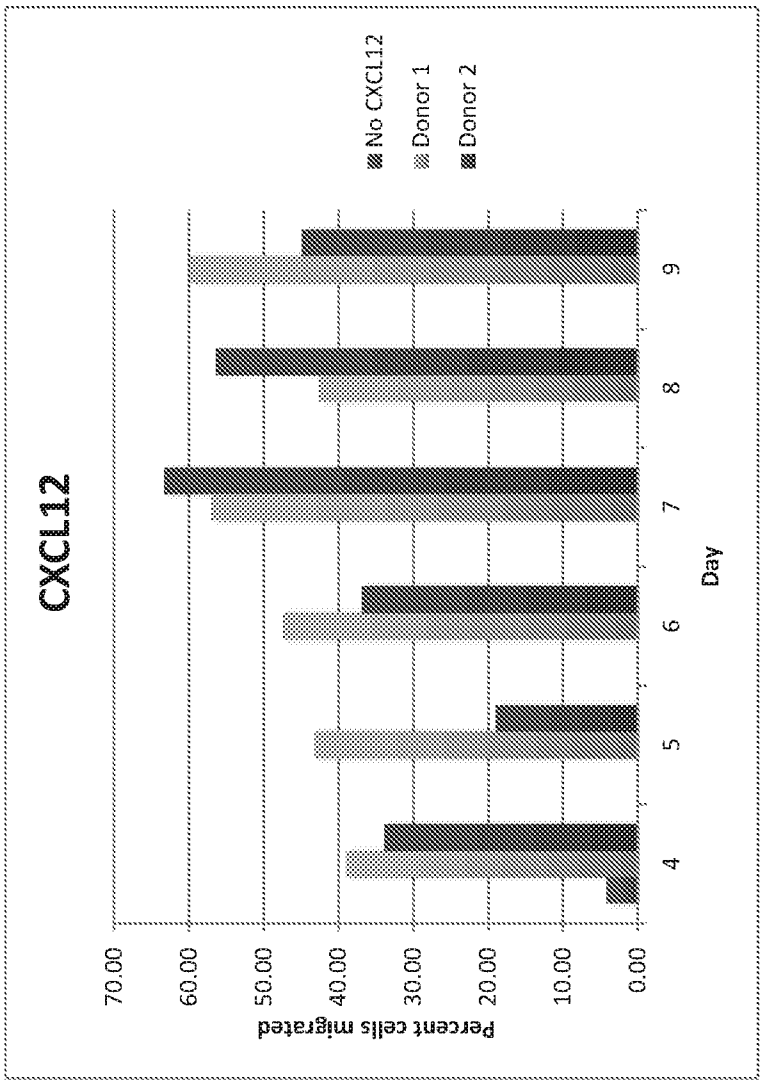


Figure 13 (Continued)

FIG. 13C

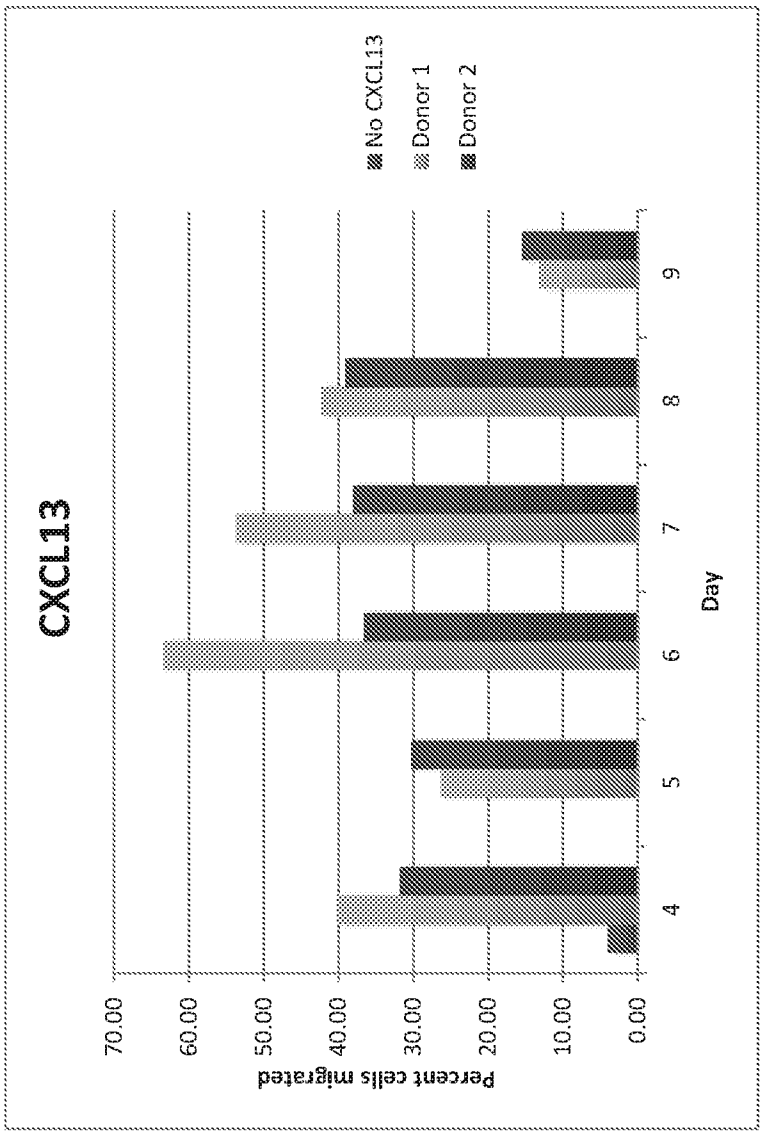


Figure 13 (Continued)

FIG. 13D

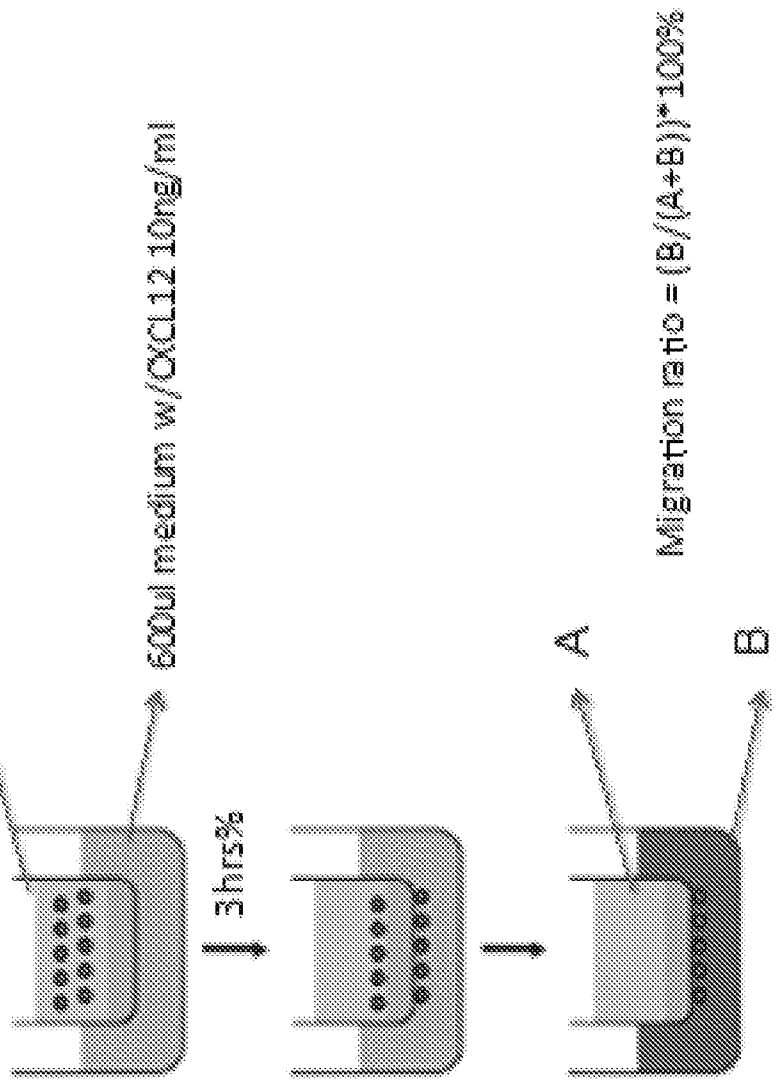


Figure 14

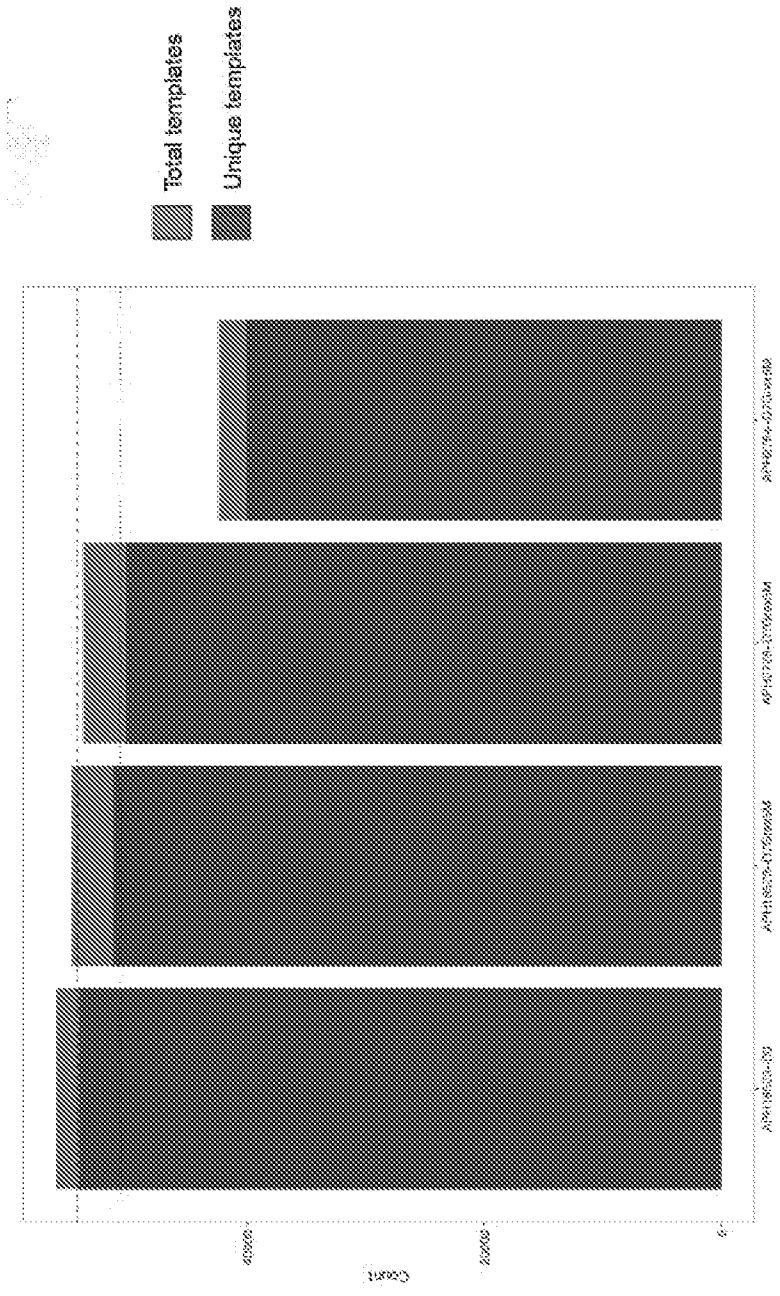


Figure 15

FIG. 15A

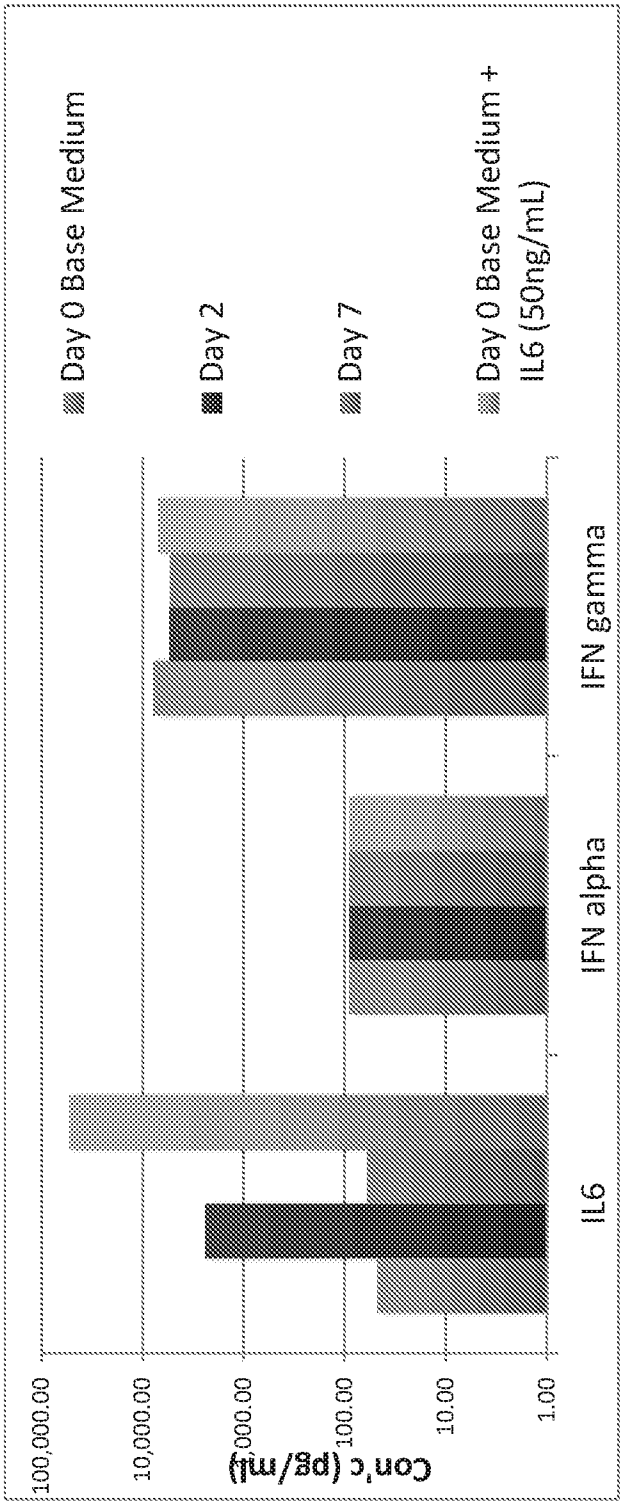


Figure 15 (Continued)

FIG. 15B

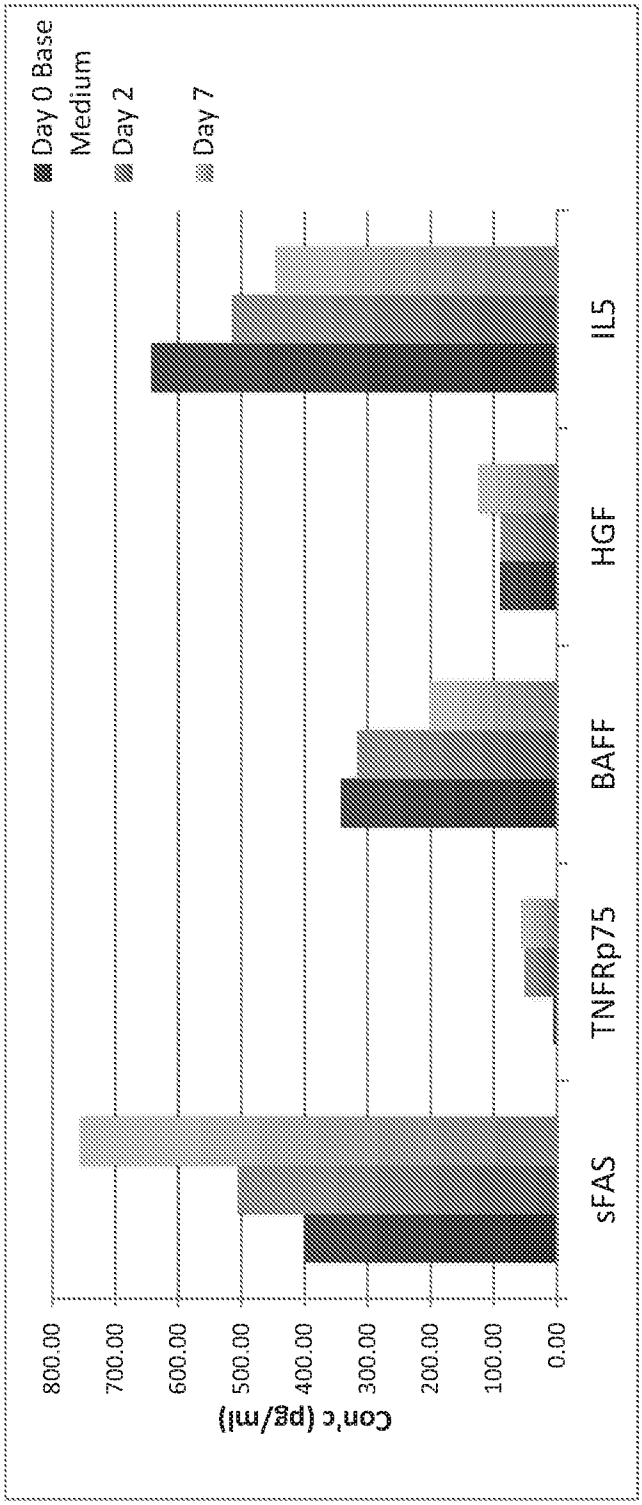


Figure 16

FIG. 16A

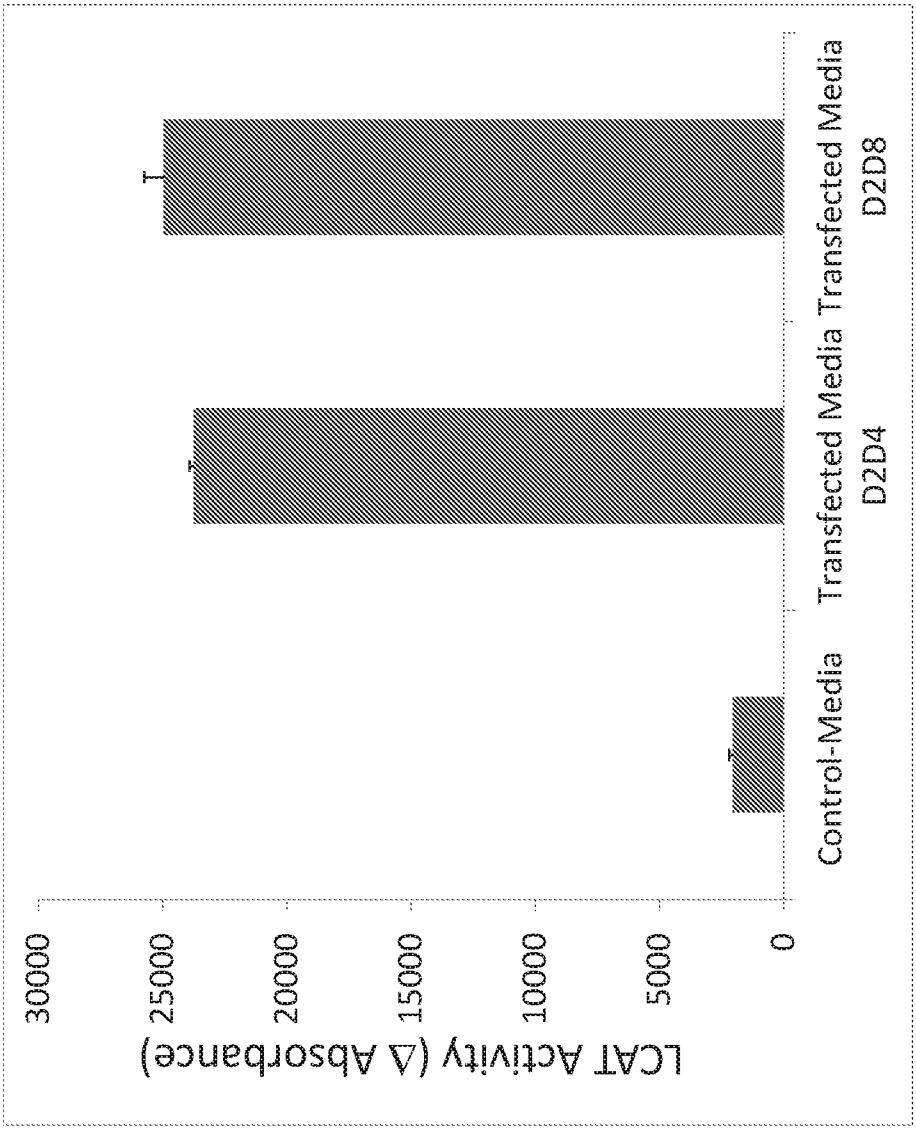


Figure 16 (Continued)

FIG. 16B

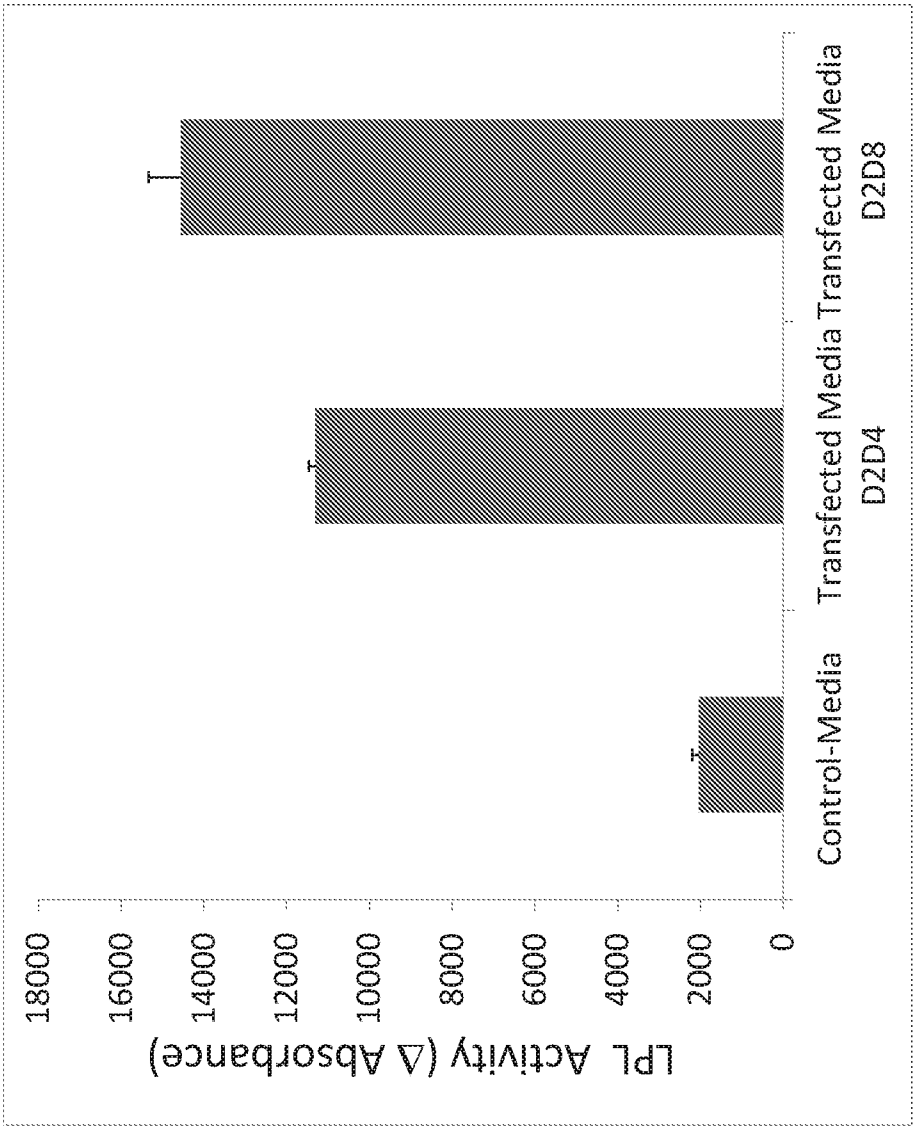
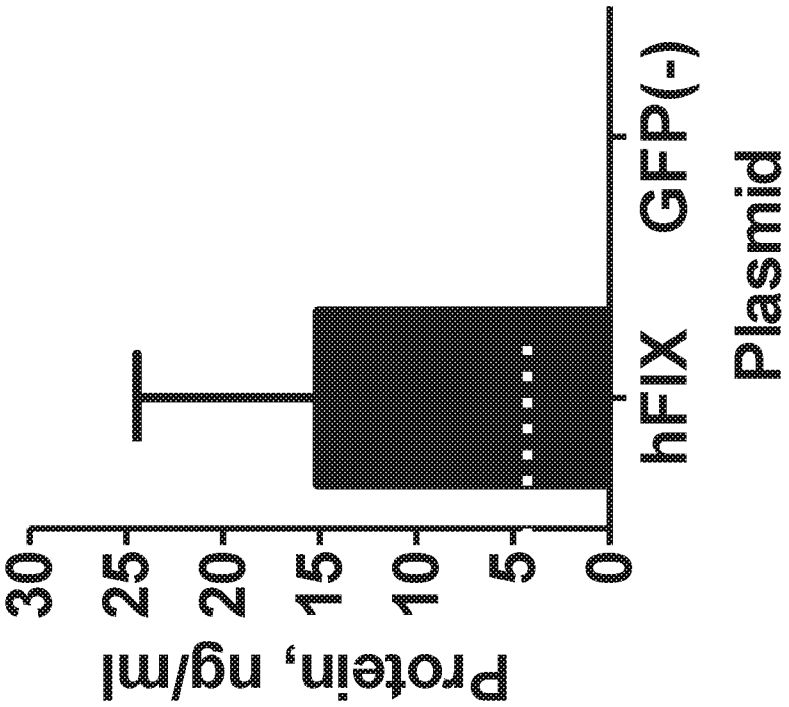


Figure 16 (Continued)

FIG. 16C



SEQUENCE LISTING

<110> Immunosoft Corporation
Scholz, Matthew Rein
Herbig, Eric J.
McIvor, R. Scott
De Laat, Rian
OLSON, Erik

<120> B CELLS FOR IN VIVO DELIVERY OF THERAPEUTIC AGENTS AND DOSAGES THEREOF

<130> IMCO-006/01WO 312423-2025

<150> US 62/491,151
<151> 2017-04-27

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<213> Artificial Sequence

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agtcagttag gacatctact ttgtgcatga cacaagtcat ttttccaaca attgtttaca 180

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