INTRACELLULAR DELIVERY OF BIOMOLECULES TO INDUCE TOLERANCE

CROSS-REREFENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Application No. 62/331,368, filed on May 3, 2016, which is hereby incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The present disclosure relates generally to methods for suppressing an immune response or inducing tolerance by delivering a compound into a cell by passing a cell suspension through a cell-deforming constriction.

BACKGROUND

[0003] The presence of antigens in the non-inflammatory and apoptotic environment of the spleen has been shown to induce anergy and tolerance. One way to ensure the consistent presentation of the antigen of interest in this tolerogenic environment is via physical interaction with a red blood cell (RBC).

[0004] However, manipulation of red blood cells to associate antigenic material is challenging given that they are irregularly shaped (biconcave), anucleate, and transcriptionally inactive. As a result, standard transfection techniques do not work and therefore initial proof of the tolerogenic potential of red blood cell has focused on conjugating materials to the surface of erythrocytes (Lorentz et al. Sci. Adv. 2015, 1:e1500112; Grimm et al. Sci Rep. 2015 Oct 29, 5:15907; Kontos et al. Proc Natl Acad Sci USA. 2013 Jan 2, 110(1):E60-8). Initial work using surface conjugation has shown promising results with model antigens and mouse models of Type 1 diabetes but has some significant drawbacks. These drawbacks include: a) the need for chemically modified antigens for attachment b) limited surface area of loading c) immunogenicity.

[0005] Thus, there is an unmet need for intracellular delivery techniques that can load antigen into the cytoplasm of red blood cells and drive a powerful immunosuppressive response for the treatment of pathogenic immune responses underlying autoimmune diseases and transplant rejection. References that describe methods of using microfluidic constrictions to deliver compounds to cells include WO2013059343, WO2015023982, WO2016070136, WO2016077761, and PCT/US2016/13113.

[0006] All references cited herein, including patent applications and publications, are incorporated by reference in their entirety.

BRIEF SUMMARY OF THE INVENTION

[0007] The present invention provides a method for suppressing an immune response in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell, and introducing the anucleate cell into the individual, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0008] Certain aspects of the present invention provide a method for suppressing an immune response in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that one or more tolerogenic factors enter the anucleate cell, and introducing the anucleate cell into the individual, wherein action of said one or more tolerogenic factors contributes to a tolerogenic environment and suppression of an immune response.

[0009] Certain aspects of the present invention provide a method for suppressing an immune response in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and one or more tolerogenic factors enter the anucleate cell, and introducing the anucleate cell into the individual, wherein processing of said antigen in a tolerogenic environment suppresses an immune response.

[0010] Certain aspects of the present invention provide a method for suppressing an immune response in an individual, comprising passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell, passing a second cell suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell, introducing the first anucleate cell and second anucleate cell into the individual, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0011] Certain aspects of the present invention provide a method for suppressing an immune response in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced

to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0012] Certain aspects of the present invention provide a method for delivering a tolerogenic factor into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the tolerogenic factor enters the cell, wherein said cell suspension is contacted with the tolerogenic factor.

[0013] Certain aspects of the present invention provide a method for introducing an antigen into a tolerogenic environment comprising delivering an anucleate cell into an individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment.

[0014] Certain aspects of the present invention provide a method for inducing tolerance to an antigen in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell, introducing the anucleate cell into the individual, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0015] Certain aspects of the present invention provide a method for inducing tolerance in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell, and introducing the anucleate cell into the individual, wherein action of said tolerogenic factor contributes to a tolerogenic environment that induces tolerance.

[0016] Certain aspects of the present invention provide a method for inducing tolerance to an antigen in an individual, comprising passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell, passing a second cell

suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell, introducing the first anucleate cell and second anucleate cell into the individual, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0017] Certain aspects of the present invention provide a method for inducing tolerance to an antigen in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0018] Certain aspects of the present invention provide a method for delivering an antigen into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the antigen enters the cell, wherein said cell suspension is contacted with the antigen. In some embodiments, processing and presentation of said antigen in a tolerogenic environment suppresses an immune response to the antigen. In some embodiments, processing and presentation of said antigen in a tolerogenic environment induces tolerance to the antigen. In some embodiments that can be combined with the previous embodiments, the tolerogenic environment is located in the spleen, liver, or lymph nodes.

[0019] In some embodiments, the constriction is contained within a microfluidic channel. In some embodiments, the channel comprises a constriction width of about 0.25 μm to about 4 μm. In some embodiments, the channel comprises a constriction width of about 4 μm, about 3.5 μm, about 3 μm, about 2.5 μm, about 2 μm, about 1 μm, about 0.5 μm, or about 0.25 μm. In some embodiments, the constriction is a pore or contained within a pore. In some embodiments, the pore is contained in a surface. In some embodiments, the surface is a filter. In some embodiments, the surface is a membrane. In some embodiments, the pore size is about 0.5 μm to about 4 μm. In some embodiments, the pore size is 4 μm, about 3 μm, about 2 μm, about 1 μm, or about 0.5 μm. In some embodiments, the constriction size is a function of the diameter of the anucleate cell. In some embodiments, the constriction size is about 10%, about 20%, about 30%,

about 40%, about 50%, about 60%, or about 70% of the cell diameter. In some embodiments, the method is performed between about -5°C and about 45°C.

[0020] Reference to a diameter of an anucleate cell means the diameter of the cell in fluid prior to being passed through a constriction, *e.g.*, as the cell approaches the constriction, unless otherwise specified.

[0021] In some embodiments that can be combined with the previous embodiments, the cell suspension comprises a mixed cell population. In some embodiments, the cell suspension is whole blood. In some embodiments, the cell suspension comprises a purified cell population. In some embodiments, the cell suspension comprises a purified anucleate cell population. In some embodiments, the cell suspension comprises mammalian cells. In some embodiments, the cell suspension comprises monkey, mouse, dog, cat, horse, rat, sheep, goat, pig, or rabbit cells. In some embodiments, the cell suspension comprises human cells. In some embodiments, the anucleate cell is a red blood cell. In some embodiments, the red blood cell is an erythrocyte. In some embodiments, the red blood cell is a reticulocyte. In some embodiments, the anucleate cell is a platelet.

[0022] In some embodiments that can be combined with the previous embodiments, the antigen is located in a cell lysate. In some embodiments, the antigen is a foreign antigen. In some embodiments, the antigen is a self-antigen. In some embodiments, the antigen is an allograft transplantation antigen. In some embodiments, the antigen is a microorganism. In some embodiments, the antigen is a lipid antigen. In some embodiments, the antigen is a carbohydrate antigen (*e.g.*, a sugar). In some embodiments, the antigen is a modified antigen. In some embodiments, the modified antigen comprises an antigen fused with a polypeptide. In some embodiments, the modified antigen comprises an antigen fused with a therapeutic agent. In some embodiments, the modified antigen comprises an antigen fused with a targeting peptide. In some embodiments, the modified antigen comprises an antigen fused with a lipid. In some embodiments, the modified antigen comprises an antigen fused with a carbohydrate (*e.g.*, a sugar). In some embodiments, the antigen is an antigen associated with transplanted tissue. In some embodiments, the antigen is associated with a virus. In some embodiments, said cell suspension is contacted with the antigen before, concurrently, or after passing through the constriction.

[0023] In some embodiments that can be combined with the previous embodiments, the tolerogenic factor comprises a polypeptide. In some embodiments, the polypeptide is IL-4, IL-

10, IL-13, IL-35, IFNα, or TGFβ. In some embodiments, the polypeptide is a therapeutic polypeptide. In some embodiments, the polypeptide is a fragment of a therapeutic polypeptide. In some embodiments, the polypeptide is a therapeutic peptide. In some embodiments, the polypeptide is conjugated to a carbohydrate (*e.g.*, a sugar). In some embodiments, said cell suspension is contacted with the tolerogenic factor before, concurrently, or after passing through the constriction. In some embodiments, the half-life of the anucleate cell is decreased. In some embodiments, the half-life of the anucleate cell is increased.

[0024] In embodiments where an immune response is suppressed, the immune response is suppressed by at least about 25%, about 50%, about 75%, about 100%, about 150%, about 200%, or more than about 200%. In some embodiments, the suppressed immune response comprises a decreased T cell response. In some embodiments, the decreased T cell response comprises decreased T cell activation. In some embodiments, the decreased T cell response comprises decreased T cell survival. In some embodiments, the decreased T cell response comprises decreased T cell proliferation. In some embodiments, the decreased T cell response comprises decreased T cell functionality. In some embodiments, the decreased T cell response comprises a change in T cell phenotype. In some embodiments, the suppressed immune response comprises uncostimulated activation of a T cell. In some embodiments, the suppressed immune response comprises an enhanced Treg response. In some embodiments, the suppressed immune response comprises a decreased B cell response. In some embodiments, the decreased B cell response comprises decreased antibody production. In some embodiments, the suppressed immune response comprises decreased cytokine production. In some embodiments, the suppressed immune response comprises a decreased autoimmune response. In some embodiments, the suppressed immune response comprises a decreased allergic response. In some embodiments, the suppressed immune response comprises a decreased immune response against the transplanted tissue. In some embodiments, the suppressed immune response comprises a decreased pathogenic immune response to the virus. In some embodiments, the suppressed immune response comprises a decreased immune response against a therapeutic agent. In some embodiments, the suppressed immune response comprises a decreased immune response against a therapeutic vehicle.

[0025] In embodiments where tolerance is induced, the tolerance may comprise a decreased T cell response. In some embodiments, the decreased T cell response comprises decreased T cell activation. In some embodiments, the decreased T cell response comprises decreased T cell

survival. In some embodiments, the decreased T cell response comprises decreased T cell proliferation. In some embodiments, the decreased T cell response comprises decreased T cell functionality. In some embodiments, the decreased T cell response comprises a change in T cell phenotype. In some embodiments, the tolerance comprises uncostimulated activation of a T cell. In some embodiments, the tolerance comprises an enhanced Treg response. In some embodiments, the tolerance comprises a decreased B cell response. In some embodiments, the decreased B cell response comprises decreased antibody production. In some embodiments, the tolerance comprises decreased cytokine production. In some embodiments, the tolerance comprises a decreased autoimmune response. In some embodiments, the tolerance comprises a decreased allergic response. In some embodiments, the tolerance comprises a decreased immune response against the transplanted tissue. In some embodiments, the tolerance comprises a decreased pathogenic immune response to the virus. In some embodiments, the tolerance comprises a decreased immune response against a therapeutic agent. In some embodiments, the tolerance comprises a decreased immune response against a therapeutic vehicle.

[0026] In some embodiments where a modified anucleate cell is administered to an individual, the method further comprises at least one (such as at least 2, 3, 4, 5, 6, or more) additional administration of a modified anucleate cell as described above. In some embodiments, the duration of time between any two administrations is at least 1 day, 1 week, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, or 1 year.

[0027] Certain aspects of the present invention relate to a system comprising the constriction, cell suspension, and antigen for use in any one of the aforementioned methods. Certain aspects of the present invention relate to a system comprising the constriction, cell suspension, and tolerogenic factor for use in any one of the aforementioned methods.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **FIGS. 1A&B** show exemplary flow cytometry histogram plots depicting fluorescence post constriction mediated delivery (SQZ) of IgG antibody (**FIG. 1A**) and dextran particles (**FIG. 1B**) to human RBCs as compared to endocytosis controls.

[0029] **FIG. 2A** shows delivery efficiency post constriction mediated delivery of IgG antibody and dextran particles to human RBCs. **FIG. 2B** shows estimated cell viability post constriction mediated delivery of IgG antibody and dextran particles to human RBCs.

[0030] **FIG. 3A** shows exemplary flow cytometry histogram plots depicting fluorescence post constriction mediated delivery of IgG antibody to mouse RBCs. **FIG. 3B** shows estimated cell viability post constriction mediated delivery of IgG antibody to mouse RBCs. **FIG. 3C** shows delivery efficiency post constriction mediated delivery of IgG antibody to mouse RBCs.

[0031] **FIGS. 4A-I** show exemplary FACS plots demonstrating delivery of dextran particles to mouse RBCs under 2psi (**FIG. 4D**), 4psi (**FIG. 4E**), 6psi (**FIG. 4F**), 10psi (**FIG. 4G**), 20psi (**FIG. 4H**), or using manual syringe pressure (**FIG. 4I**) as compared to the endocytosis (**FIG. 4A**), negative control (**FIG. 4B**), and no material control (**FIG. 4C**).

[0032] **FIG. 5A** shows estimated cell viability post constriction mediated delivery of dextran particles to mouse RBCs. **FIG. 5B** shows delivery efficiency post constriction mediated delivery of IgG antibody or dextran particles to mouse RBCs. **FIG. 5C** shows geometric mean fluorescence post constriction mediated delivery of IgG antibody or dextran particles to mouse RBCs.

[0033] **FIG. 6A** shows exemplary flow cytometry histogram plots depicting fluorescence post constriction mediated delivery of dextran particles to mouse RBCs. **FIG. 6B** shows estimated cell viability post constriction mediated delivery of dextran particles to mouse RBCs. **FIG. 6C** shows delivery efficiency post constriction mediated delivery of dextran particles to mouse RBCs. **FIG. 6D** shows geometric mean fluorescence post constriction mediated delivery of dextran particles to mouse RBCs.

[0034] **FIG. 7** shows a schematic of the treatment schedule.

[0035] **FIG. 8** shows the number of OT-I-specific T cells in mice from the four treatment groups.

[0036] **FIG. 9** shows the percent of splenic T cells that expressed high levels of IFN-γ (left) and the level of IFN-γ production (right).

[0037] **FIG. 10** shows representative flow cytograms for the proportion of OT-I T cells out of all CD8+ T cells (top panels) and the proportion of IFN-γ responsive T cells out of OT-I T cells (bottom panels).

[0038] **FIGS. 11A** and **11B** show schematics of representative treatment schedules.

[0039] **FIGS. 12A** and **12B** show schematics of representative treatment schedules.

DETAILED DESCRIPTION

[0040] The invention provides methods of inducing tolerance and/or suppressing an immune response in an individual by passing a cell suspension containing an anucleate cell through a constriction, enabling delivery of an antigen and/or a tolerogenic factor to the anucleate cell. In some embodiments the constriction is contained within a microfluidic channel. In some embodiments the constriction is a pore or contained within a pore.

[0041] Certain aspects of the present disclosure relate to methods for inducing tolerance and/or suppressing an immune response to an antigen in an individual, the methods including passing a cell suspension containing an anucleate cell through a constriction, wherein the constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell; and introducing the anucleate cell into the individual, wherein presentation of said antigen in a tolerogenic environment induces tolerance and/or suppresses an immune response to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the tolerance and/or immune suppression are antigen-specific. In some embodiments, the tolerance and/or immune suppression are non-specific, including tolerance and/or suppression of an immune response to a plurality of antigens.

[0042] Certain aspects of the present disclosure relate to methods for inducing tolerance and/or suppressing an immune response to an antigen in an individual, the methods including passing a cell suspension containing an anucleate cell through a constriction, wherein the constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and a tolerogenic factor contacted with the cell enter the anucleate cell; and introducing the anucleate cell into the individual, thereby inducing tolerance and/or suppressing an immune response to the antigen. In some embodiments, said antigen is presented in a tolerogenic environment. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of the antigen in said tolerogenic environment induces tolerance and/or suppresses an immune response to the antigen. For example, in some embodiments, the tolerogenic factor acts in a tolerogenic environment to further increase the tolerogenic nature of the environment. In some embodiments, the tolerogenic factor acts in a non-tolerogenic environment to generate a tolerogenic environment. In some embodiments, said antigen is processed in a tolerogenic environment. In some embodiments, the tolerance and/or immune suppression are antigen-specific. In some embodiments, the tolerance and/or immune suppression are non-specific, including tolerance and/or suppression of an immune response to a plurality of antigens.

[0043] Certain aspects of the present disclosure relate to methods for inducing tolerance and/or suppressing an immune response to an antigen in an individual, the method including passing a cell suspension containing an anucleate cell through a constriction, wherein the constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell; and introducing the anucleate cell into the individual, thereby inducing tolerance and/or suppressing an immune response to an antigen. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of an antigen in said tolerogenic environment induces tolerance and/or suppresses an immune response to the antigen. In some embodiments, the tolerance and/or immune suppression are antigen-specific. In some embodiments, the tolerance and/or immune suppression are non-specific, including tolerance and/or suppression of an immune response to a plurality of antigens.

I. GENERAL TECHNIQUES

[0044] The techniques and procedures described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, *et al.* eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds., 1995); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., Academic Press, 1998); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis *et al.*, eds., 1994); *Current Protocols in Immunology* (J.E. Coligan *et al.*, eds., 1991); *Short Protocols in Molecular Biology* (Ausubel *et al.*, eds., J. Wiley and Sons, 2002); *Immunobiology* (C.A. Janeway *et al.*, 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal*

Antibodies: A Practical Approach (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D. Capra, eds., Harwood Academic Publishers, 1995); and *Cancer: Principles and Practice of Oncology* (V.T. DeVita *et al.*, eds., J.B. Lippincott Company, 2011).

II. DEFINITIONS

[0045] For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any definition set forth below conflicts with any document incorporated herein by reference, the definition set forth shall control.

[0046] As used herein, the singular form "a", "an", and "the" includes plural references unless indicated otherwise.

[0047] It is understood that aspects and embodiments of the invention described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments.

[0048] The term "about" as used herein refers to the usual error range for the respective value readily known to the skilled person in this technical field. Reference to "about" a value or parameter herein includes (and describes) embodiments that are directed to that value or parameter per se.

[0049] The term "pore" as used herein refers to an opening, including without limitation, a hole, tear, cavity, aperture, break, gap, or perforation within a material. In some examples, (where indicated) the term refers to a pore within a surface of the present disclosure. In other examples, (where indicated) a pore can refer to a pore in a cell membrane.

[0050] The term "membrane" as used herein refers to a selective barrier or sheet containing pores. The term includes a pliable sheetlike structure that acts as a boundary or lining. In some examples, the term refers to a surface or filter containing pores. This term is distinct from the term "cell membrane".

[0051] The term "filter" as used herein refers to a porous article that allows selective passage through the pores. In some examples the term refers to a surface or membrane containing pores.

[0052] The term "heterogeneous" as used herein refers to something which is mixed or not uniform in structure or composition. In some examples the term refers to pores having varied sizes, shapes or distributions within a given surface.

[0053] The term "homogeneous" as used herein refers to something which is consistent or uniform in structure or composition throughout. In some examples the term refers to pores having consistent sizes, shapes, or distribution within a given surface.

[0054] The term "heterologous" as used herein refers to a molecule which is derived from a different organism. In some examples the term refers to a nucleic acid or protein which is not normally found or expressed within the given organism.

[0055] As used herein, the term "inhibit" may refer to the act of blocking, reducing, eliminating, or otherwise antagonizing the presence, or an activity of, a particular target. Inhibition may refer to partial inhibition or complete inhibition. For example, inhibiting an immune response may refer to any act leading to a blockade, reduction, elimination, or any other antagonism of an immune response. In other examples, inhibition of the expression of a nucleic acid may include, but not limited to reduction in the transcription of a nucleic acid, reduction of mRNA abundance (*e.g.,* silencing mRNA transcription), degradation of mRNA, inhibition of mRNA translation, gene editing and so forth. In other examples, inhibition of the expression of a protein may include, but not be limited to, reduction in the transcription of a nucleic acid encoding the protein, reduction in the stability of mRNA encoding the protein, inhibition of translation of the protein, reduction in stability of the protein, and so forth.

[0056] As used herein, the term "suppress" may refer to the act of decreasing, reducing, prohibiting, limiting, lessening, or otherwise diminishing the presence, or an activity of, a particular target. Suppression may refer to partial suppression or complete suppression. For example, suppressing an immune response may refer to any act leading to decreasing, reducing, prohibiting, limiting, lessening, or otherwise diminishing an immune response. In other examples, suppression of the expression of a nucleic acid may include, but not limited to reduction in the transcription of a nucleic acid, reduction of mRNA abundance (*e.g.,* silencing mRNA transcription), degradation of mRNA, inhibition of mRNA translation, and so forth. In other examples, suppression of the expression of a protein may include, but not be limited to, reduction in the transcription of a nucleic acid encoding the protein, reduction in the stability of mRNA encoding the protein, inhibition of translation of the protein, reduction in stability of the protein, and so forth.

[0057] As used herein, the term "enhance" may refer to the act of improving, boosting, heightening, or otherwise increasing the presence, or an activity of, a particular target. For example, enhancing an immune response may refer to any act leading to improving, boosting,

heightening, or otherwise increasing an immune response. In other examples, enhancing the expression of a nucleic acid may include, but not limited to increase in the transcription of a nucleic acid, increase in mRNA abundance (*e.g.*, increasing mRNA transcription), decrease in degradation of mRNA, increase in mRNA translation, and so forth. In other examples, enhancing the expression of a protein may include, but not be limited to, increase in the transcription of a nucleic acid encoding the protein, increase in the stability of mRNA encoding the protein, increase in translation of the protein, increase in the stability of the protein, and so forth.

[0058] As used herein, the term "induce" may refer to the act of initiating, prompting, stimulating, establishing, or otherwise producing a result. For example, inducing an immune response may refer to any act leading to initiating, prompting, stimulating, establishing, or otherwise producing a desired immune response. In other examples, inducing the expression of a nucleic acid may include, but not limited to initiation of the transcription of a nucleic acid, initiation of mRNA translation, and so forth. In other examples, inducing the expression of a protein may include, but not be limited to, increase in the transcription of a nucleic acid encoding the protein, increase in the stability of mRNA encoding the protein, increase in translation of the protein, increase in the stability of the protein, and so forth.

[0059] The term "homologous" as used herein refers to a molecule which is derived from the same organism. In some examples the term refers to a nucleic acid or protein which is normally found or expressed within the given organism.

[0060] The term "polynucleotide" or "nucleic acid" as used herein refers to a polymeric form of nucleotides of any length, including ribonucleotides and deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. The backbone of the polynucleotide can comprise repeating units, such as N-(2-aminoethyl)-glycine, linked by peptide bonds (*i.e.*, peptide nucleic acid). Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate (P-NH2) or a mixed phosphoramidate-phosphodiester oligomer. In addition, a double-stranded polynucleotide can be obtained from the

single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

[0061] The terms "polypeptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-expression modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a "polypeptide" refers to a protein which includes modifications, such as deletions, additions, and substitutions (generally conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0062] For any of the structural and functional characteristics described herein, methods of determining these characteristics are known in the art.

III. IMMUNE SUPPRESSION AND TOLERANCE

[0063] In certain aspects, the invention provides methods for suppressing an immune response and/or inducing tolerance in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and/or tolerogenic compound enters the anucleate cell and introducing the anucleate cell into the individual, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0064] Immunological tolerance is an immunological unresponsiveness to challenge with the antigen to which tolerance has been induced. Tolerance is demonstrated when a subject is unresponsive to subsequent challenge with the tolerance-inducing antigen. Tolerance can be mediated by several different mechanisms which include apoptosis of the autoreactive cells, induction of anergy, or deviation of lymphocyte phenotype. In some embodiments, the tolerance may include T cell non-responsiveness, causing deactivation of immune response to a specific

antigen. Tolerance can also be mediated by regulatory T cells (Tregs) through secretion of immunosuppressive cytokines and via contact-dependent interactions with cell surface molecules or cytotoxic factors. Defects in tolerogenic mechanisms can contribute to autoimmune diseases, transplantation rejection, anti-drug responses, and pathogenic responses to viral infection. Immunosuppression is a decrease in the activity of the immune response. For example, immunosuppression may include, without limitation, decreased responsiveness to challenge with antigen, decreased immune cell activation and proliferation, modulated cytokine secretion, decreased immune cell survival, or decreased immune cell effector functions. In some embodiments, the immunosuppression is specific for a given antigen. For example, an immune response against a self-antigen is suppressed.

[0065] In some embodiments, antigen is processed and presented in a tolerogenic environment. A tolerogenic environment may be a location within the body which supports the generation of a tolerogenic immune response. In some embodiments, the tolerogenic environment is located in a primary lymphoid tissue, such as the thymus. In some embodiments, the tolerogenic environment is located in a secondary lymphoid tissue. Exemplary secondary lymphoid tissues include the spleen, lymph nodes, and mucosa-associated lymphoid tissues (MALT). In some embodiments, the tolerogenic environment is located in the spleen. In some embodiments, the tolerogenic environment is located in a non-lymphoid tissue such as the liver. In some embodiments, the tolerogenic environment is induced at a site of inflammation by delivery of a tolerogenic factor. For example, an inflammatory environment is converted to a tolerogenic environment by a tolerogenic factor.

[0066] In some embodiments, the invention provides methods for delivery to an anucleate cell, wherein the cell is a mammalian cell. Anucleate cells lack a nucleus. In some embodiments, the anucleate cell is a monkey, mouse, dog, cat, horse, rat, sheep, goat pig, or rabbit cell. In some embodiments, the anucleate cell is a human cell. In some embodiments, the anucleate cell is a non-mammalian cell. In some embodiments, the anucleate cell is a chicken, frog, insect, fish, or nematode cell. In some embodiments, the anucleate cell is a red blood cell. Red blood cells (RBCs) are flexible and oval biconcave discs with cytoplasm rich in the oxygen-carrier biomolecule hemoglobin. RBCs serve as the primary means for oxygen delivery and carbon dioxide removal throughout the human body. RBCs can stay in circulation for up to 120 days, after which they are removed from the body via clearance in the spleen. Reticulocytes are anucleate immature (not yet biconcave) red blood cells and typically comprise about 1 % of the

red blood cells in the human body. Mature red blood cells are also referred to as erythrocytes. In some embodiments, the anucleate cell is a platelet. Platelets, also called thrombocytes, are a component of blood whose function involves blood clotting. Platelets are biconvex discoid (lens-shaped) structures 2–3 μm in diameter.

[0067] In some embodiments, presentation of antigen in a tolerogenic environment suppresses an immune response to the antigen or induces a tolerogenic response to the antigen. Antigens derived from apoptotic cells, such as RBCs, that are regularly cleared in the tolerogenic environment of the spleen, may drive tolerance and/or suppress an immune response to the antigens via deletion (*e.g.*, killing) or anergy of reactive T cells. In some embodiments, the tolerance and/or immune suppression is antigen-specific. RBCs have a limited life span and are unable to self-repair, leading to RBC death by eryptosis, a process similar to apoptosis, and subsequently removal from the bloodstream. In some embodiments, the antigen may be released upon apoptosis of the anucleate cell within the tolerogenic environment, where it is subsequently engulfed, processed, and presented by an antigen-presenting cell. In some embodiments, the anucleate cell containing the antigen is phagocytosed by an antigen-presenting cell, such as a macrophage, and the antigen is subsequently processed and presented by the antigen-presenting cell.

[0068] In some embodiments, the half-life of the anucleate cell can be modified. In some embodiments, the half-life of the anucleate cell is increased. For example, the anucleate may be modified to increase the time the anucleate cell circulates in the blood stream before clearance in the spleen. In some embodiments, the half-life of the anucleate cell is decreased. For example, the anucleate cell may be modified to decrease the time the anucleate cell circulates in the blood stream before clearance in the spleen. In some embodiments, an altered ratio of lipids on the anucleate cell surface decreases the half-life of the anucleate cell. For example, the presence of phosphatidylserine on the surface of the anucleate cell can be increased to decrease the half-life of the anucleate cell, such as by using any method known in the art for increasing surface phosphatidylserine (*see* Hamidi *et al., J. Control. Release*, 2007, 118(2): 145-60). Phosphatidylserine exposure on the outer cell membrane is a hallmark of apoptosis and is recognized by receptors on phagocytes in a manner that promotes engulfment. In some embodiments, the anucleate cell is incubated with lipids prior to delivery to an individual. In some embodiments, the anucleate cell is associated with a polymer to decrease the half-life of the anucleate cell.

[0069] In certain aspects, the invention provides methods for suppressing an immune response to an antigen in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell and introducing the anucleate cell into the individual, wherein presentation of said antigen in a tolerogenic environment suppresses an immune response to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the immune response is antigen-specific.

[0070] In certain aspects, the invention provides methods for suppressing an immune response in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell and introducing the anucleate cell into the individual, thereby suppressing an immune response. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of an antigen in said tolerogenic environment suppresses an immune response to the antigen. In some embodiments, the immune suppression is non-specific, including suppression of an immune response to a plurality of antigens.

[0071] In certain aspects, the invention provides methods for suppressing an immune response to an antigen in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and a tolerogenic factor enter the anucleate cell and introducing the anucleate cell into the individual, thereby suppressing an immune response to the antigen. In some embodiments, the antigen is presented in a tolerogenic environment. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the immune suppression is antigen-specific. In some embodiments, the immune suppression is non-specific, including suppression of an immune response to a plurality of antigens.

[0072] In certain aspects, the present invention provides methods for suppressing an immune response to an antigen in an individual, comprising passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby

causing a perturbation of the cell such that an antigen enters the anucleate cell, passing a second cell suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell, and introducing the first anucleate cell and second anucleate cell into the individual, thereby suppressing an immune response to the antigen. In some embodiments, the antigen is presented in a tolerogenic environment. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the first anucleate cell and second anucleate cell are introduced simultaneously. In some embodiments, the first anucleate cell and second anucleate cell are introduced sequentially. In some embodiments, the first anucleate cell is introduced to the individual before introduction of the second anucleate cell. In some embodiments, the first anucleate cell is introduced to the individual more than any of about 1 minute, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, or 24 hours before introduction of the second anucleate cell. In some embodiments, the second anucleate cell is introduced to the individual before introduction of the first anucleate cell. In some embodiments, the second anucleate cell is introduced to the individual more than any of about 1 minute, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, or 24 hours before introduction of the first anucleate cell. In some embodiments, the immune suppression is antigen-specific. In some embodiments, the immune suppression is non-specific, including suppression of an immune response to a plurality of antigens.

[0073] In certain aspects, the invention provides a method for inducing tolerance to an antigen in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell and introducing the anucleate cell into the individual, wherein presentation of said antigen in a tolerogenic environment induces tolerance to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the tolerance is antigen-specific.

[0074] In certain aspects, the invention provides methods for inducing tolerance to an antigen in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation

of the cell such that a tolerogenic factor enters the anucleate cell, and introducing the anucleate cell into the individual, thereby inducing tolerance to the antigen. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, and presentation of the antigen in said tolerogenic environment induces tolerance to the antigen. In some embodiments, the tolerance is non-specific, including tolerance to a plurality of antigens.

[0075] In certain aspects, the invention provides a method for inducing tolerance to an antigen in an individual, the method comprising passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and a tolerogenic factor enter the anucleate cell and introducing the anucleate cell into the individual, thereby inducing tolerance to the antigen. In some embodiments, the antigen is presented in a tolerogenic environment. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the tolerance is antigen-specific. In some embodiments, the tolerance is non-specific, including tolerance to a plurality of antigens.

[0076] In certain aspects, the invention provides methods for inducing tolerance to an antigen in an individual, comprising passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell, passing a second cell suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell, and introducing the first anucleate cell and second anucleate cell into the individual, thereby inducing tolerance to the antigen. In some embodiments, the antigen is presented in a tolerogenic environment. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the first anucleate cell and second anucleate cell are introduced simultaneously. In some embodiments, the first anucleate cell and second anucleate cell are introduced sequentially. In some embodiments, the first anucleate cell is introduced to the individual before introduction of the second anucleate cell. In some embodiments, the first anucleate cell is introduced to the individual more than any of about 1 minute, 5 minutes, 10

minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, or 24 hours before introduction of the second anucleate cell. In some embodiments, the second anucleate cell is introduced to the individual before introduction of the first anucleate cell. In some embodiments, the second anucleate cell is introduced to the individual more than any of about 1 minute, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, or 24 hours before introduction of the first anucleate cell. In some embodiments, the tolerance is antigen-specific. In some embodiments, the tolerance is non-specific, including tolerance to a plurality of antigens.

[0077] In certain aspects, the invention provides methods for inducing tolerance to an antigen in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein presentation of said antigen in a tolerogenic environment induces tolerance to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the tolerance is antigen-specific.

[0078] In certain aspects, the invention provides methods for suppressing an immune response to an antigen in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein presentation of said antigen in a tolerogenic environment suppresses an immune response to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the immune suppression is antigen-specific.

[0079] In certain aspects, the invention provides methods for inducing tolerance to an antigen in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises a tolerogenic factor, wherein the tolerogenic factor was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the tolerogenic factor entered the anucleate cell, thereby inducing tolerance to the antigen. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein

presentation of the antigen in said tolerogenic environment induces tolerance to the antigen. In some embodiments, the tolerance is non-specific, including tolerance to a plurality of antigens.

[0080] In certain aspects, the invention provides methods for suppressing an immune response to an antigen in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises a tolerogenic factor, wherein the tolerogenic factor was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the tolerogenic factor entered the anucleate cell, thereby suppressing an immune response to the antigen. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of the antigen in said tolerogenic environment suppresses an immune response to the antigen. In some embodiments, the immune suppression is non-specific, including suppression of an immune response to a plurality of antigens.

[0081] In certain aspects, the invention provides methods for inducing tolerance to an antigen in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen and a tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein presentation of said antigen induces tolerance to the antigen. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the tolerance is antigen-specific. In some embodiments, the tolerance is non-specific, including tolerance to a plurality of antigens.

[0082] In certain aspects, the invention provides methods for suppressing an immune response to an antigen in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen and a tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein presentation of said antigen suppresses an immune response to the antigen. In some embodiments, the tolerogenic factor generates or promotes a tolerogenic environment, wherein

presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen. In some embodiments, the antigen is processed in a tolerogenic environment. In some embodiments, the immune suppression is antigen-specific. In some embodiments, the immune suppression is non-specific, including suppression of an immune response to a plurality of antigens.

[0083] In certain aspects, the invention provides methods for introducing an antigen into a tolerogenic environment in an individual, comprising delivering an anucleate cell into the individual, wherein the anucleate cell comprises the antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, and wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell. In some embodiments, the antigen is presented in the tolerogenic environment. In some embodiments, the antigen is processed in the tolerogenic environment.

[0084] In certain aspects, the invention provides methods for generating a tolerogenic environment in an individual, comprising delivering an anucleate cell into the individual, wherein the anucleate cell comprises a tolerogenic factor, wherein the tolerogenic factor was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the tolerogenic factor entered the anucleate cell. In some embodiments, presentation of an antigen in the tolerogenic environment induces tolerance and/or suppresses an immune response to the antigen. In some embodiments, the tolerance and/or immune suppression are non-specific, including tolerance and/or suppression of an immune response to a plurality of antigens.

[0085] In some embodiments, the immune response is suppressed by at least any of about 10%, about 15%, about 20%, about 25%, about 30%, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 90%, or about 100%. In some embodiments, the suppressed immune response and/or induced tolerance comprise a decreased T cell response. For example, a decreased T cell response may include, without limitation, decreased T cell activation or proliferation, decreased T cell survival, or decreased cell functionality. In some embodiments, the decreased T cell response comprises decreased T cell activation. In some embodiments, the decreased T cell response comprises decreased T cell survival. In some embodiments, the decreased T cell response comprises decreased T cell proliferation. In some embodiments, the decreased T cell response comprises decreased T cell functionality. For example, decreased T cell functionality can include, without limitation, modulated cytokine secretion, decreased T cell

migration to sites of inflammation, and decreased T cell cytotoxic activity. In some embodiments, the suppressed immune response and/or induced tolerance comprise decreased inflammatory cytokine production and/or secretion, and/or increased anti-inflammatory cytokine production and/or secretion. In some embodiments, the suppressed immune response and/or induced tolerance comprise decreased production and/or secretion of one or more inflammatory cytokines selected from interleukin-1 (IL-1), IL-12, and IL-18, tumor necrosis factor (TNF), interferon gamma (IFN-gamma), and granulocyte-macrophage colony stimulating factor (GM-CSF). In some embodiments, the suppressed immune response and/or induced tolerance comprise decreased production and/or secretion of one or more anti-inflammatory cytokines selected from IL-4, IL-10, IL-13, IL-35, IFN-alpha and transforming growth factor-beta (TGFβ). In some embodiments, the suppressed immune response and/or induced tolerance comprises a change in T cell phenotype. For example, the T cell state may change from a pro-inflammatory phenotype to a regulatory or anti-inflammatory phenotype. In some embodiments, the suppressed immune response and/or induced tolerance comprises uncostimulated activation of a T cell, which may subsequently lead to cell death. In some embodiments, the suppressed immune response and/or induced tolerance comprise an enhanced Treg response. In some embodiments, the suppressed immune response and/or induced tolerance comprise a decreased B cell response. In some embodiments, the decreased B cell response comprises decreased antibody production.

[0086] In some embodiments, the suppressed immune response and/or induced tolerance comprise a decreased autoimmune response. For example, the decreased autoimmune response can include, without limitation, a decreased immune response or induced tolerance against an antigen associated with Type I Diabetes, Rheumatoid arthritis, Psoriasis, Multiple Sclerosis, Neurodegenerative diseases which may have an immune component such as Alzheimer's disease, ALS, Huntington's Disease, and Parkinson's Disease, Systemic Lupus Erthyromatosus, Sjogren's Disease, Crohn's disease, or Ulcerative Colitis. In some embodiments, the suppressed immune response and/or induced tolerance comprise a decreased allergic response. For example, the decreased allergic response can include a decreased immune response or induced tolerance against antigens associated with allergic asthma, atopic dermatitis, allergic rhinitis (hay fever), or food allergy. In some embodiments, the antigen is an antigen associated with transplanted tissue. In some embodiments, the suppressed immune response and/or induced tolerance comprises a decreased immune response or induced tolerance against the transplanted tissue. In

some embodiments, the antigen is associated with a virus. In some embodiments, the suppressed immune response and/or induced tolerance comprises a decreased pathogenic immune response or induced tolerance to the virus. For example, the pathogenic immune response can include the cytokine storm generated by certain viral infections. A cytokine storm is a potentially fatal immune reaction consisting of a positive feedback loop between cytokines and white blood cells.

[0087] In some embodiments, the suppressed immune response comprises a decreased immune response against a therapeutic agent. In some embodiments, the therapeutic agent is a clotting factor. Exemplary clotting factors include, without limitation, Factor VIII and Factor IX. In some embodiments, the therapeutic agent is an antibody. Exemplary therapeutic antibodies include, without limitation, anti-TNFα, anti-VEGF, anti-CD3, anti-CD20, anti-IL-2R, anti-Her2, anti-RSVF, anti-CEA, anti-IL-1beta, anti-CD15, anti-myosin, anti-PSMA, anti-40 kDa glycoprotein, anti-CD33, anti-CD52, anti-IgE, anti-CD11a, anti-EGFR, anti-C5, anti-alpha-4 integrin, anti-IL-12/IL-23, anti-IL-6R, and anti-RANKL. In some embodiments, the therapeutic agent is a growth factor. Exemplary therapeutic growth factors include, without limitation, Erythropoietin (EPO) and megakaryocyte differentiation and growth factor (MDGF). In some embodiments, the therapeutic agent is a hormone. Exemplary therapeutic hormones include, without limitation, insulin, human growth hormone, and follicle stimulating hormone. In some embodiments, the therapeutic agent is a recombinant cytokine. Exemplary therapeutic recombinant cytokines include, without limitation, IFNβ, IFNα, and GM-CSF. In some embodiments, the suppressed immune response comprises a decreased immune response against a therapeutic vehicle. In some embodiments, the therapeutic vehicle is a virus, such as an adenovirus, adeno-associated virus, baculovirus, herpes virus, or retrovirus used for gene therapy. In some embodiments, the therapeutic vehicle is a liposome. In some embodiments, the therapeutic vehicle is a nanoparticle.

IV. MICROFLUIDIC CHANNELS TO PROVIDE CELL-DEFORMING CONSTRICTIONS

[0088] In some embodiments, the invention provides methods for suppressing an immune response or inducing tolerance by passing a cell suspension through a constriction, wherein the constriction deforms the anucleate cell thereby causing a perturbation of the cell such that an antigen or compound enters the cell, wherein the constriction is contained within a microfluidic channel. In some embodiments, multiple constrictions can be placed in parallel and/or in series within the microfluidic channel. Exemplary microfluidic channels containing cell-deforming

constrictions for use in the methods disclosed herein are described in WO2013059343. Exemplary surfaces having pores for use in the methods disclosed herein are described in U.S. Provisional Application 62/214,820, filed 09/04/2015.

[0089] In some embodiments, the microfluidic channel includes a lumen and is configured such that a cell suspended in a buffer can pass through, wherein the microfluidic channel includes a constriction. The microfluidic channel can be made of any one of a number of materials, including silicon, metal (e.g., stainless steel), plastic (e.g., polystyrene, PET, PETG), ceramics, glass, crystalline substrates, amorphous substrates, or polymers (e.g., Poly-methyl methacrylate (PMMA), PDMS, Cyclic Olefin Copolymer (COC), etc.). Fabrication of the microfluidic channel can be performed by any method known in the art, including dry etching, wet etching, photolithography, injection molding, laser ablation, or SU-8 masks.

[0090] In some embodiments, the constriction within the microfluidic channel includes an entrance portion, a centerpoint, and an exit portion. In some embodiments, the length, depth, and width of the constriction within the microfluidic channel can vary. In some embodiments, the diameter of the constriction within the microfluidic channel is a function of the diameter of the cell or cluster of cells. In some embodiments, the diameter of the constriction within the microfluidic channel is about 10%, to about 99% of the diameter of the cell. In some embodiments, the constriction size is about 10%, about 15%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or about 99% of the cell diameter. In some embodiments, the constriction size is about 10%, about 15%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or about 99% of the minimum cross-sectional distance of the cell (e.g., an anucleate cell such as an RBC). In some embodiments, the channel comprises a constriction width of about 0.25 μm to about 4 μm. In some embodiments, the channel comprises a constriction width of width of about 4 μm, about 3.5 μm, about 3 μm, about 2.5 μm, about 2 μm, about 1 μm, about 0.5 μm, or about 0.25 μm (including any ranges between these values). The cross-section of the channel, the entrance portion, the centerpoint, and the exit portion can also vary. For example, the cross-sections can be circular, elliptical, an elongated slit, square, hexagonal, or triangular in shape. The entrance portion defines a constriction angle, wherein the constriction angle is optimized to reduce clogging of the channel and optimized for enhanced delivery of a compound into the cell. The angle of the exit portion can vary as well. For example, the angle of the exit portion is configured to reduce the likelihood of turbulence that can result in non-laminar flow. In some

embodiments, the walls of the entrance portion and/or the exit portion are linear. In other embodiments, the walls of the entrance portion and/or the exit portion are curved.

V. SURFACE HAVING PORES TO PROVIDE CELL DEFORMING CONSTRICTIONS

[0091] In some embodiments, the invention provides methods for suppressing an immune response or inducing tolerance by passing a cell suspension through a constriction, wherein the constriction deforms the anucleate cell thereby causing a perturbation of the cell such that an antigen compound enters the cell, wherein the constriction is a pore or contained within a pore. In some embodiments, the pore is contained in a surface. Exemplary surfaces having pores for use in the methods disclosed herein are described in U.S. Provisional Application 62/214,820, filed 09/04/2015.

[0092] The surfaces as disclosed herein can be made of any one of a number of materials and take any one of a number of forms. In some embodiments, the surface is a filter. In some embodiments, the surface is a membrane. In some embodiments, the filter is a tangential flow filter. In some embodiments, the surface is a sponge or sponge-like matrix. In some embodiments, the surface is a matrix.

[0093] In some embodiments the surface is a tortuous path surface. In some embodiments, the tortuous path surface comprises cellulose acetate. In some embodiments, the surface comprises a material selected from, without limitation, synthetic or natural polymers, polycarbonate, silicon, glass, metal, alloy, cellulose nitrate, silver, cellulose acetate, nylon, polyester, polyethersulfone, polyacrylonitrile (PAN), polypropylene, PVDF, polytetrafluorethylene, mixed cellulose ester, porcelain, and ceramic.

[0094] The surface disclosed herein can have any shape known in the art; e.g. a 3-dimensional shape. The 2-dimensional shape of the surface can be, without limitation, circular, elliptical, round, square, star-shaped, triangular, polygonal, pentagonal, hexagonal, heptagonal, or octagonal. In some embodiments, the surface is round in shape. In some embodiments, the surface 3-dimensional shape is cylindrical, conical, or cuboidal.

[0095] The surface can have various cross-sectional widths and thicknesses. In some embodiments, the surface cross-sectional width is between about 1mm and about 1m or any cross-sectional width or range of cross-sectional widths therebetween. In some embodiments, the surface has a defined thickness. In some embodiments, the surface thickness is uniform. In some embodiments, the surface thickness is variable. For example, in some embodiments, portions of

the surface are thicker or thinner than other portions of the surface. In some embodiments, the surface thickness varies by about 1% to about 90% or any percentage or range of percentages therebetween. In some embodiments, the surface is between about 0.01μm to about 5mm thick or any thickness or range of thicknesses therebetween.

[0096] In some embodiments, the constriction is a pore or contained within a pore. The cross-sectional width of the pores is related to the type of cell to be treated. In some embodiments, the pore size is a function of the diameter of the cell of cluster of cells to be treated. In some embodiments, the pore size is such that a cell is perturbed upon passing through the pore. In some embodiments, the pore size is less than the diameter of the cell. In some embodiments, the pore size is about 10% to about 99% of the diameter of the cell. In some embodiments, the pore size is about 10%, about 15%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or about 99% of the cell diameter. Optimal pore size or pore cross-sectional width can vary based upon the application and/or cell type. In some embodiments, the pore size is about 0.1 μm to about 4 μm. In some embodiments, the pore size is about 4 μm, about 3 μm, about 2 μm, about 1 μm, about 0.5 μm, about 0.25 μm, or about 0.1 μm. In some embodiments, the pore cross-sectional width is at or less than any of about 4 μm, about 3 μm, about 2 μm, about 1 μm, about 0.5 μm, about 0.25 μm, or about 0.1 μm. The entrances and exits of the pore passage may have a variety of angles. The pore angle can be selected to minimize clogging of the pore while cells are passing through. In some embodiments, the flow rate through the surface is between about 0.001 $mL/cm^2/sec$ to about 100 $L/cm^2/sec$ or any rate or range of rates therebetween. For example, the angle of the entrance or exit portion can be between about 0 and about 90 degrees. In some embodiments, the entrance or exit portion can be greater than 90 degrees. In some embodiments, the pores have identical entrance and exit angles. In some embodiments, the pores have different entrance and exit angles. In some embodiments, the pore edge is smooth, e.g. rounded or curved. A smooth pore edge has a continuous, flat, and even surface without bumps, ridges, or uneven parts. In some embodiments, the pore edge is sharp. A sharp pore edge has a thin edge that is pointed or at an acute angle. In some embodiments, the pore passage is straight. A straight pore passage does not contain curves, bends, angles, or other irregularities. In some embodiments, the pore passage is curved. A curved pore passage is bent or deviates from a straight line. In some embodiments, the pore passage has multiple curves, e.g. about 2, 3, 4, 5, 6, 7, 8, 9, 10 or more curves.

[0097] The pores can have any shape known in the art, including a 2-dimensional or 3-dimensional shape. The pore shape (e.g., the cross-sectional shape) can be, without limitation, circular, elliptical, round, square, star-shaped, triangular, polygonal, pentagonal, hexagonal, heptagonal, and octagonal. In some embodiments, the cross-section of the pore is round in shape. In some embodiments, the 3-dimensional shape of the pore is cylindrical or conical. In some embodiments, the pore has a fluted entrance and exit shape. In some embodiments, the pore shape is homogenous (i.e. consistent or regular) among pores within a given surface. In some embodiments, the pore shape is heterogeneous (i.e. mixed or varied) among pores within a given surface.

[0098] The surfaces described herein can have a range of total pore numbers. In some embodiments, the pores encompass about 10% to about 80% of the total surface area. In some embodiments, the surface contains about $1.0x10^5$ to about $1.0x10^{30}$ total pores or any number or range of numbers therebetween. In some embodiments, the surface comprises between about 10 and about $1.0x10^{15}$ pores per mm^2 surface area.

[0099] The pores can be distributed in numerous ways within a given surface. In some embodiments, the pores are distributed in parallel within a given surface. In one such example, the pores are distributed side-by-side in the same direction and are the same distance apart within a given surface. In some embodiments, the pore distribution is ordered or homogeneous. In one such example, the pores are distributed in a regular, systematic pattern or are the same distance apart within a given surface. In some embodiments, the pore distribution is random or heterogeneous. In one such example, the pores are distributed in an irregular, disordered pattern or are different distances apart within a given surface. In some embodiments, multiple surfaces are distributed in series. The multiple surfaces can be homogeneous or heterogeneous in surface size, shape, and/or roughness. The multiple surfaces can further contain pores with homogeneous or heterogeneous pore size, shape, and/or number, thereby enabling the simultaneous delivery of a range of compounds into different cell types.

[0100] In some embodiments, an individual pore has a uniform width dimension (i.e. constant width along the length of the pore passage). In some embodiments, an individual pore has a variable width (i.e. increasing or decreasing width along the length of the pore passage). In some embodiments, pores within a given surface have the same individual pore depths. In some embodiments, pores within a given surface have different individual pore depths. In some embodiments, the pores are immediately adjacent to each other. In some embodiments, the pores

are separated from each other by a distance. In some embodiments, the pores are separated from each other by a distance of about 0.001μm to about 30mm or any distance or range of distances therebetween.

[0101] In some embodiments, the surface is coated with a material. The material can be selected from any material known in the art, including, without limitation, Teflon, an adhesive coating, surfactants, proteins, adhesion molecules, antibodies, anticoagulants, factors that modulate cellular function, nucleic acids, lipids, carbohydrates, or transmembrane proteins. In some embodiments, the surface is coated with polyvinylpyrrolidone. In some embodiments, the material is covalently attached to the surface. In some embodiments, the material is non-covalently attached to the surface. In some embodiments, the surface molecules are released at the cells pass through the pores.

[0102] In some embodiments, the surface has modified chemical properties. In some embodiments, the surface is hydrophilic. In some embodiments, the surface is hydrophobic. In some embodiments, the surface is charged. In some embodiments, the surface is positively and/or negatively charged. In some embodiments, the surface can be positively charged in some regions and negatively charged in other regions. In some embodiments, the surface has an overall positive or overall negative charge. In some embodiments, the surface can be any one of smooth, electropolished, rough, or plasma treated. In some embodiments, the surface comprises a zwitterion or dipolar compound. In some embodiments, the surface is plasma treated.

[0103] In some embodiments, the surface is contained within a larger module. In some embodiments, the surface is contained within a syringe, such as a plastic or glass syringe. In some embodiments, the surface is contained within a plastic filter holder. In some embodiments, the surface is contained within a pipette tip.

VI. CELL PERTURBATIONS

[0104] In some embodiments, the invention provides methods for suppressing an immune response or inducing tolerance by passing a cell suspension through a constriction, wherein the constriction deforms the anucleate cell thereby causing a perturbation of the cell such that an antigen or compound enters the cell, wherein the perturbation in the cell is a breach in the cell that allows material from outside the cell to move into the cell (e.g., a hole, tear, cavity, aperture, pore, break, gap, perforation). The deformation can be caused by, for example, pressure induced by mechanical strain and/or shear forces. In some embodiments, the perturbation is a perturbation within the cell membrane. In some embodiments, the perturbation is transient. In

some embodiments, the cell perturbation lasts from about $1.0x10^{-9}$ seconds to about 2 hours, or any time or range of times therebetween. In some embodiments, the cell perturbation lasts for about $1.0x10^{-9}$second to about 1 second, about 1 second to about 1 minute, or about 1 minute to about 1 hour. In some embodiments, the cell perturbation lasts for between any one of about $1.0x10^{-9}$ to about $1.0x10^{-1}$, about $1.0x10^{-9}$ to about $1.0x10^{-2}$, about $1.0x10^{-9}$ to about $1.0x10^{-3}$, about $1.0x10^{-9}$ to about $1.0x10^{-4}$, about $1.0x10^{-9}$ to about $1.0x10^{-5}$, about $1.0x10^{-9}$ to about $1.0x10^{-6}$, about $1.0x10^{-9}$ to about $1.0x10^{-7}$, or about $1.0x10^{-9}$ to about $1.0x10^{-8}$ seconds. In some embodiment, the cell perturbation lasts for any one of about $1.0x10^{-8}$ to about $1.0x10^{-1}$, about $1.0x10^{-7}$ to about $1.0x10^{-1}$, about $1.0x10^{-6}$ to about $1.0x10^{-1}$, about $1.0x10^{-5}$ to about $1.0x10^{-1}$, about $1.0x10^{-4}$ to about $1.0x10^{-1}$, about $1.0x10^{-3}$ to about $1.0x10^{-1}$, or about $1.0x10^{-2}$ to about $1.0x10^{-1}$ seconds. The cell perturbations (e.g., pores or holes) created by the methods described herein are not formed as a result of assembly of protein subunits to form a multimeric pore structure such as that created by complement or bacterial hemolysins.

[0105] As the cell passes through the constriction, the constriction temporarily imparts injury to the cell membrane that causes passive diffusion of material through the perturbation. In some embodiments, the cell is only deformed for a brief period of time, on the order of 100 μs to minimize the chance of activating apoptotic pathways through cell signaling mechanisms, although other durations are possible (e.g., ranging from nanoseconds to hours). In some embodiments, the cell is deformed for about 1.0 $x10^{-9}$ seconds to about 2 hours, or any time or range of times therebetween. In some embodiments, the cell is deformed for about $1.0x10^{-9}$ second to about 1 second, about 1 second to about 1 minute, or about 1 minute to about 1 hour. In some embodiments, the cell is deformed for between any one of about $1.0x10^{-9}$ to about $1.0x10^{-1}$, about $1.0x10^{-9}$ to about $1.0x10^{-2}$, about $1.0x10^{-9}$ to about $1.0x10^{-3}$, about $1.0x10^{-9}$ to about $1.0x10^{-4}$, about $1.0x10^{-9}$ to about $1.0x10^{-5}$, about $1.0x10^{-9}$ to about $1.0x10^{-6}$, about $1.0x10^{-9}$ to about $1.0x10^{-7}$, or about $1.0x10^{-9}$ to about $1.0x10^{-8}$ seconds. In some embodiment, the cell is deformed for any one of about $1.0x10^{-8}$ to about $1.0x10^{-1}$, about $1.0x10^{-7}$ to about $1.0x10^{-1}$, about $1.0x10^{-6}$ to about $1.0x10^{-1}$, about $1.0x10^{-5}$ to about $1.0x10^{-1}$, about $1.0x10^{-4}$ to about $1.0x10^{-1}$, about $1.0x10^{-3}$ to about $1.0x10^{-1}$, or about $1.0x10^{-2}$ to about $1.0x10^{-1}$ seconds. In some embodiments, deforming the cell includes deforming the cell for a time ranging from, without limitation, about 1 μs to at least about 750 μs, e.g., at least about 1μs, 10 μs, 50μs, 100 μs, 500 μs, or 750 μs.

[0106] In some embodiments, the passage of the compound into the cell occurs simultaneously with the cell passing through the constriction and/or the perturbation of the cell. In some embodiments, passage of the compound into the cell occurs after the cell passes through the constriction. In some embodiments, passage of the compound into the cell occurs on the order of minutes after the cell passes through the constriction. In some embodiments, the passage of the compound into the cell occurs from about $1.0x10^{-2}$ seconds to at least about 30 minutes after the cell passes through the constriction. For example, the passage of the compound into the cell occurs from about $1.0x10^{-2}$ seconds to about 1 second, about 1 second to about 1 minute, or about 1 minute to about 30 minutes after the cell passes through the constriction. In some embodiments, the passage of the compound into the cell occurs about $1.0x10^{-2}$ seconds to about 10 minutes, about $1.0x10^{-2}$ seconds to about 5 minutes, about $1.0x10^{-2}$ seconds to about 1 minute, about $1.0x10^{-2}$ seconds to about 50 seconds, about $1.0x10^{-2}$ seconds to about 10 seconds, about $1.0x10^{-2}$ seconds to about 1 second, or about $1.0x10^{-2}$ seconds to about 0.1 second after the cell passes through the constriction. In some embodiments, the passage of the compound into the cell occurs about $1.0x10^{-1}$ seconds to about 10 minutes, about 1 second to about 10 minutes, about 10 seconds to about 10 minute, about 50 seconds to about 10 minutes, about 1 minute to about 10 minutes, or about 5 minutes to about 10 minutes after the cell passes through the constriction. In some embodiments, a perturbation in the cell after it passes through the constriction is corrected within the order of about five minutes after the cell passes through the constriction.

[0107] In some embodiments, the cell viability after passing through a constriction is about 5% to about 100%. In some embodiments, the cell viability after passing through the constriction is at least about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or 99%. In some embodiments, the cell viability is measured from about $1.0x10^{-2}$ seconds to at least about 10 days after the cell passes through the constriction. For example, the cell viability is measured from about $1.0x10^{-2}$ seconds to about 1 second, about 1 second to about 1 minute, about 1 minute to about 30 minutes, or about 30 minutes to about 2 hours after the cell passes through the constriction. In some embodiments, the cell viability is measured about $1.0x10^{-2}$ seconds to about 2 hours, about $1.0x10^{-2}$ seconds to about 1 hour, about $1.0x10^{-2}$ seconds to about 30 minutes, about $1.0x10^{-2}$ seconds to about 1 minute, about $1.0x10^{-2}$ seconds to about 30 seconds, about $1.0x10^{-2}$ seconds to about 1 second, or about $1.0x10^{-2}$ seconds to about 0.1 second after the cell passes through the constriction. In some embodiments, the cell viability

is measured about 1.5 hours to about 2 hours, about 1 hour to about 2 hours, about 30 minutes to about 2 hours, about 15 minutes to about 2 hours, about 1 minute to about 2 hours, about 30 seconds to about 2 hours, or about 1 second to about 2 hours after the cell passes through the constriction. In some embodiments, the cell viability is measured about 2 hours to about 5 hours, about 5 hours to about 12 hours, about 12 hours to about 24 hours, or about 24 hours to about 10 days after the cell passes through the constriction.

VII. DELIVERY PARAMETERS

[0108] A number of parameters may influence the delivery of a compound to a cell for suppressing an immune response or inducing tolerance by the methods described herein. In some embodiments, the cell suspension is contacted with the compound before, concurrently, or after passing through the constriction. The cell may pass through the constriction suspended in a solution that includes the compound to deliver, although the compound can be added to the cell suspension after the cells pass through the constriction. In some embodiments, the compound to be delivered is coated on the constriction.

[0109] Examples of parameters that may influence the delivery of the compound into the cell include, but are not limited to, the dimensions of the constriction, the entrance angle of the constriction, the surface properties of the constrictions (e.g., roughness, chemical modification, hydrophilic, hydrophobic, etc.), the operating flow speeds (e.g., cell transit time through the constriction), the cell concentration, the concentration of the compound in the cell suspension, and the amount of time that the cell recovers or incubates after passing through the constrictions can affect the passage of the delivered compound into the cell. Additional parameters influencing the delivery of the compound into the cell can include the velocity of the cell in the constriction, the shear rate in the constriction, the viscosity of the cell suspension, the velocity component that is perpendicular to flow velocity, and time in the constriction. Such parameters can be designed to control delivery of the compound. In some embodiments, the cell concentration ranges from about 10 to at least about 10^{12} cells/ml or any concentration or range of concentrations therebetween. In some embodiments, delivery compound concentrations can range from about 10 ng/ml to about 1g/mL or any concentration or range of concentrations therebetween. In some embodiments, delivery compound concentrations can range from about 1pM to at least about 2M or any concentration or range of concentrations therebetween. The composition of the cell suspension (e.g., osmolarity, salt concentration, serum content, cell concentration, pH, etc.) can impact delivery of the compound for suppressing an immune

response or inducing tolerance. In some embodiments, the aqueous solution is iso-osmolar or iso-tonic.

[0110] The temperature used in the methods of the present disclosure can be adjusted to affect compound delivery and cell viability. In some embodiments, the method is performed between about -5°C and about 45°C. For example, the methods can be carried out at room temperature (e.g., about 20°C), physiological temperature (e.g., about 37°C), higher than physiological temperature (e.g., greater than about 37°C to 45°C or more), or reduced temperature (e.g., about -5°C to about 4°C), or temperatures between these exemplary temperatures.

[0111] Various methods can be utilized to drive the cells through the constrictions. For example, pressure can be applied by a pump on the entrance side (e.g., gas cylinder, or compressor), a vacuum can be applied by a vacuum pump on the exit side, capillary action can be applied through a tube, and/or the system can be gravity fed. Displacement based flow systems can also be used (e.g., syringe pump, peristaltic pump, manual syringe or pipette, pistons, etc.). In some embodiments, the cells are passed through the constrictions by positive pressure or negative pressure. In some embodiments, the cells are passed through the constrictions by constant pressure or variable pressure. In some embodiments, pressure is applied using a syringe. In some embodiments, pressure is applied using a pump. In some embodiments, the pump is a peristaltic pump or a diaphragm pump. In some embodiments, pressure is applied using a vacuum. In some embodiments, the cells are passed through the constrictions by g-force. In some embodiments, the cells are passed through the constrictions by centrifugal force. In some embodiments, the cells are passed through the constrictions by capillary pressure.

[0112] In some embodiments, fluid flow directs the cells through the constrictions. In some embodiments, the fluid flow is turbulent flow prior to the cells passing through the constriction. Turbulent flow is a fluid flow in which the velocity at a given point varies erratically in magnitude and direction. In some embodiments, the fluid flow through the constriction is laminar flow. Laminar flow involves uninterrupted flow in a fluid near a solid boundary in which the direction of flow at every point remains constant. In some embodiments, the fluid flow is turbulent flow after the cells pass through the constriction. The velocity at which the cells pass through the constrictions can be varied. In some embodiments, the cells pass through the

constrictions at a uniform cell speed. In some embodiments, the cells pass through the constrictions at a fluctuating cell speed.

[0113] In other embodiments, a combination treatment is used to suppress an immune response or induce tolerance by passing a cell suspension through a constriction, wherein the constriction deforms the anucleate cell thereby causing a perturbation of the cell such that an antigen or compound enters the cell, e.g., the methods described herein followed by exposure to an electric field downstream of the constriction. In some embodiments, the cell is passed through an electric field generated by at least one electrode after passing through the constriction. In some embodiments, the electric field assists in delivery of compounds to a second location inside the cell. For example, the combination of a cell-deforming constriction and an electric field delivers a plasmid encoding an antibody into the cell, resulting in the de novo production of antibody. In some embodiments, one or more electrodes are in proximity to the cell-deforming constriction to generate an electric field. In some embodiments, the electric field is between about 0.1kV/m to about 100MV/m, or any number or range of numbers therebetween. In some embodiments, an integrated circuit is used to provide an electrical signal to drive the electrodes. In some embodiments, the cells are exposed to the electric field for a pulse width of between about 1ns to about 1s and a period of between about 100ns to about 10s or any time or range of times therebetween.

VIII. CELL SUSPENSIONS FOR DELIVERY TO ANUCLEATE CELLS

[0114] The cell suspension may be a mixed or purified population of cells. In some embodiments, the cell suspension is a mixed cell population, such as whole blood. In some embodiments, the cell suspension is a purified cell population, such as a purified population of anucleate cells.

[0115] The composition of the cell suspension (e.g., osmolarity, salt concentration, serum content, cell concentration, pH, etc.) can impact delivery of the compound for suppressing an immune response or inducing tolerance. In some embodiments, the suspension comprises whole blood. Alternatively, the cell suspension is a mixture of cells in a physiological saline solution or physiological medium other than blood. In some embodiments, the cell suspension comprises an aqueous solution. In some embodiments, the aqueous solution comprises cell culture medium, PBS, salts, sugars, growth factors, animal derived products, bulking materials, surfactants, lubricants, vitamins, amino acids, proteins, cell cycle inhibitors, and/or an agent that impacts actin polymerization. In some embodiments, the cell culture medium is DMEM, Opti-MEM™,

IMDM, or RPMI. Additionally, solution buffer can include one or more lubricants (pluronics or other surfactants) that can be designed, for example, to reduce or eliminate clogging of the surface and improve cell viability. Exemplary surfactants include, without limitation, poloxamer, polysorbates, sugars or sugar alcohols such as mannitol, sorbitol, animal derived serum, and albumin protein. In some embodiments, the aqueous solution is iso-osmolar or iso-tonic. In some embodiments, the aqueous solution includes plasma.

[0116] In some configurations with certain types of cells, the cells can be incubated in one or more solutions that aid in the delivery of the compound to the interior of the cell. In some embodiments, the aqueous solution comprises an agent that impacts actin polymerization. In some embodiments, the agent that impacts actin polymerization is Latrunculin A, Cytochalasin, and/or Colchicine. For example, the cells can be incubated in a depolymerization solution such as Lantrunculin A (0.1μg/ml) for 1 hour prior to delivery to depolymerize the actin cytoskeleton. As an additional example, the cells can be incubated in 10μM Colchicine (Sigma) for 2 hours prior to delivery to depolymerize the microtubule network.

[0117] In some embodiments, the cell population is enriched prior to use in the disclosed methods. For example, cells are obtained from a bodily fluid, e.g., peripheral blood, and optionally enriched or purified to concentrate B cells. Cells may be enriched by any methods known in the art, including without limitation, magnetic cell separation, fluorescent activated cell sorting (FACS), or density gradient centrifugation.

[0118] The viscosity of the cell suspension can also impact the methods disclosed herein. In some embodiments, the viscosity of the cell suspension ranges from about $8.9x10^{-4}$Pa·s to about $4.0x10^{-3}$Pa·s or any value or range of values therebetween. In some embodiments, the viscosity ranges between any one of about $8.9x10^{-4}$ Pa·s to about $4.0\ x10^{-3}$ Pa·s, about $8.9x10^{-4}$ Pa·s to about $3.0\ x10^{-3}$ Pa·s, about $8.9x10^{-4}$ Pa·s to about $2.0\ x10^{-3}$ Pa·s, or about $8.9x10^{-3}$ Pa·s to about $1.0\ x10^{-3}$ Pa·s. In some embodiments, the viscosity ranges between any one of about 0.89 cP to about 4.0 cP, about 0.89 cP to about 3.0 cP, about 0.89 cP to about 2.0 cP, or about 0.89 cP to about 1.0 cP. In some embodiments, a shear thinning effect is observed, in which the viscosity of the cell suspension decreases under conditions of shear strain. Viscosity can be measured by any method known in the art, including without limitation, viscometers, such as a glass capillary viscometer, or rheometers. A viscometer measures viscosity under one flow condition, while a rheometer is used to measure viscosities which vary with flow conditions. In some embodiments, the viscosity is measured for a shear thinning solution such as blood. In some

embodiments, the viscosity is measured between about -5°C and about 45°C. For example, the viscosity is measured at room temperature (e.g., about 20°C), physiological temperature (e.g., about 37°C), higher than physiological temperature (e.g., greater than about 37°C to 45°C or more), reduced temperature (e.g., about -5°C to about 4°C), or temperatures between these exemplary temperatures.

IX. ANTIGENS AND TOLEROGENIC FACTORS TO SUPPRESS AN IMMUNE RESPONSE OR INDUCE TOLERANCE

[0119] In some embodiments the invention provides delivery of antigens to suppress an immune response or induce tolerance, wherein the antigen is delivered to the cell by any of the methods described herein. In some embodiments, the antigen is a single antigen. In some embodiments, the antigen is a mixture of antigens. An antigen is a substance that stimulates a specific immune response, such as a cell or antibody-mediated immune response. Antigens bind to receptors expressed by immune cells, such as T cell receptors (TCRs), which are specific to a particular antigen. Antigen-receptor binding subsequently triggers intracellular signaling pathways that lead to downstream immune effector pathways, such as cell activation, cytokine production, cell migration, cytotoxic factor secretion, cell apoptosis, and antibody production.

[0120] In some embodiments, the antigen is a protein or polypeptide antigen. In some embodiments, the compound comprises a disease-associated antigen. In some embodiments, antigens are derived from foreign sources, such as bacteria, fungi, viruses, or allergens. In some embodiments, antigens are derived from internal sources, such as self-proteins (i.e. self-antigens). In some embodiments, the antigen is in a cell lysate. Self-antigens are antigens present on or in an organism's own cells. Self-antigens do not normally stimulate an immune response, but may in the context of autoimmune diseases, such as Type I Diabetes or Rheumatoid Arthritis. In some embodiments, the antigen is a therapeutic agent. In some embodiments, the antigen is a therapeutic polypeptide, or a fragment of a therapeutic polypeptide. In some embodiments, the therapeutic agent is conjugated to a carbohydrate. In some embodiments, the therapeutic agent is a clotting factor. Exemplary clotting factors include, without limitation, Factor VIII and Factor IX. For example, the antigen may be a Factor VIII protein used in the treatment of hemophilia. In some embodiments, the therapeutic agent is an antibody. Exemplary therapeutic antibodies include, without limitation, anti-TNFα, anti-VEGF, anti-CD3, anti-CD20, anti-IL-2R, anti-Her2, anti-RSVF, anti-CEA, anti-IL-1beta, anti-CD15, anti-myosin, anti-PSMA, anti-40 kDa glycoprotein, anti-CD33, anti-CD52, anti-IgE, anti-CD11a, anti-EGFR, anti-C5,

anti-alpha-4 integrin, anti-IL-12/IL-23, anti-IL-6R, and anti-RANKL. In some embodiments, the therapeutic agent is a growth factor. Exemplary therapeutic growth factors include, without limitation, Erythropoietin (EPO) and megakaryocyte differentiation and growth factor (MDGF). In some embodiments, the therapeutic agent is a hormone. Exemplary therapeutic hormones include, without limitation, insulin, human growth hormone, and follicle stimulating hormone. In some embodiments, the therapeutic agent is a recombinant cytokine. Exemplary therapeutic recombinant cytokines include, without limitation, IFNβ, IFNα, and GM-CSF.

[0121] In some embodiments, the antigen is an allograft transplantation antigen. An allograft transplant is a transfer of cells, tissues, or organs, to a recipient from a genetically non-identical donor of the same species. In some embodiments, the antigen is a modified antigen. For example, antigens may be fused with therapeutic agents or targeting peptides. In some embodiments, the modified antigen is fused with a polypeptide. In some embodiments, the modified antigen is fused with a lipid. In some embodiments, the antigen is fused with a carbohydrate. In some embodiments, the antigen is a non-protein antigen, such as a lipid, glycolipid, or polysaccharide. In some embodiments, the antigen is a whole microorganism, such as an intact bacterium.

[0122] In some embodiments, the antigen is associated with a virus. In some embodiments, the antigen is a viral antigen. Exemplary viral antigens include SARS-CoV antigens and influenza antigens. In some embodiments, the compound comprises cell lysate from tissue infected with an unknown pathogen. In some embodiments, the antigen is a therapeutic vehicle. In some embodiments, the therapeutic vehicle is a virus, such as an adenovirus, adeno-associated virus, baculovirus, herpes virus, or retrovirus used for gene therapy. In some embodiments, the therapeutic vehicle is a liposome. In some embodiments, the therapeutic vehicle is a nanoparticle.

[0123] In some embodiments, the antigen is associated with a microorganism; for example a bacterium. In some embodiments, the suppressed immune response and/or induced tolerance comprises a decreased pathogenic immune response or induced tolerance to the microorganism; for example, a bacterium. In some embodiments, the microorganism is part of the microbiome of the individual. In some embodiments, the microorganism may benefit the microbiome of the individual.

[0124] In certain aspects, the invention provides methods for delivering an antigen into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell

through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the antigen enters the cell, wherein said cell suspension is contacted with the antigen. In some embodiments, presentation of the antigen in a tolerogenic environment suppresses an immune response to the antigen. In some embodiments, presentation of said antigen in a tolerogenic environment induces tolerance to the antigen. In some embodiments, the antigen is delivered to the anucleate cell in vitro, ex vivo, or in vivo.

[0125] In some embodiments the invention provides delivery of tolerogenic factors to suppress an immune response or induce tolerance, wherein the tolerogenic factor is delivered to the cell by any of the methods described herein. In some embodiments, the tolerogenic factor enhances suppression of an immune response to an antigen and/or enhances the induction of tolerance to an antigen. For example, the tolerogenic factor may promote tolerogenic presentation of the antigen by an antigen-presenting cell. In some embodiments, the tolerogenic factor is introduced simultaneously with the antigen. In some embodiments, the tolerogenic factor and antigen are introduced sequentially. In some embodiments, the tolerogenic factor comprises a polypeptide. In some embodiments, the polypeptide is IL-4, IL-10, IL-13, IL-35, IFNα, or TGFβ. In some embodiments, the polypeptide is a therapeutic polypeptide. In some embodiments, the polypeptide is a fragment of a therapeutic polypeptide. In some embodiments, the polypeptide is conjugated to a carbohydrate. In some embodiments, the tolerogenic factor is a nucleic acid. In some embodiments, the nucleic acid can include, without limitation, mRNA, DNA, miRNA, or siRNA. For example, the tolerogenic factor can include siRNA to knock down expression of inflammatory genes. In some embodiments, the tolerogenic factor is a DNA sequence that binds NFκB and prevents NFκB activation and downstream signaling. In some embodiments, the tolerogenic factor is a small molecule.

[0126] In some embodiments, the tolerogenic factor modulates expression and/or activity of an immunomodulatory agent (such as an immunostimulatory agent (*e.g.*, a costimulatory molecule), an immunosuppressive agent, or an inflammatory or anti-inflammatory molecule). In some embodiments, the tolerogenic factor inhibits expression and/or activity of an immunostimulatory agent (*e.g.*, a costimulatory molecule), enhances expression and/or activity of an immunosuppressive molecule, inhibits expression and/or activity of an inflammatory molecule, and/or enhances expression and/or activity of an anti-inflammatory molecule. In some embodiments, the tolerogenic factor inhibits the activity of a costimulatory molecule. Interaction between costimulatory molecules and their ligands is important to sustain and integrate TCR

signaling to stimulate optimal T cell proliferation and differentiation. In some embodiments, the tolerogenic factor decreases expression of a costimulatory molecule. Exemplary costimulatory molecules expressed on antigen-presenting cells include, without limitation, CD40, CD80, CD86, CD54, CD83, CD79, or ICOS Ligand. In some embodiments, the costimulatory molecule is CD80 or CD86. In some embodiments, the tolerogenic factor inhibits the expression of a nucleic acid that expresses or modulates expression of the costimulatory molecule. In some embodiments, the tolerogenic factor deletes a nucleic acid that expresses or modulates expression of the costimulatory molecule. In some embodiments, deletion of the nucleic acid that expresses or modulates expression of the costimulatory molecule is achieved via gene editing. In some embodiments, the tolerogenic factor inhibits the costimulatory molecule. In some embodiments, the tolerogenic factor is a siRNA that inhibits the costimulatory molecule. In some embodiments, the tolerogenic factor increases the activity of a transcriptional regulator that suppresses expression of the costimulatory molecule. In some embodiments, the tolerogenic factor increases the activity of a protein inhibitor that suppresses expression of the costimulatory molecule. In some embodiments, the tolerogenic factor comprises nucleic acid encoding a suppressor of the costimulatory molecule. In some embodiments, the tolerogenic factor degrades the costimulatory molecule. In some embodiments, the tolerogenic factor labels the costimulatory molecule for destruction. For example, the tolerogenic factor may enhance ubiquitination of the costimulatory molecule, thereby targeting it for destruction.

[0127] In some embodiments, the tolerogenic factor enhances the expression and/or activity of an immunosuppressive molecule. In some embodiments, the immunosuppressive molecule is a co-inhibitory molecule, a transcriptional regulator, or an immunosuppressive molecule. Co-inhibitory molecules negatively regulate the activation of lymphocytes. Exemplary co-inhibitory molecules include, without limitation, PD-L1, PD-L2, HVEM, B7-H3, TRAIL, immunoglobulin-like transcripts (ILT) receptors (ILT2, ILT3, ILT4), FasL, CTLA4, CD39, CD73, and B7-H4. In some embodiments, the co-inhibitory molecule is PD-L1 or PD-L2. In some embodiments, the tolerogenic factor increases the activity of the co-inhibitory molecule. In some embodiments, the tolerogenic factor increases expression of a co-inhibitory molecule. In some embodiments, the tolerogenic factor encodes the co-inhibitory molecule. In some embodiments, the tolerogenic factor increases the activity of the co-inhibitory molecule. In some embodiments, the tolerogenic factor increases the activity of a transcriptional regulator that enhances expression of the co-inhibitory molecule. In some embodiments, the tolerogenic factor

increases the activity of a polypeptide that increases expression of the co-inhibitory molecule. In some embodiments, the tolerogenic factor comprises nucleic acid encoding an enhancer of the co-inhibitory molecule. In some embodiments, the tolerogenic factor inhibits an inhibitor of a co-inhibitory molecule.

[0128] In some embodiments, the tolerogenic factor increases expression and/or activity of an immunosuppressive molecule. Exemplary immunosuppressive molecules include, without limitation, arginase-1 (ARG1), indoleamine 2,3-dioxygenase (IDO), Prostaglandin E2 (PGE2), inducible nitric-oxide synthase (iNOS), nitric oxide (NO), nitric-oxide synthase 2 (NOS2), thymic stromal lymphopoietin (TSLP), vascular intestinal peptide (VIP), hepatocyte growth factor (HGF), transforming growth factor beta (TGFβ), IFNα, IL-4, IL-10, IL-13, and IL-35. In some embodiments, the immunosuppressive molecule is NO or IDO. In some embodiments, the tolerogenic factor encodes the immunosuppressive molecule. In some embodiments, the tolerogenic factor increases the activity of the immunosuppressive molecule. In some embodiments, the tolerogenic factor increases the activity of a transcriptional regulator that enhances expression of the immunosuppressive molecule. In some embodiments, the tolerogenic factor increases the activity of a polypeptide that enhances expression of the immunosuppressive molecule. In some embodiments, the tolerogenic factor comprises nucleic acid encoding an enhancer of the immunosuppressive molecule. In some embodiments, the tolerogenic factor inhibits a negative regulator of an immunosuppressive molecule.

[0129] In some embodiments, the tolerogenic factor inhibits expression and/or activity of an inflammatory molecule. In some embodiments, the inflammatory molecule is an inflammatory transcription factor. In some embodiments, the tolerogenic factor inhibits the inflammatory transcription factor. In some embodiments, the tolerogenic factor decreases expression of an inflammatory transcription factor. In some embodiments, the inflammatory transcription factor is NF-κB, an interferon regulatory factor (IRF), or a molecule associated with the JAK-STAT signaling pathway. The NF-κB pathway is a prototypical proinflammatory signaling pathway that mediates the expression of proinflammatory genes including cytokines, chemokines, and adhesion molecules. Interferon regulatory factors (IRFs) constitute a family of transcription factors that can regulate the expression of proinflammatory genes. The JAK-STAT signaling pathway transmits information from extracellular cytokine signals to the nucleus, resulting in DNA transcription and expression of genes involved in immune cell proliferation and differentiation. The JAK-STAT system consists of a cell surface receptor, Janus kinases (JAKs),

and Signal Transducer and Activator of Transcription (STAT) proteins. Exemplary JAK-STAT molecules include, without limitation, JAK1, JAK2, JAK 3, Tyk2, STAT1, STAT2, STAT3, STAT4, STAT5 (STAT5A and STAT5B), and STAT6. In some embodiments, the tolerogenic factor enhances expression of a suppressor of cytokine signaling (SOCS) protein. SOCS proteins may inhibit signaling through the JAK-STAT pathway. In some embodiments, the tolerogenic factor inhibits the expression of a nucleic acid encoding the inflammatory transcription factor. In some embodiments, the tolerogenic factor deletes a nucleic acid encoding the inflammatory transcription factor. In some embodiments, the tolerogenic factor increases the activity of a transcriptional regulator that suppresses expression of the inflammatory transcription factor. In some embodiments, the tolerogenic factor increases the activity of a protein inhibitor that suppresses expression of the inflammatory transcription factor. In some embodiments, the tolerogenic factor comprises nucleic acid encoding a suppressor of the inflammatory transcription factor.

[0130] In some embodiments, the tolerogenic factor enhances expression and/or activity of an anti-inflammatory molecule. In some embodiments, the anti-inflammatory molecule is an anti-inflammatory transcription factor. In some embodiments, the tolerogenic factor enhances the anti-inflammatory transcription factor. In some embodiments, the tolerogenic factor increases expression of an anti-inflammatory transcription factor. In some embodiments, the tolerogenic factor enhances expression of nucleic acid encoding the anti-inflammatory transcription factor. In some embodiments, the tolerogenic factor decreases the activity of a transcriptional regulator that suppresses expression of the anti-inflammatory transcription factor. In some embodiments, the tolerogenic factor decreases the activity of a protein inhibitor that suppresses expression of the anti-inflammatory transcription factor. In some embodiments, the tolerogenic factor comprises nucleic acid encoding an enhancer of the anti-inflammatory transcription factor.

[0131] In certain aspects, the invention provides methods for delivering a tolerogenic factor into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the tolerogenic factor enters the cell, wherein said cell suspension is contacted with the tolerogenic factor. In some embodiments, the tolerogenic factor is delivered to the anucleate cell in vitro, ex vivo, or in vivo.

[0132] In some embodiments, the tolerogenic factor comprises a nucleic acid. In some embodiments, the tolerogenic factor is a nucleic acid. Exemplary nucleic acids include, without

limitation, recombinant nucleic acids, DNA, recombinant DNA, cDNA, genomic DNA, RNA, siRNA, mRNA, saRNA, miRNA, lncRNA, tRNA, guide RNA, and shRNA. In some embodiments, the nucleic acid is homologous to a nucleic acid in the cell. In some embodiments, the nucleic acid is heterologous to a nucleic acid in the cell. In some embodiments, the tolerogenic factor is a plasmid. In some embodiments, the nucleic acid is a therapeutic nucleic acid. In some embodiments, the nucleic acid encodes a therapeutic polypeptide. In some embodiments, the tolerogenic factor comprises a nucleic acid encoding siRNA, mRNA, miRNA, lncRNA, tRNA, or shRNA. For example, the tolerogenic factor can include siRNA to knock down expression of inflammatory genes. In some embodiments, the tolerogenic factor is a DNA sequence that binds NFκB and prevents NFκB activation and downstream signaling.

[0133] In some embodiments, the tolerogenic factor comprises a polypeptide. In some embodiments, the tolerogenic factor is a polypeptide. In some embodiments, the protein or polypeptide is a therapeutic protein, antibody, fusion protein, antigen, synthetic protein, reporter marker, or selectable marker. In some embodiments, the protein is a gene-editing protein or nuclease such as a zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), mega nuclease, CRE recombinase, transposase, RNA-guided endonuclease (e.g., CAS9 enzyme), DNA-guided endonuclease, or integrase enzyme. In some embodiments, the fusion proteins can include, without limitation, chimeric protein drugs such as antibody drug conjugates or recombinant fusion proteins such as proteins tagged with GST or streptavidin. In some embodiments, the compound is a transcription factor. Exemplary transcription factors include, without limitation, Oct5, Sox2, c-Myc, Klf-4, T-bet, GATA3, FoxP3, and RORγt. In some embodiments, the polypeptide is IL-4, IL-10, IL-13, IL-35, IFNα, or TGFβ. In some embodiments, the polypeptide is a therapeutic polypeptide. In some embodiments, the polypeptide is a fragment of a therapeutic polypeptide. In some embodiments, the polypeptide is a peptide nucleic acid (PNA).

[0134] In some embodiments, the tolerogenic factor comprises a protein-nucleic acid complex. In some embodiments, the tolerogenic factor is a protein-nucleic acid complex. In some embodiments, protein-nucleic acid complexes, such as clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9, are used in genome editing applications. These complexes contain sequence-specific DNA-binding domains in combination with nonspecific DNA cleavage nucleases. These complexes enable targeted genome editing, including adding, disrupting, or changing the sequence of a specific gene. In some embodiments, a disabled

CRISPR is used to block or induce transcription of a target gene. In some embodiments, the tolerogenic factor contains a Cas9 protein and a guide RNA or donor DNA. In some embodiments, the tolerogenic factor includes a nucleic acid encoding for a Cas9 protein and a guide RNA or donor DNA. In some embodiments, the gene editing complex targets expression of a costimulatory molecule (e.g., CD80 and/or CD86).

[0135] In some embodiments, the tolerogenic factor comprises a chimeric antigen receptor (CAR). In some embodiments, the tolerogenic factor is a chimeric antigen receptor (CAR). In some embodiments, the CAR is a fusion of an extracellular recognition domain (e.g., an antigen-binding domain), a transmembrane domain, and one or more intracellular signaling domains. Upon antigen engagement, the intracellular signaling portion of the CAR can initiate an immunosuppression or tolerogenic-related response in an immune cell. In some embodiments, the CAR is a chimeric T cell antigen receptor. In some embodiments, the CAR antigen-binding domain is a single-chain antibody variable fragment (scFv). In some embodiments, the tolerogenic factor encodes a modified TCR containing cytoplasmic signaling domains that trigger production of immunosuppressive cytokines upon binding to antigen. In some embodiments, the tolerogenic factor encodes a chimeric antigen receptor containing cytoplasmic signaling domains that trigger production of immunosuppressive cytokines upon binding to antigen.

[0136] In some embodiments, the tolerogenic factor comprises a small molecule. In some embodiments, the tolerogenic factor is a small molecule. In some embodiments, the small molecule inhibits the activity of a costimulatory molecule, enhances the activity of a co-inhibitory molecule, and/or inhibits the activity of an inflammatory molecule. Exemplary small molecules include, without limitation, pharmaceutical agents, metabolites, or radionuclides. In some embodiments, the pharmaceutical agent is a therapeutic drug and/or cytotoxic agent. In some embodiments, the compound comprises a nanoparticle. Examples of nanoparticles include gold nanoparticles, quantum dots, carbon nanotubes, nanoshells, dendrimers, and liposomes. In some embodiments, the nanoparticle contains or is linked (covalently or noncovalently) to a therapeutic molecule. In some embodiments, the nanoparticle contains a nucleic acid, such as mRNA or cDNA.

[0137] In some embodiments, the compound to deliver is purified. In some embodiments, the compound is at least about 60% by weight (dry weight) the compound of interest. In some embodiments, the purified compound is at least about 75%, 90%, or 99% the compound of

interest. In some embodiments, the purified compound is at least about 90%, 91%, 92%, 93%, 94%, 95%, 98%, 99%, or 100% (w/w) the compound of interest. Purity is determined by any known methods, including, without limitation, column chromatography, thin layer chromatography, HPLC analysis, NMR, mass spectrometry, or SDS-PAGE. Purified DNA or RNA is defined as DNA or RNA that is free of exogenous nucleic acids, carbohydrates, and lipids.

[0138] In some embodiments, the compound is an intermediate compound. The intermediate compound may be a molecular entity that is formed from preceding intermediates and reacts further to give the final reaction product. In some embodiments, the intermediate compound is a protein precursor, or pro-protein, that is cleaved by an enzyme to produce the mature, functional form of the protein. In some embodiments, the intermediate compound is an inactive enzyme precursor, or zymogen, that requires modification or cleavage to produce the active enzyme.

X. APPLICATIONS

[0139] In some aspects, the invention provides methods of treating a patient by introducing an anucleate cell, modified by passing through a constriction such that a compound enters the cell, to the patient. In some embodiments, the treatment comprises multiple (such as any of 2, 3, 4, 5, 6, or more) steps of introducing such modified anucleate cells to the patient. In some embodiments, the cell is isolated from a patient, modified according to the methods disclosed, and introduced back into the patient. For example, a population of anucleate cells is isolated from a patient, passed through the constriction to achieve delivery of a compound, and then re-infused into the patient to augment a therapeutic immune response. In some embodiments, the cell is isolated from an individual, modified according to the disclosed methods, and introduced back into the individual. For example, a population of anucleate cells is isolated from an individual, passed through the constriction to achieve delivery of a compound, and then re-infused into the patient to suppress an immune response or induce tolerance in the individual.

[0140] In some embodiments, an anucleate cell is isolated from a universal blood donor (e.g. an O- blood donor) and then stored and/or frozen for later constriction-mediated delivery and introduction into an individual. In some embodiments, an antigen is isolated from an individual, delivered to an anucleate cell isolated from a universal donor, and introduction into the individual. In some embodiments, an anucleate cell is isolated from a blood donor and then stored and/or frozen for later constriction mediated delivery and introduction into an individual with a matched blood type to the blood donor. In some embodiments, an antigen is isolated from

an individual, delivered to an anucleate cell isolated from a blood donor, and introduction into the individual.

[0141] In some embodiments, the invention provides methods of treating an individual by introducing the cell, modified by passing through a constriction such that a compound enters the cell, to the individual. In some embodiments, the cell is an autologous cell. For example, the anucleate cell is isolated from an individual (e.g, a patient), modified according to the methods disclosed, and introduced back into the individual. In some embodiments, the cell is isolated from an individual, modified according to the disclosed methods, and introduced back into the same individual. In some embodiments, the cell is an allogeneic cell. For example, the cell is isolated from a different individual, modified according to the methods disclosed, and introduced into the first individual (e.g., the patient). In some embodiments, a pool of cells from multiple individuals is modified according to the methods disclosed, and introduced into the first individual (e.g., the patient). In some embodiments, the cell is isolated from an individual, modified according to the disclosed methods, and introduced into a different individual. In some embodiments, a population of cells is isolated from an individual (the patient) or different individual, passed through the constriction to achieve delivery of a compound, and then re-infused into the patient to augment a therapeutic response.

[0142] In some embodiments, the treatment comprises multiple (such as any of 2, 3, 4, 5, 6, or more) steps of administering modified anucleate cells as described herein to the individual. For example, in some embodiments, there is provided a method of treating an individual by administering a cell, modified by passing through a constriction such that a compound enters the cell, to the individual 2, 3, 4, 5, 6, or more times. In some embodiments, the duration of time between any two consecutive administrations of the cell is at least about 1 day (such at least about any of 2 days, 3 days, 4 days, 5 days, 6 days, 1 week, 2 weeks, 3weeks, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year, or longer, including any ranges between these values).

[0143] Any of the methods described above are carried out in vitro, ex vivo, or in vivo. For in vivo applications, the device may be implanted in a vascular lumen, e.g., an in-line stent in an artery or vein. In some embodiments, the methods are used as part of a bedside system for ex vivo treatment of patient cells and immediate reintroduction of the cells into the patient. Such methods could be employed as a means of suppressing an immune response or inducing tolerance in an individual. In some embodiments, the method can be implemented in a typical

hospital laboratory with a minimally trained technician. In some embodiments, a patient operated treatment system can be used. In some embodiments, the method is implemented using an in-line blood treatment system, in which blood is directly diverted from a patient, passed through the constriction, resulting in compound delivery to blood cells, and directly transfused back into the patient after treatment.

XI. SYSTEMS AND KITS

[0144] In some aspects, the invention provides a system comprising the constriction, cell suspension, and compound for use in the methods disclosed herein. The system can include any embodiment described for the methods disclosed above, including microfluidic channels or a surface having pores to provide cell-deforming constrictions, cell suspensions, cell perturbations, delivery parameters, compounds, and/or applications etc. In some embodiment, the cell-deforming constrictions are sized for delivery to anucleate cells. In some embodiments, the delivery parameters, such as operating flow speeds, cell and compound concentration, velocity of the cell in the constriction, and the composition of the cell suspension (e.g., osmolarity, salt concentration, serum content, cell concentration, pH, etc.) are optimized for maximum response of a compound for suppressing an immune response or inducing tolerance.

[0145] Also provided are kits or articles of manufacture for use in delivering a compound to suppress an immune response or induce tolerance. In some embodiments, the kits comprise the compositions described herein (e.g. a microfluidic channel or surface containing pores, cell suspensions, and/or compounds) in suitable packaging. Suitable packaging materials are known in the art, and include, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

[0146] The present disclosure also provides kits comprising components of the methods described herein and may further comprise instruction(s) for performing said methods to suppress an immune response or induce tolerance. The kits described herein may further include other materials, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for performing any methods described herein; e.g., instructions for suppressing an immune response or inducing tolerance.

XII. EXEMPLARY EMBODIMENTS

[0147] Embodiment 1. A method for suppressing an immune response in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0148] Embodiment 2. A method for suppressing an immune response in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said tolerogenic factor contributes to a tolerogenic environment that suppresses an immune response.

[0149] Embodiment 3. A method for suppressing an immune response in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and a tolerogenic factor enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0150] Embodiment 4. A method for suppressing an immune response in an individual, comprising:

a. passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell,

b. passing a second cell suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell,

c. introducing the first anucleate cell and second anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0151] Embodiment 5. A method for suppressing an immune response in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0152] Embodiment 6. A method for suppressing an immune response in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen and a tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

[0153] Embodiment 7. A method for delivering a tolerogenic factor into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the tolerogenic factor enters the cell, wherein said cell suspension is contacted with the tolerogenic factor.

[0154] Embodiment 8. A method for introducing an antigen into a tolerogenic environment comprising delivering an anucleate cell into an individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment.

[0155] Embodiment 9. A method for introducing an antigen and a tolerogenic factor into a tolerogenic environment comprising delivering an anucleate cell into an individual, wherein the anucleate cell comprises the antigen and the tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a

constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment.

[0156] Embodiment 10. A method for inducing antigen-specific tolerance in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0157] Embodiment 11. A method for inducing tolerance in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said tolerogenic factor contributes to a tolerogenic environment that induces tolerance.

[0158] Embodiment 12. A method for inducing tolerance in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and a tolerogenic factor enter the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0159] Embodiment 13. A method for inducing antigen-specific tolerance in an individual, comprising:

a. passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell,

b. passing a second cell suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell,

c. introducing the first anucleate cell and second anucleate cell into the individual, wherein said antigen and said tolerogenic factors are processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0160] Embodiment 14. A method for inducing antigen-specific tolerance in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0161] Embodiment 15. A method for inducing antigen-specific tolerance in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen and a tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

[0162] Embodiment 16. A method for delivering an antigen into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the antigen enters the cell, wherein said cell suspension is contacted with the antigen.

[0163] Embodiment 17. A method for delivering an antigen and a tolerogenic factor into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor enter the cell, wherein said cell suspension is contacted with the antigen and the tolerogenic factor.

[0164] Embodiment 18. The method of embodiment 16 or 17, wherein processing and presentation of said antigen in a tolerogenic environment suppresses an immune response to the antigen.

[0165] Embodiment 19. The method of embodiment 16 or 17, wherein processing and presentation of said antigen in a tolerogenic environment induces tolerance to the antigen.

[0166] Embodiment 20. The method of any one of embodiments 1, 4, 5, 8, 10, and 13-19 , wherein at least one additional antigen is introduced into the cell.

[0167] Embodiment 21. The method of any one of embodiments 2-4. 6, 7, 9, 11, 12, 13, 15, and 17 , wherein at least one additional tolerogenic factor is introduced into the cell.

[0168] Embodiment 22. The method of any one of embodiments 1-6, 8-15, 18, and 19, wherein the tolerogenic environment is located in the spleen, liver, or lymph nodes.

[0169] Embodiment 23. The method of any one of embodiments 1-22, wherein the constriction is contained within a microfluidic channel.

[0170] Embodiment 24. The method of any one of embodiments 1-23, wherein the channel comprises a constriction width of about 0.25 μm to about 4 μm.

[0171] Embodiment 25. The method of any one of embodiments 1-24, wherein the channel comprises a constriction width of about 4 μm, 3.5 μm, about 3 μm, about 2.5 μm, about 2 μm, about 1.5 μm , about 1 μm, about 0.5 μm, or about 0.25 μm.

[0172] Embodiment 26. The method of any one of embodiments 1-22, wherein the constriction is a pore or contained within a pore.

[0173] Embodiment 27. The method of embodiment 26, wherein the pore is contained in a surface.

[0174] Embodiment 28. The method of embodiment 27, wherein the surface is a filter.

[0175] Embodiment 29. The method of embodiment 27, wherein the surface is a membrane.

[0176] Embodiment 30. The method of any one of embodiments 26-29, wherein the pore size is about 0.5 μm to about 4 μm.

[0177] Embodiment 31. The method of any one of embodiments 26-30, wherein the pore size is 4 μm, about 3 μm, about 2 μm, about 1 μm, or about 0.5 μm.

[0178] Embodiment 32. The method of any one of embodiments 1-31, wherein the constriction size is a function of the diameter of the anucleate cell.

[0179] Embodiment 33. The method of any one of embodiments 1-32, wherein the constriction size is about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, or about 70% of the cell diameter.

[0180] Embodiment 34. The method of any one of embodiments 1-33, wherein the method is performed between about -5°C and about 45°C.

[0181] Embodiment 35. The method of any one of embodiments 1-4, 7, 10-13, and 16-34, wherein the cell suspension comprises a mixed cell population.

[0182] Embodiment 36. The method of embodiment 35, wherein the cell suspension is whole blood.

[0183] Embodiment 37. The method of any one of embodiments 1-4, 7, 10-13, and 16-34, wherein the cell suspension comprises a purified cell population.

[0184] Embodiment 38. The method of embodiment 37, wherein the cell suspension comprises a purified anucleate cell population.

[0185] Embodiment 39. The method of any one of embodiments 1-4, 7, 10-13, and 16-38, wherein the cell suspension comprises mammalian cells.

[0186] Embodiment 40. The method of any one of embodiments 1-4, 7, 10-13, and 16-39, wherein the cell suspension comprises monkey, mouse, dog, cat, horse, rat, sheep, goat, pig, or rabbit cells.

[0187] Embodiment 41. The method of any one of embodiments 1-4, 7, 10-13, and 16-39, wherein the cell suspension comprises human cells.

[0188] Embodiment 42. The method of any one of embodiments 1-41, wherein the anucleate cell is a red blood cell.

[0189] Embodiment 43. The method of embodiment 42, wherein the red blood cell is an erythrocyte.

[0190] Embodiment 44. The method of embodiment 42, wherein the red blood cell is a reticulocyte.

[0191] Embodiment 45. The method of any one of embodiments 1-41, wherein the anucleate cell is a platelet.

[0192] Embodiment 46. The method of any one of embodiments 1-45, wherein the anucleate cell is a mammalian cell.

[0193] Embodiment 47. The method of any one of embodiments 1-46, wherein the anucleate cell is a monkey, mouse, dog, cat, horse, rat, sheep, goat, pig, or rabbit cell.

[0194] Embodiment 48. The method of any one of embodiments 1-46, wherein the anucleate cell is a human cell.

[0195] Embodiment 49. The method of any one of embodiments 1-5, 8-14, and 22-48, wherein the anucleate cell is from the individual.

[0196] Embodiment 50. The method of any one of embodiments 1-5, 8-14, and 22-48, wherein the anucleate cell is from a different individual.

[0197] Embodiment 51. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is located in a cell lysate.

[0198] Embodiment 52. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is a foreign antigen.

[0199] Embodiment 53. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is a self-antigen.

[0200] Embodiment 54. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is an allograft transplantation antigen.

[0201] Embodiment 55. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is a protein or polypeptide.

[0202] Embodiment 56. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is a lysate.

[0203] Embodiment 57. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is a microorganism.

[0204] Embodiment 58. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is a lipid antigen.

[0205] Embodiment 59. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is a carbohydrate antigen.

[0206] Embodiment 60. The method of any one of embodiments 1, 4, 5, 8-10, and 13-59 wherein the antigen is a modified antigen.

[0207] Embodiment 61. The method of embodiment 60, wherein the modified antigen comprises an antigen fused with a polypeptide.

[0208] Embodiment 62. The method of embodiment 60 or 61, wherein the modified antigen comprises an antigen fused with a therapeutic agent.

[0209] Embodiment 63. The method of embodiment 60 or 61, wherein the modified antigen comprises an antigen fused with a targeting peptide.

[0210] Embodiment 64. The method of embodiment 60 or 61, wherein the modified antigen comprises an antigen fused with a lipid.

[0211] Embodiment 65. The method of embodiment 60 or 61, wherein the modified antigen comprises an antigen fused with a carbohydrate.

[0212] Embodiment 66. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is an antigen associated with transplanted tissue.

[0213] Embodiment 67. The method of any one of embodiments 1, 4, 5, 8-10, and 13-50, wherein the antigen is associated with a virus.

[0214] Embodiment 68. The method of any one of embodiments 1, 4, 10, 13, and 16-67, wherein said cell suspension is contacted with the antigen before, concurrently, or after passing through the constriction.

[0215] Embodiment 69. The method of any one of embodiments 2-4, 7, 11-13, and 22-68, wherein the tolerogenic factor comprises a polypeptide.

[0216] Embodiment 70. The method of embodiment 69, wherein the polypeptide is IL-4, IL-10, IL-13, IL-35, IFNα, or TGFβ.

[0217] Embodiment 71. The method of embodiment 69, wherein the polypeptide is a therapeutic polypeptide.

[0218] Embodiment 72. The method of embodiment 69, wherein the polypeptide is a fragment of a therapeutic polypeptide.

[0219] Embodiment 73. The method of embodiment 69, wherein the polypeptide is a therapeutic peptide.

[0220] Embodiment 74. The method of embodiment 69, wherein the polypeptide is conjugated to a carbohydrate.

[0221] Embodiment 75. The method of any one of embodiments 2-4, 7, 11-13, and 22-74, wherein said cell suspension is contacted with the tolerogenic factor before, concurrently, or after passing through the constriction.

[0222] Embodiment 76. The method of any one of embodiment 1-75, wherein the half-life of the anucleate cell is decreased.

[0223] Embodiment 77. The method of any one of embodiment 1-75, wherein the half-life of the anucleate cell is increased.

[0224] Embodiment 78. The method of any one of embodiment 1-6, 18, and 22-77, wherein the immune response is suppressed by at least about 10%, about 15%, about 20%, about 25%,

about 30%, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 90%, or about 100%.

[0225] Embodiment 79. The method of any one of embodiments 1-6, 18, and 22-78, wherein the suppressed immune response comprises decreased production and/or secretion of one or more inflammatory cytokines.

[0226] Embodiment 80. The method of embodiment 79, wherein the one or more inflammatory cytokines are selected from the group consisting of interleukin-1 (IL-1), IL-12, and IL-18, tumor necrosis factor (TNF), interferon gamma (IFN-gamma), and granulocyte-macrophage colony stimulating factor (GM-CSF).

[0227] Embodiment 81. The method of any one of embodiments 1-6, 18, and 22-78, wherein the suppressed immune response comprises increased production and/or secretion of one or more anti-inflammatory cytokines.

[0228] Embodiment 82. The method of embodiment 81, wherein the one or more anti-inflammatory cytokines are selected from the group consisting of IL-4, IL-10, IL-13, IL-35, IFN-α and transforming growth factor-beta (TGFβ).

[0229] Embodiment 83. The method of any one of embodiments 1-6, 18, and 22-78, wherein the suppressed immune response comprises a decreased T cell response.

[0230] Embodiment 84. The method of embodiment 83, wherein the decreased T cell response comprises decreased T cell activation.

[0231] Embodiment 85. The method of embodiment 83 or 84, wherein the decreased T cell response comprises decreased T cell survival.

[0232] Embodiment 86.The method of any one of embodiments 83-85, wherein the decreased T cell response comprises decreased T cell proliferation.

[0233] Embodiment 87. The method of any one of embodiments 83-86, wherein the decreased T cell response comprises decreased T cell functionality.

[0234] Embodiment 88. The method of any one of embodiments 83-87, wherein the decreased T cell response comprises a change in T cell phenotype.

[0235] Embodiment 89. The method of any one of embodiments 1-6, 18, and 22-88, wherein the suppressed immune response comprises uncostimulated activation of a T cell.

[0236] Embodiment 90. The method of any one of embodiments 1-6, 18, and 22-89, wherein the suppressed immune response comprises an enhanced Treg response.

[0237] Embodiment 91. The method of any one of embodiments 1-6, 18, and 22-90, wherein the suppressed immune response comprises a decreased B cell response.

[0238] Embodiment 92. The method of embodiment 91, wherein the decreased B cell response comprises decreased antibody production.

[0239] Embodiment 93. The method of any one of embodiments 1-6, 18, and 22-92, wherein the suppressed immune response comprises decreased cytokine production.

[0240] Embodiment 94. The method of any one of embodiments 1-6, 18, and 22-93, wherein the suppressed immune response comprises a decreased autoimmune response.

[0241] Embodiment 95. The method of any one of embodiments 1-6, 18, and 22-94, wherein the suppressed immune response comprises a decreased allergic response.

[0242] Embodiment 96. The method of any one of embodiments 1-6, 18, and 22-95, wherein the suppressed immune response comprises a decreased immune response against the transplanted tissue.

[0243] Embodiment 97. The method of any one of embodiments 1-6, 18, and 22-95, wherein the suppressed immune response comprises a decreased pathogenic immune response to the virus.

[0244] Embodiment 98. The method of any one of embodiments 1-6, 18, and 22-95 wherein the suppressed immune response comprises a decreased immune response against a therapeutic agent.

[0245] Embodiment 99. The method of any one of embodiments 1-6, 18, and 22-95, wherein the suppressed immune response comprises a decreased immune response against a therapeutic vehicle.

[0246] Embodiment 100. The method of any one of embodiments 10-15 and 19-77, wherein the tolerance comprises decreased production and/or secretion of one or more inflammatory cytokines.

[0247] Embodiment 101. The method of embodiment 100, wherein the one or more inflammatory cytokines are selected from the group consisting of interleukin-1 (IL-1), IL-12, and IL-18, tumor necrosis factor (TNF), interferon gamma (IFN-gamma), and granulocyte-macrophage colony stimulating factor (GM-CSF).

[0248] Embodiment 102. The method of any one of embodiments 10-15 and 19-77, wherein the tolerance comprises increased production and/or secretion of one or more anti-inflammatory cytokines.

[0249] Embodiment 103. The method of embodiment 102, wherein the one or more anti-inflammatory cytokines are selected from the group consisting of IL-4, IL-10, IL-13, IL-35, IFN-α and transforming growth factor-beta (TGFβ).

[0250] Embodiment 104. The method of any one of embodiments 10-15 and 19-77, wherein the tolerance comprises a decreased T cell response.

[0251] Embodiment 105. The method of embodiment 104, wherein the decreased T cell response comprises decreased T cell activation.

[0252] Embodiment 106. The method of embodiment 104 or 105, wherein the decreased T cell response comprises decreased T cell survival.

[0253] Embodiment 107. The method of any one of embodiments 104-106, wherein the decreased T cell response comprises decreased T cell proliferation.

[0254] Embodiment 108. The method of any one of embodiments 104-107, wherein the decreased T cell response comprises decreased T cell functionality.

[0255] Embodiment 109. The method of any one of embodiments 104-108, wherein the decreased T cell response comprises a change in T cell phenotype.

[0256] Embodiment 110. The method of any one of embodiments 10-15, 19-77, and 104-109, wherein the tolerance comprises uncostimulated activation of a T cell.

[0257] Embodiment 111. The method of any one of embodiments 10-15, 19-77, and 104-110, wherein the tolerance comprises an enhanced Treg response.

[0258] Embodiment 112. The method of any one of embodiments 10-15, 19-77, and 104-111, wherein the tolerance comprises a decreased B cell response.

[0259] Embodiment 113. The method of embodiment 112, wherein the decreased B cell response comprises decreased antibody production.

[0260] Embodiment 114. The method of any one of embodiments 10-15, 19-77, and 104-113, wherein the tolerance comprises decreased cytokine production.

[0261] Embodiment 115. The method of any one of embodiments 10-15, 19-77, and 104-114, wherein the tolerance comprises a decreased autoimmune response.

[0262] Embodiment 116. The method of any one of embodiments 10-15, 19-77, and 104-115, wherein the tolerance comprises a decreased allergic response.

[0263] Embodiment 117. The method of any one of embodiments 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased immune response against the transplanted tissue.

[0264] Embodiment 118. The method of any one of embodiments 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased pathogenic immune response to the virus.

[0265] Embodiment 119. The method of any one of embodiments 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased immune response against a therapeutic agent.

[0266] Embodiment 120. The method of any one of embodiments 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased immune response against a therapeutic vehicle.

[0267] Embodiment 121. The method of any one of embodiments 1-120, wherein the method is repeated at least 1, 2, 3, 4, 5, or 6 times.

[0268] Embodiment 122. The method of embodiment 121, wherein the duration of time between any two repetitions of the method is at least 1 day, 1 week, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, or 1 year.

[0269] Embodiment 123. A system comprising the constriction, cell suspension, and antigen for use in the methods of any one of embodiments 1, 4, 10, 13, and 16-120.

[0270] Embodiment 124. A system comprising the constriction, cell suspension, and tolerogenic factor for use in the methods of any one of embodiments 2-4, 7, 11-13, and 22-120.

EXAMPLES

[0271] The following examples are given for the purpose of illustrating various embodiments of the disclosure and are not meant to limit the present disclosure in any fashion. One skilled in the art will appreciate readily that the present disclosure is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. Changes therein and other uses which are encompassed within the spirit of the disclosure as defined by the scope of the claims will occur to those skilled in the art.

Example 1: Constriction-mediated delivery of dextran and IgG antibody to human RBCs

INTRODUCTION

[0272] In order to evaluate filter-mediated delivery of molecules into anucleate cells, human RBCs mixed with fluorescent dextran particles or IgG antibody were passed through syringe filters containing pores of defined sizes, and intracellular particle delivery was evaluated via FACS analysis.

Materials and Methods

[0273] 2μm diameter pore size polycarbonate membrane filters with a commercial filter and holder combination (COTS filters) were obtained from STERLITECH™. Human RBCs were separated from whole blood or leukoreduction collar using a Ficoll gradient separation method

and resuspended at the desired concentration (50-500M/mL) in Optimem. The polycarbonate membrane filter was suspended in Optimem using forceps to wet the membrane filer. The plastic filter holder was uncapped, the filter was placed onto the inside surface with the shiny-side facing upwards, and the filter holder was recapped. Dextran particles and IgG antibody were suspended at 0.1mg/mL. 1mL of cells mixed with dextran particles or IgG antibody was added into the filter holder at room temperature. A manual finger push on the syringe was used to drive the cells through the filter. The filter flow-through was collected into a 15ml Falcon tube or FACS tube. Before FACS analysis all samples were centrifuged and washed three times at 400 rcf for 4 minutes at 4°C to remove extracellular dextrans. To prepare for FACS analysis the following steps were taken. FACS buffer was prepared (PBS + final concentration: 1% FBS + 2mM EDTA) and 400 µL of FACS buffer was added per tube and the cells were resuspended. The flow cytometry forward scatter, side scatter and fluorescent channel voltages were adjusted to visualize all cell events and ≥10,000 events/sample were recorded. Cells mixed with dextran particles or IgG antibody, but not passed through the filters, were used as a control for delivery via endocytosis. These cells had contact with dye but were not passed through the filters. All other steps were the same for the endocytosis controls.

Results

[0274] RBCs mixed with 10kDa Alexa Fluor® 647 dextran particles or 150kDa IgG antibody conjugated to Alexa Fluor® 647 were passed through 2µm sized filter pores. Exemplary flow cytometry histogram plots depicting increased delivery of fluorescent dextran and IgG post constriction mediated delivery (SQZ) versus the endocytosis control are shown in **FIGS. 1A&B**. Delivery efficiency, as indicated by percentage of cells positive for the dextran particles of IgG antibody post-filter delivery, is shown in **FIG. 2A**. Delivery efficiency of IgG antibody was 88.6%, and delivery efficiency of dextran particles was 95.6% post-filter delivery, compared to <10% delivery efficiency for both molecules in endocytosis controls.

[0275] Cell viability was measured post-filter delivery. Estimated cell viability, as indicated by percentage of cells present within the FSC and SSC gate post-filter delivery, is shown in **FIG. 2B**. Estimated viability of cells delivered IgG antibody was 68.8%, and estimated viability of cells delivered dextran particles was 63.3% post-filter delivery.

[0276] Constriction mediated delivery of dextran particles and IgG antibody into human RBCs was achieved.

Example 2: Constriction-mediated delivery of dextran and IgG antibody to mouse RBCs

INTRODUCTION

[0277] In order to evaluate filter-mediated delivery of molecules into anucleate cells, mouse RBCs mixed with fluorescent dextran particles or IgG antibody were passed through syringe filters containing pores of defined sizes, and intracellular particle delivery was evaluated via FACS analysis.

Materials and Methods

[0278] 1μm and 2μm diameter pore size polycarbonate membrane filters with a commercial filter and holder combination (COTS filters) were obtained from STERLITECH™. Whole blood was spun down and cells were resuspended at the desired concentration (100-500 M/ml) in Optimem. The polycarbonate membrane filter was suspended in Optimem using forceps to wet the membrane filer. The plastic filter holder was uncapped, the filter was placed onto the inside surface with the shiny-side facing upwards, and the filter holder was recapped. Dextran particles and IgG antibody were suspended at 0.1mg/mL. 1mL of cells mixed with dextran particles or IgG antibody was added into the filter holder at room temperature. A manual finger push on the syringe was used to drive the cells through the filter. Alternatively, a controlled pressure system is used to drive 200μL of cells mixed with dextran particles or IgG antibody through the filter. The filter flow-through was collected into a 15ml Falcon tube or FACS tube. Before FACS analysis all samples were centrifuged and washed three times at 1000 rfc for 10 minutes at 24°C to remove extracellular dextrans. To prepare for FACS analysis the following steps were taken. FACS buffer was prepared (PBS + final concentration: 1% FBS + 2mM EDTA) and 400 μL of FACS buffer was added per tube and the cells were resuspended. The flow cytometry forward scatter, side scatter and fluorescent channel voltages were adjusted to visualize all cell events and ≥10,000 events/sample were recorded. Cells mixed with dextran particles or IgG antibody, but not passed through the filters, were used as a control for delivery via endocytosis. These cells had contact with dye but were not passed through the filters. All other steps were the same for the endocytosis controls. The NC (No contact) samples did not have contact with dye but were subjected to the same steps as the experimental samples but were not passed through the filters.

Results

[0279] Mouse RBCs mixed with IgG antibody (150kDa) were passed through 1μm and 2μm sized filter pores using manual syringe pressure. Exemplary flow cytometry histogram plots depicting fluorescence post constriction mediated delivery (SQZ) versus the endocytosis (Endo), negative (NC), and no material controls control are shown in **FIG. 3A**. The 'no material' control

did not have contact with IgG antibody but was subjected to the same steps as the experimental samples and was passed through the filter.

[0280] Cell viability was measured post-filter delivery. Estimated cell viability, as indicated by percentage of cells present within the FSC and SSC gate post-filter delivery of IgG antibody using manual syringe pressure, is shown in **FIG. 3B**, with filter-mediated delivery decreasing the estimated viability by ~70% vs. controls, but leading to 4-12-fold higher delivery efficiency of IgG antibody, as shown in **FIG. 3C**.

[0281] RBCs mixed with 70kDa Alexa Fluor® 488 dextran particles and IgG antibody (150kDa) conjugated to Alexa Fluor® 647 were passed through 2μm sized filter pores using a controlled pressure system under 2psi, 4psi, 6psi, 10psi, 20psi, or using manual syringe pressure. Exemplary flow cytometry histogram plots are shown in **FIGS. 4A-I,** depicting fluorescence post constriction mediated delivery (SQZ – **FIGS. 4D-I**) versus the endocytosis (Endo – **FIG. 4A**), negative (NC – **FIG. 4B**), and no material control (**FIG. 4C**). These data showed that filter-mediated delivery led to up to 55% increases in dextran and IgG delivery, relative to Endo, NC and no material controls. Cell viability was also measured post-filter delivery. Estimated cell viability, as indicated by percentage of cells present within the FSC and SSC gate post-filter delivery of dextran particles is shown in **FIG. 5A**. Delivery efficiency, as indicated by percentage of cells positive for dextran particles or IgG antibody post-filter delivery is shown in **FIG. 5B**. The geometric mean fluorescence post-filter delivery of dextran particles or IgG antibody is shown in **FIG. 5C**.

[0282] RBCs mixed with 70kDa Alexa Fluor® 488 dextran particles were passed through 2μm sized filter pores using a controlled pressure system under 10psi, 12psi, 14psi, 16psi, or 18psi. Exemplary flow cytometry histogram plots depicting fluorescence post constriction mediated delivery (SQZ) versus the endocytosis (Endo), and negative (NC) controls are shown in **FIGS. 6A**. Cell viability was measured post-filter delivery. Estimated cell viability, as indicated by percentage of cells present within the FSC and SSC gate post-filter delivery of dextran particles is shown in **FIG. 6B**. Delivery efficiency, as indicated by percentage of cells positive for dextran particles post-filter delivery is shown in **FIG. 6C**. The geometric mean fluorescence post-filter delivery of dextran particles is shown in **FIG. 6D**.

[0283] Constriction mediated delivery of dextran particles and IgG antibody into mouse RBCs was achieved.

Example 3: Constriction-mediated delivery of antigen to RBCs to induce tolerance

[0284] A series of experiments are undertaken to demonstrate induction of tolerance using model antigen and transgenic T cells.

[0285] RBCs are mixed with OVA antigen and passed through a constriction within a microfluidic channel, or through a surface containing pores. Pressure, temperature, and buffer composition are optimized to achieve delivery. OVA loaded RBCs are then introduced into a CD45.1 mouse with CFSE labeled adoptively transferred OT-I cells. Alternatively, the OT-I T cells are activated ex-vivo prior to transfer into the CD45.1 mouse. 9 days later, to allow for tolerogenic presentation and subsequent T cell deletion, mice are challenged with soluble OVA and LPS. 4 days after challenge spleen and the draining lymph nodes are analyzed for OT-I proliferation.

Example 4: Constriction-mediated delivery of antigen to RBCs to induce tolerance

[0286] In order to determine the ability of RBCs containing antigen delivered by SQZ for the induction of *in vivo* antigen-dependent tolerance, the number of antigen-specific T cells and the levels of inflammatory cytokine IFN-γ were measured by flow cytometry.

Materials and Methods

[0287] C57BL/6J recipient mice (CD45.1) were adoptively transferred with 1M OVA-specific OT-I T cells per mouse from female OT-I donor mice (CD45.2) at day 0. On days 1 (prime) and 6 (boost), RBCs from C57BL/6J mice were harvested and RBCs were incubated with full OVA protein or OVA was delivered by filter-mediated SQZ conditions, followed by injection of 20-125M RBCs/mouse into recipient mice. Control animals received no injection (naïve), or injection with either PBS (challenge control) or free OVA (10 μg/animal – tolerance control). Antigen challenge occurred on day 15, with intradermal injection of 10 μg OVA protein+50 ng LPS/mouse in challenged animals, compared to naïve (no antigen or LPS). OT-I T cell response to antigen challenge was measured by flow cytometry for the number of OT-I T cells and IFN-γ intracellular staining on day 19. A schematic of the treatment schedule is shown in FIG. 7, and the treatment groups are summarized in Table 1.

Table 1

Group	Day 0	Days 1 & 5	Day 15
Naïve	OT-I adoptive transfer		
Challenge Control	OT-I adoptive transfer	PBS	OVA+LPS

Tolerance Control	OT-I adoptive transfer	OVA protein	OVA+LPS
RBC-Endo	OT-I adoptive transfer	RBCs incubated with OVA without constriction	OVA+LPS
RBC-SQZ	OT-I adoptive transfer	RBCs SQZ'd with OVA with constriction	OVA+LPS

Results

[0288] OT-I-specific T cell numbers were measured in mice challenged with antigen (OVA) and adjuvant (LPS) for groups treated with either RBCs incubated with OVA protein (RBC-Endo) or intracellular OVA protein delivery by SZQ (RBC-SQZ) (FIG. 8). These conditions were compared to animals that did not see antigen, adjuvant or RBCs (naïve), or mice treated with free OVA protein (tolerance control) or PBS (challenge control) followed by antigen+adjuvant challenge. OT-I T cell numbers in both the draining lymph nodes and spleen were significantly inhibited (****P<0.0001) in mice primed/boosted with RBCs SQZ'd with OVA protein compared to the challenge control. These mice saw OT-I T cell numbers similar to naïve mice or the tolerance control, whereas treatment with RBCs that were incubated with OVA protein were no different that challenge control.

[0289] To assess the functional effect of SQZ RBC-mediated tolerance on T cell function, the % of splenic T cells that expressed high levels of IFN-γ (FIG. 9, left) and the level of IFN-γ production per cell (FIG. 9, righ; based on all CD44hi cells) of OT-I T cells restimulated with SIINFEKL peptide (SEQ ID NO: 1) (active OVA epitope) were assessed for all groups by intracellular cytokine staining. OT-I T cells from mice treated with OVA SQZ'd RBCs showed a modest but significant decrease in high IFN-γ expressing cells (**P<0.005) and a large decrease of IFN-γ production (****P<0.0001) compared to challenge control, with a larger decrease than the tolerance control (Free Ova; **P<0.005). However, RBCs incubated with OVA protein did not see a decrease in IFN-γ production relative to challenge control.

[0290] Representative flow cytograms (FIG. 10, summarized in Table 2) for the proportion of OT-I T cells out of all CD8+ T cells and the proportion of IFN-γ responsive T cells out of OT-I T cells show that the RBC-SQZ samples had fewer OT-Is and lower IFNg response relative to challenge control and RBC-Endo conditions.

Table 2

Group	% OT-I Specific Cells	% IFN-γ Expressing

Naïve	0.49	7.07
Challenge Control	8.13	76.0
Tolerance Control	1.38	64.2
RBC-Endo	11.7	75.8
RBC-SQZ	1.45	57.1

Example 5: CD4+/CD8+ mediated tolerance

[0291] To determine the ability of red blood cells (RBCs) containing antigen delivered by SQZ induce CD4- and/or CD8-mediated T cell antigen-dependent tolerance *in vivo*, the number of antigen-specific T cells and the levels of inflammatory cytokine IFN-γ are measured by flow cytometry.

Materials and Methods

[0292] C57BL/6J recipient mice (CD45.1+) are adoptively transferred with 1M OVA-specific CD8+ OT-I and 2-4M CD4+ OT-II T cells (both CD45.2+) per mouse from female donor mice at day 0. On days 1 (prime) and 6 (boost), RBCs from C57BL/6J mice are harvested and RBCs are incubated with full OVA protein or OVA is delivered by SQZ conditions, followed by injection of 20-125M RBCs/mouse into recipient mice. Control animals receive injection with either PBS (challenge control) or free OVA (10 μg/animal – tolerance control). Antigen challenge occurs on day 15, with intradermal injection of 10 μg OVA protein+50 ng LPS/mouse in challenged animals. Draining lymph nodes and spleens of each mouse are analyzed on day 19 and OT-I and OT-II T cell response to antigen challenge is measured by flow cytometry for the number of OT-I and OT-II T cells and IFN-γ intracellular staining and ELISpot assays. A schematic of a representative treatment schedule is shown in FIG. 7, and the treatment groups are summarized in Table 3.

Table 3

Group	**Day 0**	**Days 1 & 5**	**Day 8**
Challenge Control	OT-I and OT-II adoptive transfer	PBS	OVA + LPS
Tolerance Control	OT-I and OT-II adoptive transfer	OVA protein	OVA + LPS
RBC-Endo	OT-I and OT-II adoptive transfer	RBCs incubated with OVA without constriction	OVA + LPS
RBC-SQZ	OT-I and OT-II adoptive transfer	RBCs incubated with OVA with constriction	OVA + LPS

Example 6A: Prophylaxis in murine type-I diabetes model

Introduction

[0293] To determine the ability of RBCs containing antigen delivered by SQZ induce antigen-dependent tolerance in a prophylaxis *in vivo* model of murine type-I diabetes, the levels of blood glucose will be measured weekly over time after tolerance induction.

Materials and Methods

[0294] NOD/ShiltJ mice are treated with RBCs at 10 weeks of age. On days 0 (prime) and 5 (boost), RBCs from NOD/ShiltJ mice are harvested and RBCs are incubated with insulin B-chain peptide 9-23 ($InsB_{9-23}$: CKKGSSHLVEALYLVCGERG, SEQ ID NO: 2) or $InsB_{9-23}$ is delivered by SQZ conditions, followed by injection of 20-125M RBCs/mouse into recipient mice. Control animals receive injection with PBS (challenge control). Blood glucose measurements are carried out weekly, starting on Day 7 after treatment (age 11 weeks). Mice are considered diabetic when their blood glucose level exceeded 260 mg/dL as measured by Bayer Contour Diabetes Meter/Glucose Test Strip. A schematic of a representative treatment schedule is shown in FIG. 11A, and the treatment groups are summarized in Table 4.

Table 4

Group	**Days 0 and 5**
Challenge control	PBS
APC-Endo	APCs incubated with $InsB_{9-23}$
APC-SQZ	APCs SQZ'd with $InsB_{9-23}$

Example 6B: Therapy in murine type-I diabetes model

Introduction

[0295] To determine the ability of RBCs containing antigen delivered by SQZ induce antigen-dependent tolerance in a treatment *in vivo* model of murine type-I diabetes, the levels of blood glucose will be measured weekly over time after tolerance induction in mice.

Materials and Methods

[0296] To induce rapid onset of T1D in recipients, CD4 T cells are harvested from BDC2.5 NOD mice, activated *ex vivo* with p31 mimetope peptide (YVRPLWVRME, SEQ ID NO: 3) for

4 days, and adoptively transferred (5M cells/mouse) into normoglycemic NOD recipients. Tolerance induction begins 8 hours after adoptive transfer, and is repeated every 3 days for a total of 3 doses. Recipients are treated with PBS (challenge control) or 20-125M RBCs incubated with p31 (RBC-Endo) or delivered by SQZ with p31 (RBC-SQZ). Blood glucose is measured every day by Bayer Contour Diabetes Meter/Glucose Test Strip. Mice are considered diabetic when their blood glucose level exceeded 260 mg/dL. A schematic of a representative treatment schedule is shown in FIG. 11B, and the treatment groups are summarized in Table 5.

Table 5

Group	**Days 0 and 5**
Challenge control	PBS
APC-Endo	APCs incubated with $InsB_{9-23}$
APC-SQZ	APCs SQZ'd with $InsB_{9-23}$

Example 7A: Prophylaxis in murine MS-type autoimmune disorder

Introduction

[0297] To determine the ability of RBCs containing antigen delivered by SQZ induce antigen-dependent tolerance in an *in vivo* prophylaxis model of a murine MS-type autoimmune disorder, the clinical score of mobility will be assessed daily over time.

Materials and Methods

[0298] Female C57BL/6 mice (10-12 wks of age) are treated with RBCs prior to induction of experimental autoimmune encephalomyelitis (EAE). On days -7 (prime) and -2 (boost), RBCs from C57BL/6 mice are harvested and RBCs are incubated with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}: MEVGWYRSPFSRVVHLYRNGKGS, SEQ ID NO: 4) without constriction, or $OVA_{323-339}$ peptide (negative control: ISQAVHAAHAEINEAGRGS, SEQ ID NO: 5) or MOG_{35-55} peptide is delivered to RBCs by SQZ conditions, followed by injection of 20-125M RBCs/mouse into recipient mice. On Day 0, EAE is induced by administration of MOG_{35-55} in CFA and pertussis toxin in PBS (Hooke kit). Mice are scored daily starting on Day 7, with clinical score defined as follows: 1, limp tail; 2, partial hind leg paralysis; 3, complete hind leg paralysis; 4, complete hind and partial front leg paralysis; and 5,

moribund. A schematic of a representative treatment schedule is shown in FIG. 12A, and the treatment groups are summarized in Table 6.

Table 6

Group	**Day -7**	**Day 0 (Induction of EAE)**
Challenge control	APCs SQZ'd with $OVA_{323-339}$	MOG_{35-55} + pertussis toxin
APC-Endo	APCs incubated with MOG_{35-55}	MOG_{35-55} + pertussis toxin
APC-SQZ	APCs SQZ'd with MOG_{35-55}	MOG_{35-55} + pertussis toxin

Example 7B: Therapy in murine MS-type autoimmune disorder

Introduction

[0299] To determine the ability of RBCs containing antigen delivered by SQZ induce antigen-dependent tolerance in an *in vivo* model of an established murine MS-type autoimmune disorder, the clinical score of mobility will be assessed daily over time.

Materials and Methods

[0300] In female C57BL/6 mice (10-12 wks of age) experimental autoimmune encephalomyelitis (EAE) is induced by administration of MOG_{35-55} in CFA and pertussis toxin in PBS (Hooke kit) on Day 0. Mice are then treated with RBCs on the day of onset of EAE, which occurs once mice are scored ≥ 1 based on the below mobility criteria. On days ~11/12 (prime) and ~17/18 (boost), RBCs from C57BL/6 mice are harvested and RBCs are incubated with myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}: MEVGWYRSPFSRVVHLYRNGKGS, SEQ ID NO: 4) without constriction, or $OVA_{323-339}$ peptide (negative control: ISQAVHAAHAEINEAGRGS, SEQ ID NO: 5) or MOG_{35-55} peptide is delivered to RBCs by SQZ conditions, followed by injection of 20-125M RBCs/mouse into recipient mice. Mice are scored daily starting on Day 19, with clinical score defined as follows: 1, limp tail; 2, partial hind leg paralysis; 3, complete hind leg paralysis; 4, complete hind and partial front leg paralysis; and 5, moribund. A schematic of a representative treatment schedule is shown in FIG. 12B, and the treatment groups are summarized in Table 7.

Table 7

Group	**Day -7**	**Day 0 (Induction of EAE)**
Challenge control	APCs SQZ'd with $OVA_{323-339}$	MOG_{35-55} + pertussis toxin
APC-Endo	APCs incubated with MOG_{35-55}	MOG_{35-55} + pertussis toxin
APC-SQZ	APCs SQZ'd with MOG_{35-55}	MOG_{35-55} + pertussis toxin

Sequence Listing

SEQ ID NO	Sequence	Description
1	SIINFEKL	CD8+ active OVA epitope
2	CKKGSSHLVEALYLVCGERG	B-chain peptide 9-23 ($InsB_{9-23}$)
3	YVRPLWVRME	p31 mimetope peptide
4	MEVGWYRSPFSRVVHLYRNGKGS	myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55})
5	ISQAVHAAHAEINEAGRGS	$OVA_{323-339}$ peptide

CLAIMS

What is claimed is:

1. A method for suppressing an immune response in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

2. A method for suppressing an immune response in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said tolerogenic factor contributes to a tolerogenic environment that suppresses an immune response.

3. A method for suppressing an immune response in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and a tolerogenic factor enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

4. A method for suppressing an immune response in an individual, comprising:

a. passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell,

b. passing a second cell suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell,

c. introducing the first anucleate cell and second anucleate cell into the individual, wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

5. A method for suppressing an immune response in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

6. A method for suppressing an immune response in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen and a tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment suppresses an immune response to the antigen.

7. A method for delivering a tolerogenic factor into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the tolerogenic factor enters the cell, wherein said cell suspension is contacted with the tolerogenic factor.

8. A method for introducing an antigen into a tolerogenic environment comprising delivering an anucleate cell into an individual, wherein the anucleate cell comprises an antigen,

wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment.

9. A method for introducing an antigen and a tolerogenic factor into a tolerogenic environment comprising delivering an anucleate cell into an individual, wherein the anucleate cell comprises the antigen and the tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment.

10. A method for inducing antigen-specific tolerance in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

11. A method for inducing tolerance in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said tolerogenic factor contributes to a tolerogenic environment that induces tolerance.

12. A method for inducing tolerance in an individual, the method comprising:

a. passing a cell suspension comprising an anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen and a tolerogenic factor enter the anucleate cell; and

b. introducing the anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

13. A method for inducing antigen-specific tolerance in an individual, comprising:

a. passing a first cell suspension comprising a first anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that an antigen enters the anucleate cell,

b. passing a second cell suspension comprising a second anucleate cell through a constriction, wherein said constriction deforms the cell thereby causing a perturbation of the cell such that a tolerogenic factor enters the anucleate cell,

c. introducing the first anucleate cell and second anucleate cell into the individual,

wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

14. A method for inducing antigen-specific tolerance in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen, wherein the antigen was introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen entered the anucleate cell, wherein said antigen is processed in a tolerogenic environment, wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

15. A method for inducing antigen-specific tolerance in an individual, the method comprising introducing an anucleate cell into the individual, wherein the anucleate cell comprises an antigen and a tolerogenic factor, wherein the antigen and the tolerogenic factor were introduced to the anucleate cell by passing the anucleate cell through a constriction, wherein said constriction deformed the cell thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor entered the anucleate cell, wherein said antigen is

processed in a tolerogenic environment, and wherein presentation of said antigen in said tolerogenic environment induces tolerance to the antigen.

16. A method for delivering an antigen into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the antigen enters the cell, wherein said cell suspension is contacted with the antigen.

17. A method for delivering an antigen and a tolerogenic factor into an anucleate cell, the method comprising passing a cell suspension comprising the anucleate cell through a constriction, wherein said constriction deforms the anucleate cell, thereby causing a perturbation of the cell such that the antigen and the tolerogenic factor enter the cell, wherein said cell suspension is contacted with the antigen and the tolerogenic factor.

18. The method of claim 16 or 17, wherein processing and presentation of said antigen in a tolerogenic environment suppresses an immune response to the antigen.

19. The method of claim 16 or 17, wherein processing and presentation of said antigen in a tolerogenic environment induces tolerance to the antigen.

20. The method of any one of claims 1, 4, 5, 8, 10, and 13-19 , wherein at least one additional antigen is introduced into the cell.

21. The method of any one of claims 2-4. 6, 7, 9, 11, 12, 13, 15, and 17 , wherein at least one additional tolerogenic factor is introduced into the cell.

22. The method of any one of claims 1-6, 8-15, 18, and 19, wherein the tolerogenic environment is located in the spleen, liver, or lymph nodes.

23. The method of any one of claims 1-22, wherein the constriction is contained within a microfluidic channel.

24. The method of any one of claims 1-23, wherein the channel comprises a constriction width of about 0.25 μm to about 4 μm.

25. The method of any one of claims 1-24, wherein the channel comprises a constriction width of about 4 μm, 3.5 μm, about 3 μm, about 2.5 μm, about 2 μm, about 1.5 μm , about 1 μm, about 0.5 μm, or about 0.25 μm.

26. The method of any one of claims 1-22, wherein the constriction is a pore or contained within a pore.

27. The method of claim 26, wherein the pore is contained in a surface.

28. The method of claim 27, wherein the surface is a filter.

29. The method of claim 27, wherein the surface is a membrane.

30. The method of any one of claims 26-29, wherein the pore size is about 0.5 μm to about 4 μm.

31. The method of any one of claims 26-30, wherein the pore size is 4 μm, about 3 μm, about 2 μm, about 1 μm, or about 0.5 μm.

32. The method of any one of claims 1-31, wherein the constriction size is a function of the diameter of the anucleate cell.

33. The method of any one of claims 1-32, wherein the constriction size is about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, or about 70% of the cell diameter.

34. The method of any one of claims 1-33, wherein the method is performed between about -5°C and about 45°C.

35. The method of any one of claims 1-4, 7, 10-13, and 16-34, wherein the cell suspension comprises a mixed cell population.

36. The method of claim 35, wherein the cell suspension is whole blood.

37. The method of any one of claims 1-4, 7, 10-13, and 16-34, wherein the cell suspension comprises a purified cell population.

38. The method of claim 37, wherein the cell suspension comprises a purified anucleate cell population.

39. The method of any one of claims 1-4, 7, 10-13, and 16-38, wherein the cell suspension comprises mammalian cells.

40. The method of any one of claims 1-4, 7, 10-13, and 16-39, wherein the cell suspension comprises monkey, mouse, dog, cat, horse, rat, sheep, goat, pig, or rabbit cells.

41. The method of any one of claims 1-4, 7, 10-13, and 16-39, wherein the cell suspension comprises human cells.

42. The method of any one of claims 1-41, wherein the anucleate cell is a red blood cell.

43. The method of claim 42, wherein the red blood cell is an erythrocyte.

44. The method of claim 42, wherein the red blood cell is a reticulocyte.

45. The method of any one of claims 1-41, wherein the anucleate cell is a platelet.

46. The method of any one of claims 1-45, wherein the anucleate cell is a mammalian cell.

47. The method of any one of claims 1-46, wherein the anucleate cell is a monkey, mouse, dog, cat, horse, rat, sheep, goat, pig, or rabbit cell.

48. The method of any one of claims 1-46, wherein the anucleate cell is a human cell.

49. The method of any one of claims 1-5, 8-14, and 22-48, wherein the anucleate cell is from the individual.

50. The method of any one of claims 1-5, 8-14, and 22-48, wherein the anucleate cell is from a different individual.

51. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is located in a cell lysate.

52. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is a foreign antigen.

53. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is a self-antigen.

54. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is an allograft transplantation antigen.

55. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is a protein or polypeptide.

56. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is a lysate.

57. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is a microorganism.

58. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is a lipid antigen.

59. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is a carbohydrate antigen.

60. The method of any one of claims 1, 4, 5, 8-10, and 13-59 wherein the antigen is a modified antigen.

61. The method of claim 60, wherein the modified antigen comprises an antigen fused with a polypeptide.

62. The method of claim 60 or 61, wherein the modified antigen comprises an antigen fused with a therapeutic agent.

63. The method of claim 60 or 61, wherein the modified antigen comprises an antigen fused with a targeting peptide.

64. The method of claim 60 or 61, wherein the modified antigen comprises an antigen fused with a lipid.

65. The method of claim 60 or 61, wherein the modified antigen comprises an antigen fused with a carbohydrate.

66. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is an antigen associated with transplanted tissue.

67. The method of any one of claims 1, 4, 5, 8-10, and 13-50, wherein the antigen is associated with a virus.

68. The method of any one of claims 1, 4, 10, 13, and 16-67, wherein said cell suspension is contacted with the antigen before, concurrently, or after passing through the constriction.

69. The method of any one of claims 2-4, 7, 11-13, and 22-68, wherein the tolerogenic factor comprises a polypeptide.

70. The method of claim 69, wherein the polypeptide is IL-4, IL-10, IL-13, IL-35, IFNα, or TGFβ.

71. The method of claim 69, wherein the polypeptide is a therapeutic polypeptide.

72. The method of claim 69, wherein the polypeptide is a fragment of a therapeutic polypeptide.

73. The method of claim 69, wherein the polypeptide is a therapeutic peptide.

74. The method of claim 69, wherein the polypeptide is conjugated to a carbohydrate.

75. The method of any one of claims 2-4, 7, 11-13, and 22-74, wherein said cell suspension is contacted with the tolerogenic factor before, concurrently, or after passing through the constriction.

76. The method of any one of claim 1-75, wherein the half-life of the anucleate cell is decreased.

77. The method of any one of claim 1-75, wherein the half-life of the anucleate cell is increased.

78. The method of any one of claim 1-6, 18, and 22-77, wherein the immune response is suppressed by at least about 10%, about 15%, about 20%, about 25%, about 30%, about 40%, about 50%, about 60%, about 70%, about 75%, about 80%, about 90%, or about 100%.

79. The method of any one of claims 1-6, 18, and 22-78, wherein the suppressed immune response comprises decreased production and/or secretion of one or more inflammatory cytokines.

80. The method of claim 79, wherein the one or more inflammatory cytokines are selected from the group consisting of interleukin-1 (IL-1), IL-12, and IL-18, tumor necrosis factor (TNF), interferon gamma (IFN-gamma), and granulocyte-macrophage colony stimulating factor (GM-CSF).

81. The method of any one of claims 1-6, 18, and 22-78, wherein the suppressed immune response comprises increased production and/or secretion of one or more anti-inflammatory cytokines.

82. The method of claim 81, wherein the one or more anti-inflammatory cytokines are selected from the group consisting of IL-4, IL-10, IL-13, IL-35, IFN-α and transforming growth factor-beta (TGFβ).

83. The method of any one of claims 1-6, 18, and 22-78, wherein the suppressed immune response comprises a decreased T cell response.

84. The method of claim 83, wherein the decreased T cell response comprises decreased T cell activation.

85. The method of claim 83 or 84, wherein the decreased T cell response comprises decreased T cell survival.

86. The method of any one of claims 83-85, wherein the decreased T cell response comprises decreased T cell proliferation.

87. The method of any one of claims 83-86, wherein the decreased T cell response comprises decreased T cell functionality.

88. The method of any one of claims 83-87, wherein the decreased T cell response comprises a change in T cell phenotype.

89. The method of any one of claims 1-6, 18, and 22-88, wherein the suppressed immune response comprises uncostimulated activation of a T cell.

90. The method of any one of claims 1-6, 18, and 22-89, wherein the suppressed immune response comprises an enhanced Treg response.

91. The method of any one of claims 1-6, 18, and 22-90, wherein the suppressed immune response comprises a decreased B cell response.

92. The method of claim 91, wherein the decreased B cell response comprises decreased antibody production.

93. The method of any one of claims 1-6, 18, and 22-92, wherein the suppressed immune response comprises decreased cytokine production.

94. The method of any one of claims 1-6, 18, and 22-93, wherein the suppressed immune response comprises a decreased autoimmune response.

95. The method of any one of claims 1-6, 18, and 22-94, wherein the suppressed immune response comprises a decreased allergic response.

96. The method of any one of claims 1-6, 18, and 22-95, wherein the suppressed immune response comprises a decreased immune response against the transplanted tissue.

97. The method of any one of claims 1-6, 18, and 22-95, wherein the suppressed immune response comprises a decreased pathogenic immune response to the virus.

98. The method of any one of claims 1-6, 18, and 22-95 wherein the suppressed immune response comprises a decreased immune response against a therapeutic agent.

99. The method of any one of claims 1-6, 18, and 22-95, wherein the suppressed immune response comprises a decreased immune response against a therapeutic vehicle.

100. The method of any one of claims 10-15 and 19-77, wherein the tolerance comprises decreased production and/or secretion of one or more inflammatory cytokines.

101. The method of claim 100, wherein the one or more inflammatory cytokines are selected from the group consisting of interleukin-1 (IL-1), IL-12, and IL-18, tumor necrosis factor (TNF), interferon gamma (IFN-gamma), and granulocyte-macrophage colony stimulating factor (GM-CSF).

102. The method of any one of claims 10-15 and 19-77, wherein the tolerance comprises increased production and/or secretion of one or more anti-inflammatory cytokines.

103. The method of claim 102, wherein the one or more anti-inflammatory cytokines are selected from the group consisting of IL-4, IL-10, IL-13, IL-35, IFN-α and transforming growth factor-beta (TGFβ).

104. The method of any one of claims 10-15 and 19-77, wherein the tolerance comprises a decreased T cell response.

105. The method of claim 104, wherein the decreased T cell response comprises decreased T cell activation.

106. The method of claim 104 or 105, wherein the decreased T cell response comprises decreased T cell survival.

107. The method of any one of claims 104-106, wherein the decreased T cell response comprises decreased T cell proliferation.

108. The method of any one of claims 104-107, wherein the decreased T cell response comprises decreased T cell functionality.

109. The method of any one of claims 104-108, wherein the decreased T cell response comprises a change in T cell phenotype.

110. The method of any one of claims 10-15, 19-77, and 104-109, wherein the tolerance comprises uncostimulated activation of a T cell.

111. The method of any one of claims 10-15, 19-77, and 104-110, wherein the tolerance comprises an enhanced Treg response.

112. The method of any one of claims 10-15, 19-77, and 104-111, wherein the tolerance comprises a decreased B cell response.

113. The method of claim 112, wherein the decreased B cell response comprises decreased antibody production.

114. The method of any one of claims 10-15, 19-77, and 104-113, wherein the tolerance comprises decreased cytokine production.

115. The method of any one of claims 10-15, 19-77, and 104-114, wherein the tolerance comprises a decreased autoimmune response.

116. The method of any one of claims 10-15, 19-77, and 104-115, wherein the tolerance comprises a decreased allergic response.

117. The method of any one of claims 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased immune response against the transplanted tissue.

118. The method of any one of claims 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased pathogenic immune response to the virus.

119. The method of any one of claims 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased immune response against a therapeutic agent.

120. The method of any one of claims 10-15, 19-77, and 104-116, wherein the tolerance comprises a decreased immune response against a therapeutic vehicle.

121. The method of any one of claims 1-120, wherein the method is repeated at least 1, 2, 3, 4, 5, or 6 times.

122. The method of claim 121, wherein the duration of time between any two repetitions of the method is at least 1 day, 1 week, 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, or 1 year.

123. A system comprising the constriction, cell suspension, and antigen for use in the methods of any one of claims 1, 4, 10, 13, and 16-120.

124. A system comprising the constriction, cell suspension, and tolerogenic factor for use in the methods of any one of claims 2-4, 7, 11-13, and 22-120.