# ERYTHROCYTE-BINDING THERAPEUTICS

## CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Serial No. 13/397,202 filed February 15, 2012, which is hereby incorporated by reference herein.

## TECHNICAL FIELD OF THE INVENTION

The Technical Field relates to medical compositions and uses for ligands or antibodies that bind erythrocytes. Specific uses include immunotolerization, drug delivery, and cancer therapies.

## BACKGROUND

The rejection of transplanted tissue and autoimmune diseases are pathological conditions that involve an immunorejection of a foreign biomolecule because of its antigenic nature. Many drugs and clinical processes are involved in suppressing or treating immunorejection. Vaccines take advantage of this process by stimulating an immune response to antigens on pathogenic biomolecules to build up the immune system response against proteins or other biomolecules that carry the antigen.

## SUMMARY OF THE INVENTION

Tolerogenesis is a process of creating immunological tolerance to a substance. A patient, either human or non-human, that is treated to create tolerance of a substance will have a reduced adapative immune response to the substance. The reduction in an adaptive immune response can be measured by analyzing the amounts of circulating antibodies reactive to the substance, or by analyzing T-cell reactions to the substance and tolerizing agent. Compositions and methods for tolerization are provided herein. Many of the embodiments involve administering a fusion molecule that has a tolerizing antigen combined with an erythrocyte binding moiety. The fusion molecule binds to erythrocytes and begins a process of presenting the tolerizing antigen to the immune system in a manner that creates tolerance.

Peptides that specifically bind to erythrocytes (also known as red blood cells) have been discovered. These peptide ligands are erythrocyte-binding moieties that bind specifically to erythrocytes even in the presence of other factors present in blood. These ligands may be used in a variety of ways.

An embodiment of the invention is a pharmaceutically acceptable composition comprising a molecular fusion that comprises a tolerogenic antigen and an erythrocyte-binding moiety that specifically binds an erythrocyte in the patient, wherein the erythrocyte-binding moiety specifically binds to a biomolecule chosen from the group consisting of Band 3 (CD233), aquaporin-1, Glut-1, Kidd antigen, RhAg/Rh50 (CD241), Rh (CD240), Rh30CE (CD240CE), Rh30D (CD240D), Kx, glycophorin B (CD235b), glycophorin C (CD235c), glycophorin D (CD235d), Kell (CD238), Duffy/DARCi (CD234), CR1 (CD35), DAF (CD55), Globoside, CD44, ICAM-4 (CD242), Lu/B-CAM (CD239), XG1/XG2 (CD99), EMMPRIN/neurothelin (CD147), JMH, Glycosyltransferase, Cartwright, Dombrock, C4A/CAB, Scianna, MER2, stomatin, BA-1 (CD24), GPIV (CD36), CD108, CD139, and H antigen (CD173).

An embodiment of the invention is a pharmaceutically acceptable composition comprising: an erythrocyte-binding moiety joined to a domain that specifically binds a target, e.g., a protein that comprises a tolerogenic antigen. One or both of these domains may be a peptidic ligand or an antibody or antibody fragment.

Another embodiment is a pharmaceutically acceptable composition for use in antibody depletion or otherwise removing antibodies from circulation in a patient. The composition has an erythrocyte-binding moiety joined to an antigen, e.g., a native auto-antigen.or an antigen for a therapeutic protein.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the experimental scheme and results for the molecular fusion of ERY 1 and ovalbumin (OVA), wherein the ERY1-OVA fusion binds the equatorial periphery of mouse erythrocytes with high affinity; Panel (a) Schematic of conjugation of ERY1 peptide to ovalbumin (OVA), resulting in binding to erythrocyte-surface glycophorin-A; Panel (b) Binding of each OVA conjugate and intermediate, characterized by flow cytometry; black filled histogram, ERY1-OVA; empty histogram, SMCC-OVA; dotted histogram, MIS-OVA; ERY1 = erythrocyte-binding peptide WMVLPWLPGTLD (SEQ ID NO:1), MIS = mismatch peptide PLLTVGMDLWPW (SEQ ID NO:2), SMCC = sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, used to conjugate ERY1 to OVA; Panel (c) Equilibrium binding of ERY1-OVA to erythrocytes demonstrating the low dissociation constant of ERY1-OVA ($R^2 = 0.97$, one-site binding), determined by flow cytometry.

Figure 2 is a montage of results showing that erythrocyte-binding induces tolerance to antigen challenge: Panel (a) The OTI $CD8^+$ T cell adoptive transfer tolerance model, displaying experimental protocol for experimental as well as challenge and naive control groups (n = 5); Panel (b) Flow cytometric detection of OTI $CD8^+$ T cell populations ($CD3\varepsilon^+$ $CD8\alpha^+$ $CD45.2^+$); Panel (c) OTI $CD8^+$ T cell population quantification in the draining lymph nodes (inguinal and popliteal) 4 d following antigen challenge in $CD45.1^+$ mice (** $P < 0.01$); Panel (d) Flow cytometric detection of IFNγ-expressing OTI $CD8^+$ T cells; Panel (e) IFNγ-expressing OTI $CD8^+$ T cells in the draining lymph nodes 4 d following antigen challenge and restimulation with SIINFEKL peptide (SEQ ID NO:3) (** $P < 0.01$); Panel (f) IFNγ concentrations in lymph node cell culture media 4 d following restimulation with SIINFEKL peptide (SEQ ID NO:3), determined by ELISA (** $P < 0.01$); Panel (g) IL-10 concentrations in lymph node cell culture media 4 d following restimulation with OVA, determined by ELISA (* $P < 0.05$). Data represent median ± min to max; Panel (h) OVA-specific serum IgG titers at day 19, (* $P < 0.05$) data represent mean ± SE; Panel (i) The combination OTI and OVA-expressing EL4 thymoma (E.G7-OVA) tumor tolerance model, displaying experimental protocol for experimental as well as control groups (n = 4, 3, respectively); Panel (j) Quantification of non-proliferating (generation 0) OTI $CD8^+$ T cells circulating in blood 5 d following adoptive transfer; data represent median ± min to max (** $P < 0.01$); Panel (k) Growth profile of E.G7-OVA tumors, subcutaneously injected 9 d following OTI adoptive transfer, data represent mean ± SE (* $P < 0.05$).

Figure 3 is a bar graph showing how erythrocyte binding attenuates antigen-specific humoral responses in C57BL/6 mice. OVA-specific IgG detection in serum 19 days following two administrations of 1 μg OVA or 1 μg ERY1-OVA 6 d apart in C57BL/6 mice (* $P \leq 0.05$).

Figure 4 presents experimental results wherein 8-arm PEG-ERY1 binds erythrocytes in vitro and in vivo; Panel (a) 8-arm PEG-ERY1 (black filled histogram), but not 8-arm PEG-MIS (grey filled histogram) or 8-arm PEG-pyridyldisulfide bind to mouse erythrocytes following in vitro incubation; Panel (b) 8-arm PEG-ERY1 (black filled histogram), but not 8-arm PEG-MIS (grey filled histogram) bind to circulating erythrocytes upon intravenous injection.

Figure 5 presents experimental results depicting erythrocyte cell-surface half-life of 8-arm PEG-ERY1 (filled circles) and 8-arm PEG-MIS (empty boxes), determined by flow cytometry.

Figure 6 presents experimental results as a bar graph showing binding of peptidic

ligands to human erythrocytes.

Figure 7 presents experimental results as a flow cytometry histogram. Mouse erythrocytes were incubated with TER119-SIINFEKL (grey filled histogram) or albumin (empty histogram). Fluorescent signal comes from the anti-6xHis-PE antibody used in detection of the scFv.

Figure 8 is a plot of experimental results showing proliferating OT-I CD8 T cells in the spleen following intravenous administration of: saline, OVA, ERY1-OVA, or TER119-SIINFEKL. **P<0.01, ***P<0.001

Figure 9 is a plot of experimental results showing phenotypic characterization of OTI CD8 T cells following adoptive transfer and intravenous administration of: saline, OVA, ERY1-OVA, or TER119-SIINFEKL. Left panel: apoptotic OTI cell induction marked by Annexin-V binding in flow cytometry; Right panel: exhausted OTI cell induction marked by expression of PD-1 in flow cytometry.

Figure 10 is a plot of experimental results showing characterization of inflammatory (IFNγ+) OTI T cells in lymph nodes draining the site of challenge from mice tolerized with various injection formulations, as analyzed by flow cytometry.

Figure 11 is a plot of experimental results showing asparaginase (ASNase)-specific serum IgG titers from mice receiving 2 or 6 doses of ERY1-ASNase or wild-type ASNase, 21 days following administration, as determined by ELISA.

Figure 12 is a plot of experimental results showing proliferation of CD4 T cells from the transgenic NODBDC2.5 mouse in co-culture with splenic DCs, with various media additives.

Figure 13 is a plot of experimental results showing quantification of non-proliferating NODBDC2.5 CD4 T cells following adoptive transfer and intravenous administration of TER119-ChrA, free 1040-p31 peptide, or saline, as determined by flow cytometry.

## DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Molecular designs are provided for tolerogenesis. The protein or other molecular antigen to which tolerance is sought is formed as a conjugate with an erythrocyte binding moiety. The moiety may comprise a peptide ligand, an antibody, an aptamer, or an antibody fragment. The conjugate, also referred to as a molecular fusion, may be a fusion protein or may involve a linker, e.g., a polymer or polymer micelle or polymer nanoparticle conjugate.

Peptides that specifically bind erythrocytes are described herein. These are provided as peptidic ligands having sequences that specifically bind, or as antibodies or fragments

thereof that provide specific binding, to erythrocytes. The peptides may be prepared as molecular fusions with therapeutic agents, tolerizing antigens, or targeting peptides. The therapeutic agents may advantageously have an increased circulating half-life in vivo when they are part of the fusion. Immunotolerance may be created by use of the fusions and choice of an antigen on a substance for which tolerance is desired. The term antigen, in this context, means the full size antigen, an antigenic fragment thereof, or a mimetic of the tolerogenic antigen. Fusions with targeting peptides direct the fusions to the target, for instance a tumor, where the erythrocyte-binding ligands reduce or entirely eliminate blood flow to the tumor by recruiting erythrocytes to the target.

*Peptidic sequences that specifically bind erythrocytes*

Peptides that specifically bind erythrocytes have been discovered and reported by the Applicant in PCT/US2011/047078., included a peptide referred to as ERY1 that specifically binds to an erythrocyte. Six peptides (ERY19, ERY59, ERY64, ERY123, ERY141 and ERY162) that bind specifically to human erythrocytes were also reported. Example 9 details how peptidic ligands (ERY64, ERY123, and ERY141) were made part of a fusion protein with a fluorescent protein reporter and retained their specific binding properties. An embodiment of the invention is a substantially pure polypeptide comprising an amino acid sequence of ERY1, or one of the human erythrocyte binding peptides, or a conservative substitution thereof, or a nucleic acid encoding the same. Such polypeptides specifically bind erythrocytes and are a ligand for the same. Ligand is a term that refers to a chemical moiety that has specific binding to a target molecule. A target refers to a predetermined molecule, tissue, or location that the user intends to bind with the ligand. Thus targeted delivery to a tissue refers to delivering a molecule or other material such as a cell to the intended target tissue. Accordingly, embodiments include molecules or compositions comprising at least one of the ligands disclosed herein that are used to bind an erythrocyte. The binding activity of a polypeptide to an erythrocyte may be determined simply by following experimental protocols as described herein. Using such methods, the binding strengths of polypeptide variants relative to ERY1 or a human erythrocyte binding peptide under given physiological conditions can be determined, e.g., sequences made using conservative substitutions, addition or removal of flanking groups, or changes or additions for adjusting sequence solubility in aqueous solution. Peptidic ligands may be generated specifically for one or more of the following targets or group of targets: an erythrocyte surface molecule (protein, glycoprotein), Band 3 (CD233), a glycophorin, more particularly glycophorin A (CD235a), glycophorin B

(CD235b), glycophorin C (CD235c) & glycophorin D (CD235d), aquaporin-1, Glut-1, Kidd antigen, RhAg/Rh50 (CD241), Rh (CD240), Rh30CE (CD240CE), Rh30D (CD240D), Kx, Kell (CD238), Duffy/DARC (CD234), CR1 (CD35), DAF (CD55), Globoside, CD44, ICAM-4 (CD242), Lu/B-CAM (CD239), XG1/XG2 (CD99), EMMPRIN/neurothelin (CD147), JMH, Glycosyltransferase, Cartwright, Dombrock, C4A/CAB, Scianna, MER2, stomatin, BA-1 (CD24), GPIV (CD36), CD108, CD139, and H antigen (CD173). Erythrocytes cell-surface proteins, either purified preparations or a mixture of them, can be screened for peptidic ligands. Erythrocyte cell-surface proteins may be recombinantly expressed in their full form or as partial domains fused to tags that enhance expression or stability of the protein. The recombinant protein target may then be immobilized on a plate or bead, and used as the affinity target for the library screening process. Erythrocyte cell-surface proteins may also be isolated from cellular preparations of whole blood, from which purified or complex mixtures of membrane proteins may be immobilized on a solid matrix (bead, plate, or other), and used as affinity targets for the screening process. The entire screening process may be performed in the presence of a high concentration of serum albumin (e.g., 50 mg/mL) and at 37°C to reduce non-specific binding events and to select for peptides with favorable binding characteristics in blood serum. Peptidic ligands are most preferably selected from Band 3 (CD233), glycophorin B (CD235b), glycophorin C (CD235c) and glycophorin D (CD235d).

Peptidic ligands were observed to bind the erythrocyte cell surfaces without altering cell morphology and without cytoplasmic translocation. The ligands distributed across the cell surface and were free of clustering. Glycophorin-A (GYPA) was a specific protein indentified as the target of ERY-1. ERY-1 was reactive only with mouse and rat species. Peptidic ligands that specifically bound human erythrocytes were determined to be specific for human erythrocytes and not other speciesA phage clone displaying a high-affinity peptide, WMVLPWLPGTLD (SEQ ID NO:1 herein termed ERY1) towards the mouse erythrocyte cell surface was reported . Other experiments identified binding ligands for human erythrocytes as shown in Tables 1-2. Six sequences bound specifically to human erythrocytes. A seventh sequence, named ERY50, bound human erythrocytes and also bound epithelial/endothelial cells.

Table 1: Peptidic ligands that bind human erythrocytes

| Peptide Name | Human Erythrocyte Binding Peptide Sequence | Sequence Identifier |
|---|---|---|
| ERY19 | GQSGQPNSRWIYMTPLSPGIYRGSSGGS | SEQ ID NO:4 |
| ERY50 | GQSGQSWSRAILPLFKIQPVGSSGGS | SEQ ID NO:5 |
| ERY59 | GQSGQYICTSAGFGEYCFIDGSSGGS | SEQ ID NO:6 |
| ERY64 | GQSGQTYFCTPTLLGQYCSVGSSGGS | SEQ ID NO:7 |
| ERY123 | GQSGHWHCQGPFANWVGSSGGS | SEQ ID NO:8 |
| ERY141 | GQSGQFCTVIYNTYTCVPSSGSSGGS | SEQ ID NO:9 |
| ERY162 | GQSGQSVWYSSRGNPLRCTGGSSGGS | SEQ ID NO:10 |

Underlined sequence portions indicate linker sequences

Table 2: Peptidic ligands that bind mouse or human erythrocytes

| Peptide | | Sequence Identifier |
|---|---|---|
| ERY19' | PNSRWIYMTPLSPGIYR | SEQ ID NO:11 |
| ERY50'* | SWSRAILPLFKIQPV | SEQ ID NO:12 |
| ERY59' | YICTSAGFGEYCFID | SEQ ID NO:13 |
| ERY64' | TYFCTPTLLGQYCSV | SEQ ID NO:14 |
| ERY123' | HWHCQGPFANWV | SEQ ID NO:15 |
| ERY141' | FCTVIYNTYTCVPSS | SEQ ID NO:16 |
| ERY162' | SVWYSSRGNPLRCTG | SEQ ID NO:17 |
| ERY1** | WMVLPWLPGTLD | SEQ ID NO:1 |

*not specific for erythrocytes
**for mouse

Embodiments of the invention include peptides that that specifically bind the surface of erythrocytes. The sequences were not optimized for minimum length. Such optimization is within the skill of the art and may be practiced using techniques described herein. For example, Kenrick et al. (Protein Eng. Des. Sel. (2010) 23(1):9-17) screened from a 15 residue library, and then identified minimal binding sequences 7 residues in length. Getz (ACS Chem. Biol., May 26, 2011 identified minimal binding domains as small as 5 residues in length. The erythrocyte binding peptides may be present in repeats of the same sequences, e.g., between 2 and 20 repeats; artisans will immediately appreciate that all the ranges and values within the explicitly stated ranges are contemplated. Moreover, the peptides may be present in combination, with two or more distinct sequences being in the same peptide or being part of a single molecular fusion.

The number of consecutive residues that provide specific binding is expected to be between about 4 and 12 residues. Accordingly, all peptides of four consecutive residues in length found in Table 2 are disclosed, as well as all peptides of, e.g., 5, 6, 7, or 8 consecutive

residues. This number is based on the number of residues for other peptidic protein-binding ligands. Embodiments of the invention include minimum length sequences for one of the erythrocyte-binding SEQ IDs set for the herein, including Table 1. Accordingly, certain embodiments are directed to a composition comprising a peptide, or an isolated (or purified) peptide, comprising a number of consecutive amino acid sequences between 4 and 12 consecutive amino acid residues of a sequence chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:1, and conservative substitutions thereof, wherein said sequence specifically binds an erythrocyte. Alternatively the number of consecutive residues may be chosen to be between about 5 and about 18; artisans will immediately appreciate that all the ranges and values within the explicitly stated ranges are contemplated, e.g., 7, 8, 9, 10, or from 8 to 18. The erythrocyte-binding sequence may have, e.g., a conservative substitution of at least one and no more than two amino acids of the sequences, or 1, 2, or 3 substitutions, or between 1 and 5 substitutions. Moreover, the substitution of L-amino acids in the discovered sequence with D-amino acids can be frequently accomplished, as in Giordano. The peptide or composition may, in some embodiments, consist essentially of a sequence chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:1. The peptide may be limited in length, e.g., having a number of residues between about 10 and about 100; artisans will immediately appreciate that all the ranges and values within the explicitly stated ranges are contemplated, e.g., about 10 to about 50 or about 15 to about 80. A peptide erythrocyte-binding moiety may be provided that comprises a peptide ligand that has a dissociation constant of between about 10 μM and 0.1 nM as determined by equilibrium binding measurements between the peptide and erythrocytes; artisans will immediately appreciate that all the ranges and values within the explicitly stated ranges are contemplated, e.g., from about 1 μM to about 1nM. The peptide may further comprise a therapeutic agent. The therapeutic agent may be, e.g., a protein, a biologic, an antibody fragment, an ScFv, or a peptide. The peptide may further comprise a tolerogenic antigen, e.g., a human protein used in a human deficient in that protein (e.g., blood factors such as factor VIII or factor IX), proteins with nonhuman glycosylation, synthetic proteins not naturally found in humans, human food allergens, or human autoimmune antigens.

Polypeptides of various lengths may be used as appropriate for the particular application. In general, polypeptides that contain the polypeptide ligand sequences will exhibit specific binding if the polypeptide is available for interaction with erythrocytes *in*

*vivo*. Peptides that have the potential to fold can be tested using methods described herein. Accordingly, certain embodiments are directed to polypeptides that have a polypeptide ligand but do not occur in nature, and certain other embodiments are directed to polypeptides having particular lengths, e.g., from 6 to 3000 residues, or 12-1000, or 12-100, or 10-50; artisans will immediately appreciate that every value and range within the explicitly articulated limits is contemplated.

Certain embodiments provide various polypeptide sequences and/or purified or isolated polypeptides. A polypeptide is a term that refers to a chain of amino acid residues, regardless of post-translational modification (e.g., phosphorylation or glycosylation) and/or complexation with additional polypeptides, synthesis into multisubunit complexes, with nucleic acids and/or carbohydrates, or other molecules. Proteoglycans therefore also are referred to herein as polypeptides. As used herein, a "functional polypeptide" is a polypeptide that is capable of promoting the indicated function. Polypeptides can be produced by a number of methods, many of which are well known in the art. For example, polypeptides can be obtained by extraction (e.g., from isolated cells), by expression of a recombinant nucleic acid encoding the polypeptide, or by chemical synthesis. Polypeptides can be produced by, for example, recombinant technology, and expression vectors encoding the polypeptide introduced into host cells (e.g., by transformation or transfection) for expression of the encoded polypeptide.

There are a variety of conservative changes that can generally be made to an amino acid sequence without altering activity. These changes are termed conservative substitutions or mutations; that is, an amino acid belonging to a grouping of amino acids having a particular size or characteristic can be substituted for another amino acid. Substitutes for an amino acid sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, methionine, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations are not expected to substantially affect apparent molecular weight as determined by polyacrylamide gel electrophoresis or isoelectric point. Conservative substitutions also include substituting optical isomers of the sequences for other optical isomers, specifically D amino acids for L amino acids for one or more residues of a sequence. Moreover, all of the amino acids in a sequence may undergo a D to L isomer substitution. Exemplary conservative

substitutions include, but are not limited to, Lys for Arg and vice versa to maintain a positive charge; Glu for Asp and vice versa to maintain a negative charge; Ser for Thr so that a free --OH is maintained; and Gln for Asn to maintain a free $NH_2$. Moreover, point mutations, deletions, and insertions of the polypeptide sequences or corresponding nucleic acid sequences may in some cases be made without a loss of function of the polypeptide or nucleic acid fragment. Substitutions may include, e.g., 1, 2, 3, or more residues. The amino acid residues described herein employ either the single letter amino acid designator or the three-letter abbreviation. Abbreviations used herein are in keeping with the standard polypeptide nomenclature, J. Biol. Chem., (1969), 243, 3552-3559. All amino acid residue sequences are represented herein by formulae with left and right orientation in the conventional direction of amino-terminus to carboxy-terminus.

In some cases a determination of the percent identity of a peptide to a sequence set forth herein may be required. In such cases, the percent identity is measured in terms of the number of residues of the peptide, or a portion of the peptide. A polypeptide of, e.g., 90% identity, may also be a portion of a larger peptide

The term purified as used herein with reference to a polypeptide refers to a polypeptide that has been chemically synthesized and is thus substantially uncontaminated by other polypeptides, or has been separated or purified from other most cellular components by which it is naturally accompanied (e.g., other cellular proteins, polynucleotides, or cellular components). An example of a purified polypeptide is one that is at least 70%, by dry weight, free from the proteins and naturally occurring organic molecules with which it naturally associates. A preparation of a purified polypeptide therefore can be, for example, at least 80%, at least 90%, or at least 99%, by dry weight, the polypeptide. Polypeptides also can be engineered to contain a tag sequence (e.g., a polyhistidine tag, a myc tag, or a FLAG® tag) that facilitates the polypeptide to be purified or marked (e.g., captured onto an affinity matrix, visualized under a microscope). Thus a purified composition that comprises a polypeptide refers to a purified polypeptide unless otherwise indicated. The term isolated indicates that the polypeptides or nucleic acids of the invention are not in their natural environment. Isolated products of the invention may thus be contained in a culture supernatant, partially enriched, produced from heterologous sources, cloned in a vector or formulated with a vehicle, etc.

Polypeptides may include a chemical modification; a term that, in this context, refers to a change in the naturally-occurring chemical structure of amino acids. Such modifications may be made to a side chain or a terminus, e.g., changing the amino-terminus or carboxyl

terminus. In some embodiments, the modifications are useful for creating chemical groups that may conveniently be used to link the polypeptides to other materials, or to attach a therapeutic agent.

Specific binding, as that term is commonly used in the biological arts, refers to a molecule that binds to a target with a relatively high affinity compared to non-target tissues, and generally involves a plurality of non-covalent interactions, such as electrostatic interactions, van der Waals interactions, hydrogen bonding, and the like. Specific binding interactions characterize antibody-antigen binding, enzyme-substrate binding, and specifically binding protein-receptor interactions; while such molecules may bind tissues besides their targets from time to time, such binding is said to lack specificity and is not specific binding. The peptide ERY1 and its derivatives and the human erythrocyte binding peptides and their derivatives may bind non-erythrocytes in some circumstances but such binding has been observed to be non-specific, as evidenced by the much greater binding of the peptides to the erythrocytes as opposed to other cells or proteins.

Thus, embodiments include a ligand that binds with specificity to an erythrocyte and does not specifically bind other blood components, e.g., one or more of: blood proteins, albumin, fibronectin, platelets, white blood cells, substantially all components found in a blood sample taken from a typical human. In the context of a blood sample, the term "substantially all" refers to components that are typically present but excludes incidental components in very low concentrations so that they do not effectively reduce the titer of otherwise bioavailable ligands.

*Antibody peptides*

In addition to peptides that bind erythrocytes, proteins are also presented herein, specifically antibodies and especially single chain antibody fragments. Techniques for raising an antibody against an antigen are well known. The term antigen, in this context, refers to a site recognized by a host immune system that responds to the antigen. Antigen selection is known in the arts of raising antibodies, among other arts. Embodiments include use of these peptides in a molecular fusion and other methods presented herein. Antigens may be recombinantly expressed in their full form or as partial domains fused to tags that enhance expression or stability of the protein. The recombinant protein antigen may then be immobilized on a plate or bead, and used as the affinity target for the library screening process. Antigens may also be isolated from cellular preparations of whole blood, from which purified or complex mixtures of membrane proteins may be immobilized on a solid

matrix (bead, plate, or other), and used as affinity targets for the screening process.

Artisans reading this disclosure will be able to create antibodies that specifically bind erythrocytes. Examples 4-6 relate to making antibodies or fragments thereof. Numerous parallel techniques are used to discover new antibodies or antibody fragments or to make compositions comprising such fragments active towards erythrocytes, including but not limited to phage display, yeast display, and bacterial display. Additional erythrocyte binding antibodies or antibody fragments, or scFvs may be generated specifically for one or more of the following targets or group of targets (collectively referred to herein as the Erythrocyte Target Group) chosen from the group consisting of Band 3 (CD233), aquaporin-1, Glut-1, Kidd antigen, RhAg/Rh50 (CD241), Rh (CD240), Rh30CE (CD240CE), Rh30D (CD240D), Kx, glycophorin A (CD235a), glycophorin B (CD235b), glycophorin C (CD235c), glycophorin D (CD235d), Kell (CD238), Duffy/DARC (CD234), CR1 (CD35), DAF (CD55), Globoside, CD44, ICAM-4 (CD242), Lu/B-CAM (CD239), XG1/XG2 (CD99), EMMPRIN/neurothelin (CD147), JMH, Glycosyltransferase, Cartwright, Dombrock, C4A/CAB, Scianna, MER2, stomatin, BA-1 (CD24), GPIV (CD36), CD108, CD139, and H antigen (CD173).

Additionally, new erythrocyte-specific antibodies may be generated by immunization of mice with intact, or protein preparations of, human erythrocytes. Following immunization with the target formulated in adjuvant, mice are sacrificed at pre-determined time points and their antibody repertoires are sequenced and/or their B cells isolated for hybridoma-based screening. These antibodies or antibody fragments may be optimized by several aforementioned *in vitro* techniques, for improved binding and/or stability parameters. Additional erythrocyte binding antibodies or antibody fragments may be generated by using antibody hybridoma clones previously discovered to bind erythrocytes. They may be generated, for instance, by using the method used to generate the TER119 scFv. As a template, or as an antibody source, the following hybridoma clones may be used, among others: BRIC 4, 5, 6, 10, 14, 18, 39, 66, 68, 69, 87, 108, 110, 111, 125, 126, 128, 145, 155, 157, 163, 170, 198, 203, 216, 220, 221, 222, 229, 230, 231, 235, or 256; BRAC 17, 18; BGRL 1, 2, 11, 100; BRAD 3; BIRMA D6, D10, K3, 84B; 6A7; COE; or KZ1.

The term peptide is used interchangeably with the term polypeptide herein. Antibodies and antibody fragments are peptides. The term antibody fragment refers to a portion of an antibody that retains the antigen-binding function of the antibody. The fragment may literally be made from a portion of a larger antibody or alternatively may be synthesized *de novo*. Antibody fragments include, for example, a single chain variable

fragment (scFv) An scFv is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulin, connected with a linker peptide, e.g., about 10 to about 50 amino acids. The linker can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. The term scFv includes divalent scFvs, diabodies, triabodies, tetrabodies and other combinations of antibody fragments. Antibodies have an antigen-binding portion referred to as the paratope. The term peptide ligand refers to a peptide that is not part of a paratope.

The binding of erythrocytes, by antibodies and fragments thereof, by peptidic binding ligands, and by aptamers, may be performed without covalent bonding to the erythrocytes. The binding may be performed *in vivo* and without an *ex vivo* erythrocyte binding event. This binding event, including the resultant association of an antigen with the erythrocyte, can be performed without causing the erythrocytes to be apoptotic. The process may be performed so that the surface molecules of erythrocytes are not crosslinked to each other by a crosslinking agent and/or the binding event, and are not crosslinked by covalent bonds.

*Bi-specific protein constructs to bind antigens to erythrocytes*

When creating protein constructs that bind to erythrocytes, a main design option is to create a single protein or molecular fusion that contains both the antigen of interest and the erythrocyte-binding moiety.

An alternative design that may be more efficient in production and use is a bi-specific protein or molecular construct that contains one domain that binds to erythrocytes fused to an additional domain that binds directly or indirectly to the antigen of interest. The actual antigen of interest is not included in this molecular fusion, only a moiety that binds directly or indirectly to the antigen of interest. As such, there is no requirement to modify or engineer the antigen of interest. One example of such a design is a bi-specific antibody or antibody fragment, with one antibody domain specific for erythrocytes recombinantly fused to a second antibody domain that specifically binds a biomolecule having the antigen of interest. Multiple different bi-specific antibody constructs are implemented, including but not limited to tandem scFv's, diabodies, and tandem scFv-IgG molecules (62). Other non-proteinaceous scaffolds are also used, including polymeric nanoparticles or other multivalent polymer conjugates, which are engineered for dual specificity by harboring a domain that binds to the antigen of interest, and another domain that binds to erythrocytes.

The affinity moieties of the bi-specific construct may be any combination of peptide domains and/or antibody fragment domains. Peptides that bind to erythrocytes or the target

protein antigen are obtained with conventional peptide library screening techniques, including, e.g., bead or multiplexed peptide library screening, phage display, bacterial display, and yeast display. The engineered peptide is characterized for specificity and affinity to its binding target using standard biochemical assays, e.g., ELISA, surface plasmon resonance, and flow cytometric-based methods. Antibodies or antibody fragments that bind to erythrocytes or the target protein antigen are obtained using conventional techniques, e.g., hybridoma-based selection methods, phage display, yeast display, bacterial display, ribosomal display, or direct antibody gene or proteomics-based sequencing methods of immunized animals. The engineered antibody or antibody fragment is characterized for specificity and affinity to its binding target using standard biochemical assays, e.g., ELISA, surface plasmon resonance, and flow cytometric-based methods.

Following discovery and characterization of each affinity moiety (peptide, antibody, and/or antibody domain), they are fused to form a bi-specific molecular construct using standard recombinant DNA techniques such as assembly PCR or direct gene synthesis, then the fusion protein is expressed in a suitable host. Bi-specific constructs are also created by chemically conjugating two individual moieties together in any combination deemed suitable for bi-specific binding (peptide-peptide, peptide-antibody fragment, etc). Two antibody domains are used to create bi-specific antibodies using various state-of-the-art architectures (1). Additionally, a synthetic polymeric scaffold (PEG, PPG, or other) is used to create stable linkages between each binding moiety and improve flexibility of the construct. Branched polymers are also used to increase the affinity of binding to the erythrocyte, to the protein antigen, or both, by avidity effects.

Characterization of binding efficacy of the bi-specific construct is performed using standard *in vitro* biochemical binding affinity assays, such as ELISA and surface plasmon resonance. *In vivo* performance of the bi-specific construct is performed by injecting the bi-specific construct or the bispecific construct and the antigen into an animal model such as mice or rats, then sampling blood and testing for both erythrocyte and antigen binding using similar *in vitro* assays.

Embodiments include a pharmaceutically acceptable composition comprising: an erythrocyte-binding moiety joined to a domain that specifically binds a target. The target may comprise a protein, with the protein further comprising a tolerogenic antigen. The erythrocyte-binding moiety may comprises a peptide ligand, an antibody, an antibody fragment, a single chain antigen binding domain (ScFv) or an aptamer. The domain may be chosen from the group consisting of an antibody, an antibody fragment, and a single chain

antigen binding domain (ScFv), a peptide ligand, and an apatamer. An embodiment is a composition that comprises a member of the group chosen from a molecular fusion, a tandem scFv, a diabody, and a tandem scFv-IgG molecule, with the erythrocyte-binding moiety and the domain being part of said member. A single fusion protein or nucleic acid encoding the same may comprise the erythrocyte-binding domain and the other domain that binds the target.

*Therapeutic depletion of antigen-specific antibodies using erythrocyte-binding antigens*

The induction of antigen-specific antibodies is detrimental to treatment and can cause dangerous side-effects during protein replacement therapies, in the pathogenesis of autoimmunity, and during biologics treatment in general (63-68). Therapeutic interventions capable of depleting circulating antigen-specific antibodies in the blood would have positive clinical impact, in that depletion of such drug-specific antibodies would enable future treatment with the drug while diminishing the risk of adverse immunological side-effects due to drug-antibody complexes. In the example case of hemophilia-A, approximately 30% of patients induce anti-drug antibodies towards the standard therapeutic protein drug Factor VIII, thus making further treatment with Factor VIII dangerous and inefficacious. A therapeutic intervention depleting Factor VIII-specific circulating antibodies in such a patient re-enables the therapeutic Factor VIII treatment regimen, and obviates the need to switch the patient to other more expensive and less effective treatment alternatives. In the example case of systemic lupus erythematosis (SLE, lupus), an autoimmune disease mediated mainly by the induction of antibodies towards native auto-antigens in the body, a therapeutic treatment capable of depleting auto-antigen-specific antibodies circulating in the blood would greatly decrease the pathogenesis and progression of disease onset.

In order to deplete antigen-specific antibodies, antigens are engineered to bind to circulating erythrocytes following administration. Molecular constructs of an erythrocyte-biding moiety fused to the antigen of interest are created to form the erythrocyte-binding antigen. Alternatively, a bi-specific construct may be implemented to create an erythrocyte-binding formulation of the antigen, as described above. In this manner, upon injection of the erythrocyte-binding antigen, any circulating antibody specific for the antigen will bind to the engineered antigen, and thus be closely associated with a circulating erythrocyte *in situ*. During the close physical association of an antigen-specific antibody to a naturally circulating cell such as the erythrocyte, dangerous inflammatory immune effects are inhibited by regulatory signals present on the erythrocyte. Erythrocytes express several regulatory

molecules on their surface, including CD55 and CD59, among others (67). Thus, antigen-specific antibodies remain bound to erythrocytes and traffic to zones of natural clearance of erythrocytes, during which they are cleared as well. The antigen-decorated erythrocyte serves as a cellular safe-deposit and a depletion carrier for the antigen-specific antibody.

Depletion of antigen-specific antibodies is characterized *in vivo* following administration of the engineered antigen construct, by periodic sampling of blood from test animals and performing antigen-specific antibody titration techniques, such as ELISAs. Specific deletion or inactivation of the antibody-secreting immune cell (B cell, plasma cell, or other) is also characterized by isolation of immune cells from the blood, bone marrow, spleen, or lymphatic system of recipient mice and performing antigen-specific in vitro restimulations B-cell ELISpots assays, and multi-parameter flow cytometry. In autoimmunity-related studies, relevant mouse models of autoimmune disorders are used to characterize the effects of erythrocyte-binding antigens in auto-antigen depletion.

Uses of the compositions include removing antibodies from circulation and/or binding them to red blood cells. An embodiment is a pharmaceutically acceptable composition comprising: an erythrocyte-binding moiety joined to an antigen. The antigen may be a native auto-antigen, e.g., a lupus erythematosis antigen. The antigen may alternatively be an antigen for a therapeutic protein that is administered to a patient, e.g., a Factor VIII antigen.

*Aptamers for specific binding of erythrocytes*

In addition to peptide ligands that bind erythrocytes, nucleotide aptamer ligands for erythrocyte surface components are taught. Accordingly, aptamers are to be made and used as described herein for other erythrocyte-binging moieties. DNA and RNA aptamers may be used to provide non-covalent erythrocyte binding. As they are only composed of nucleotides, aptamers are promising biomolecular targeting moieties in that screening methodologies are well established, they are readily chemically synthesized, and pose limited side-effect toxicity and/or immunogenicity due to their rapid clearance *in vivo* (Keefe, Pai, et al., 2010). Furthermore, due to the non-canonical nature of the nucleotide-target protein interaction, any productive agonist signaling upon target binding *in vivo* is unlikely, thus contributing low immunogenicity and toxicity. As such, numerous aptamer-based molecules are currently in human clinical trials for a number of clinical indications, including leukemia, macular degeneration, thrombosis, and type 2 diabetes (Keefe, Pai, et al., 2010). Aptamers have also been used as targeting agents to deliver drug payloads to specific tissues *in vivo*, in applications such as cancer chemotherapy and fluorescence or radiological tumor detection

techniques (Rockey, Huang, et al., 2011; Savla, Taratula, et al., 2011).

Aptamers are oligonucleic acids or peptides that bind to a specific target molecule. Aptamers are usually created to bind a target of interest by selecting them from a large random sequence pool. Aptamers can be classified as DNA aptamers, RNA aptamers, or peptide aptamers. Nucleic acid aptamers are nucleic acid species that have been engineered through repeated rounds of in vitro selection or Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method (Archemix, Cambridge, MA, USA) (Sampson, 2003) to specifically bind to targets such as small molecules, proteins, nucleic acids, cells, tissues and organisms. Peptide aptamers typically have a short variable peptide domain, attached at both ends to a protein scaffold. Peptide aptamers are proteins that are designed to interfere with other protein interactions inside cells. They consist of a variable peptide loop attached at both ends to a protein scaffold. This double structural constraint greatly increases the binding affinity of the peptide aptamer to be comparable to an antibody. The variable loop length is typically composed of about ten to about twenty amino acids, and the scaffold is a protein which has good solubility and is compact. For example the bacterial protein Thioredoxin-A is a scaffold protein, with the variable loop being inserted within the reducing active site, which is a -Cys-Gly-Pro-Cys- loop in the wild protein, the two Cysteines lateral chains being able to form a disulfide bridge.

Some techniques for making aptamers are detailed in Lu et al., Chem Rev 2009:109(5):1948-1998, and also in US 7,892,734, US 7,811,809, US 2010/0129820, US 2009/0149656, US 2006/0127929, and US 2007/0111222. Example 8 further details materials and methods for making and using aptamers for use with the embodiments disclosed herein.

*Molecular fusion*

A molecular fusion may be formed between a first peptidic erythrocyte binding ligand and a second peptide. The fusion comprises the peptides conjugated directly or indirectly to each other. The peptides may be directly conjugated to each other or indirectly through a linker. The linker may be a peptide, a polymer, an aptamer, a nucleic acid, or a particle. The particle may be, e.g., a microparticle, a nanoparticle, a polymersome, a liposome, or a micelle. The polymer may be, e.g., natural, synthetic, linear, or branched. A fusion protein that comprises the first peptide and the second peptide is an example of a molecular fusion of the peptides, with the fusion protein comprising the peptides directly joined to each other or with intervening linker sequences and/or further sequences at one or both ends. The

conjugation to the linker may be through covalent bonds. Other bonds include ionic bonds. Methods include preparing a molecular fusion or a composition comprising the molecular fusion, wherein the molecular fusion comprises peptides that specifically bind to erythrocytes and a therapeutic agent, tolerizing antigen, or other substance.

The term molecular fusion, or the term conjugated, refers to direct or indirect association by chemical bonds, including covalent, electrostatic ionic, charge-charge. The conjugation creates a unit that is sustained by chemical bonding. Direct conjugation refers to chemical bonding to the agent, with or without intermediate linkers or chemical groups. Indirect conjugation refers to chemical linkage to a carrier. The carrier may largely encapsulate the agent, e.g., a polymersome, a liposome or micelle or some types of nanoparticles, or have the agent on its surface, e.g., a metallic nanoparticle or bead, or both, e.g., a particle that includes some of the agent in its interior as well as on its exterior. The carrier may also encapsulate an antigen for immunotolerance. For instance a polymersome, liposome, or a particle may be made that encapsulates the antigen. The term encapsulate means to cover entirely, effectively without any portion being exposed, for instance, a polymersome may be made that encapsulates an antigen or an agent. Examples of therapeutic agents are single-chain variable fragments (scFv), antibody fragments, small molecule drugs, bioactive peptides, bioactive proteins, and bioactive biomolecules.

Conjugation may be accomplished by covalent bonding of the peptide to another molecule, with or without use of a linker. The formation of such conjugates is within the skill of artisans and various techniques are known for accomplishing the conjugation, with the choice of the particular technique being guided by the materials to be conjugated. The addition of amino acids to the polypeptide (C- or N-terminal) which contain ionizable side chains, i.e. aspartic acid, glutamic acid, lysine, arginine, cysteine, histidine, or tyrosine, and are not contained in the active portion of the polypeptide sequence, serve in their unprotonated state as a potent nucleophile to engage in various bioconjugation reactions with reactive groups attached to polymers, i.e. homo- or hetero-bi-functional PEG (e.g., Lutolf and Hubbell, *Biomacromolecules* 2003;4:713-22, Hermanson, *Bioconjugate Techniques*, London. Academic Press Ltd; 1996). In some embodiments, a soluble polymer linker is used, and may be adminsited to a patient in a pharmaceutically acceptable form. Or a drug may be encapsulated in polymerosomes or vesicles or covalently attached to the peptide ligand.

An embodiment is a conjugation of a non-protein therapeutic agent and a peptide ligand, antibody, antibody fragment, or aptamer that binds specifically to an erythrocyte. Application of the erythrocyte binding peptide methodology is not restricted to polypeptide

therapeutics; rather it may be translated into other drug formulations, such as small molecules and polymeric particles. In the long history of small molecules and their application in medicine, short circulation half-lives and poor bioavailability have consistently plagued their efficacy *in vivo*. Polymeric micelles and nanoparticles represent a relatively newer generation of drug class, yet their pharmacokinetic behavior remains sub-optimal for reasons that include a high clearance rate via the action of the reticuloendothelial system (Moghimi and Szebeni, 2003). The erythrocyte-binding design can be extended to these other drug classes to increase their circulation half-lives and clinical efficacy.

The conjugate may comprise a particle. The erythrocyte binding peptide may be attached to the particle. An antigen, agent, or other substance may be in or on the particle. Examples of nanoparticles, micelles, and other particles are found at, e.g., US 2008/0031899, US 2010/0055189, US 2010/0003338, which applications are hereby incorporated by reference herein for all purposes, including combining the same with a ligand as set forth herein; in the case of conflict, however, the instant specification controls.

Nanoparticles may be prepared as collections of particles having an average diameter of between about 10 nm and about 200 nm, including all ranges and values between the explicitly articulated bounds, e.g., from about 20 to about 200, and from about 20 to about 40, to about 70, or to about 100 nm, depending on the polydispersity which is yielded by the preparative method. Various nanoparticle systems can be utilized, such as those formed from copolymers of poly(ethylene glycol) and poly(lactic acid), those formed from copolymers of poly(ethylene oxide) and poly(beta-amino ester), and those formed from proteins such as serum albumin. Other nanoparticle systems are known to those skilled in these arts. See also Devalapally et al., *Cancer Chemother Pharmacol.*, 07-25-06; Langer et al., *International Journal of Pharmaceutics*, 257:169-180 (2003); and Tobío et al., *Pharmaceutical Research*, 15(2):270-275 (1998).

Larger particles of more than about 200 nm average diameter incorporating the cartilage tissue-binding ligands may also be prepared, with these particles being termed microparticles herein since they begin to approach the micron scale and fall approximately within the limit of optical resolution. For instance, certain techniques for making microparticles are set forth in U.S. Patent Nos. 5,227,165, 6,022,564, 6,090,925, and 6,224,794.

Functionalization of nanoparticles to employ targeting capability requires association of the targeting polypeptide with the particle, e.g., by covalent binding using a bioconjugation technique, with choice of a particular technique being guided by the particle or nanoparticle,

or other construct, that the polypeptide is to be joined to. In general, many bioconjugation techniques for attaching peptides to other materials are well known and the most suitable technique may be chosen for a particular material. For instance, additional amino acids may be attached to the polypeptide sequences, such as a cysteine in the case of attaching the polypeptide to thiol-reactive molecules.

To create a multimeric molecule capable of displaying multiple different bioactive molecules, a commercially available 8-arm PEG dendrimer was chemically modified to include reactive groups for facile conjugation reactions. The 8-arm PEG-pyridyldisulfide contained the pyridyldisulfide group that reacts readily with thiolates from small molecules and/or cysteine-containing peptides or proteins, resulting in a disulfide-bond between the attached bioactive moiety and the 8-arm PEG scaffold. The multimeric architecture of the 8-arm PEG allowed the conjugation of different peptides or molecules to the scaffold, thus creating a hetero-functionalized biomolecule with multiple activities by virtue of its attached moieties. Heterofunctionalized fluorescent 8-arm PEG constructs, capable of binding erythrocytes *in vitro* (Fig. 4A) and *in vivo* (Fig. 4B) were created. This binding was sequence specific to the ERY1 peptide, as conjugates harboring the non-specific MIS peptide demonstrated little to no binding to erythrocytes. The binding *in vivo* was long-lived, as fluorescent 8-arm PEG-ERY1-ALEXAFLUOR647 was detected on circulating erythrocytes 5 h following intravenous administration, and displayed a cell-surface half-life of 2.2 h (Fig. 5). To demonstrate the induction of tolerance in an autoimmune diabetic mouse model, an 8-arm PEG conjugated with both ERY1 and the diabetes antigen chromogranin-A (CrA) was created. The modular nature of the 8-arm PEG-pyridyldisulfide scaffold made it possible to co-conjugate different of thiol-containing molecules by sequentially adding stoichiometrically defined quantities of the molecules.

The molecular fusion may comprise a polymer. The polymer may be branched or linear. The molecular fusion may comprise a dendrimer. In general, soluble hydrophilic biocompatbile polymers may be used so that the conjugate is soluble and is bioavailable after introduction into the patient. Examples of soluble polymers are polyvinyl alcohols, polyethylyene imines, and polyethylene glycols (a term including polyethylene oxides) having a molecular weight of at least 100, 400, or between 100 and 400,000 (with all ranges and values between these explicit values being contemplated). Solubility in this context refers to a solubility in water or physiological saline of at least 1 gram per liter. Domains of biodegradable polymers may also be used, e.g., polylactic acid, polyglycolic acid, copolymers of polylactic and polyglycolic acid, polycaprolactones, polyhydroxy butyric acid,

polyorthoesters, polyacetals, polydihydropyrans, and polycyanoacylates.

In some embodiments, a polypeptide-polymer association, e.g., a conjugate, is prepared and introduced into the body as a purified composition in a pharmaceutically acceptable condition, or with a pharmaceutical excipient. The site of introduction may be, e.g., systemic, or at a tissue or a transplantation site.

Artisans may prepare fusion proteins using techniques known in these arts. Embodiments include preparing fusion proteins, isolating them, and administering them in a pharmaceutically acceptable form with or without other agents, e.g., in combination with an interleukin of TGF-beta. Embodiments include a vector for, and methods of, transfecting a cell to thereby engineer the cell to make the fusion protein in vivo, with the cell being transfected in vitro, ex vivo, or in vivo, and with the cell being a member of a tissue implant or distinct therefrom. The following U.S. patent applications are hereby incorporated by reference herein for all purposes, including the purposes of making fusion proteins, with the instant specification controlling in case of conflict: 5227293, 5358857, 5885808, 5948639, 5994104, 6512103, 6562347, 6905688, 7175988, 7704943, US 2002/0004037, US 2005/0053579, US 2005/0203022, US 2005/0250936, US 2009/0324538.

Embodiments of a molecular fusion include, for example, a molecular fusion that comprises a tolerogenic antigen and an erythrocyte-binding moiety that specifically binds an erythrocyte in the patient and thereby links the antigen to the erythrocyte, wherein the molecular fusion is administered in an amount effective to produce immunotolerance to a substance that comprises the tolerogenic antigen. Embodiments include, for example, a composition comprising an erythrocyte-binding moiety that specifically binds an erythrocyte joined to a carrier chosen from the group consisting of a polymer, a branched polymer, and a particle, wherein the carrier is joined to a therapeutic agent. The particle may be, e.g., a microparticle, a nanoparticle, a polymersome, a liposome, or a micelle. The erythrocyte-binding moiety may comprises a peptide comprising at least 5 consecutive amino acid residues of a sequence chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:1, and conservative substitutions thereof, wherein said sequence specifically binds an erythrocyte. The erythrocyte-binding moiety may comprise an antibody, antibody fragment, aptamer, scFv or peptide ligand. Embodiments of a molecular fusion include an erythrocyte binding moiety and a tolerogenic antigen, an antibody, an antibody fragment, an ScFv, a small molecule drug, a particle, a protein, a peptide, or an aptamer.

In a preferred aspect of the invention there is provided a molecular fusion that

comprises a tolerogenic antigen and an erythrocyte-binding moiety selected from Band 3 (CD233), glycophorin B (CD235b), glycophorin C (CD235c) and glycophorin D (CD235d). The molecular fusion is particularly for use in producing immunotolerance. A preferred molecular fusion is one that comprises a tolerogenic antigen selected from: a protein, a portion of a protein, a human protein or portion thereof, a nonhuman protein or portion thereof, a glycan, a glycan of a protein that comprises nonhuman glycosylation, a human autoimmune antigen, a protein therapeutic for a human or a portion thereof, and a portion of a human food, proteins deficient by genetic disease, proteins with nonhuman glycosylation, nonhuman proteins, synthetic proteins not naturally found in humans, human food antigens, human transplantation antigens, and human autoimmune antigens; and an erythrocyte-binding moiety selected from Band 3 (CD233), glycophorin B (CD235b), glycophorin C (CD235c), glycophorin D (CD235d).

*Erythrocyte binding ligands for improved pharmacokinetics*

As many drugs are systemically delivered to the blood circulatory system, the answer to the problem of effective drug delivery often focuses on maintaining the drug in the blood for extended periods of time. Thus, the development of long-circulating (long half-life) therapeutics that remain biologically available in the blood for extended time periods will represent a new generation of drugs engineered for efficacy, safety, and economic feasibility.

Embodiments of the invention include molecular fusions of an erythrocyte-binding peptide and a therapeutic agent. Molecular fusions between peptides that specifically bind to erythrocytes and a therapeutic agent or other substance provide an increased circulation time (circulating half-life in blood in vivo) for the agent/substance. The increase may be, for instance from about 1.5-fold to 20-fold increase in serum half-life, artisans will immediately appreciate that all the ranges and values within the explicitly stated ranges are contemplated, e.g., about 3-fold or about 6-fold or between about 3 fold and about 6-fold.

The molecular fusions may be accomplished by, for instance, recombinant addition of the peptide or adding the peptide by chemical conjugation to a reactive site on the therapeutic agent or associated molecule or particle. As solid-phase peptide synthesis can be used to synthesize high yields of pure peptide with varying terminal reactive groups, there exist multiple conjugation strategies for the attachment of the peptide onto the therapeutic. Though this functionalization method differs with the recombinant method used with proteins, the effect (erythrocyte binding leading to increased circulation half life) is postulated to be the same.

One embodiment of the invention involves functionalization of therapeutic agents with short peptide ligands that specifically bind to erythrocytes as tools for the improvement of pharmacokinetic parameters of the therapeutic agents. This half-life extension methodology takes into consideration pivotal parameters in therapeutic drug design, namely simplicity in manufacturing, modularity, and the ability to tune the extension effect. Using standard recombinant DNA techniques, proteins are easily altered at the amino acid level to contain new or altered functionalities. Generally, relying the use of shorter peptide domains for function is preferable to using larger polypeptide domains, for reasons that include ease in manufacturing, correct folding into a functional therapeutic protein, and minimal biophysical alterations to the original therapeutic itself. Polypeptides, e.g., ERY1, a human erythrocyte binding ligand, or antibodies or antibody fragments, may be engineered to bind specifically to erythrocytes and conjugated to a therapeutic agent to extend bioavailability, e.g., as measured by the circulating half-life of the agent.

The results reported herein provide opportunities to make molecular fusions to improve pharmacokinetic parameters of the therapeutic agents such as insulin, pramlintide acetate, growth hormone, insulin-like growth factor-1, erythropoietin, type 1 alpha interferon, interferon α2a, interferon α2b, interferon β1a, interferon β1b, interferon γ1b, β-glucocerebrosidase, adenosine deaminase, granulocyte colony stimulating factor, granulocyte macrophage colony stimulating factor, interleukin 1, interleukin 2, interleukin 11, factor VIIa, factor VIII, factor IX, exenatide, L-asparaginase, rasburicase, tumor necrosis factor receptor, and enfuvirtide.

Attempts by others to create passive half-life improvement methods focus on increasing the apparent hydrodynamic radius of the drug. The kidney's glomerular filtration apparatus is the primary site in the body where blood components are filtered. The main determinant of filtration is the hydrodynamic radius of the molecule in the blood; smaller molecules (<80 kDa) are filtered out of the blood to a higher extent than larger molecules. Researchers have used this generalized rule to modify drugs to exhibit a larger hydrodynamic radius and thus longer half-life, mainly via chemical conjugation to large molecular weight water-soluble polymers, such as polyethylene glycol (PEG). The success of this method is evident in the numerous PEGylated protein and small molecule therapeutics currently offered in the clinic (Pasut and Veronese, 2009; Fishburn, 2008). Though effective in many cases in increasing circulation half-life, especially as the hydrodynamic radius of the graft or fusion increases (Gao, Liu, et al., 2009), these methods offer challenges in manufacturing and

maintenance of biological effector function. Heterogeneities in conjugation reactions can cause complex product mixtures with varying biological activities, due mostly to the utilization of site-unspecific chemistries. Extensive biochemical characterization often follows precise purification methods to retain a homogenous therapeutic product (Huang, Gough, et al., 2009; Bailon, Palleroni, et al., 2001; Dhalluin, Ross, et al., 2005). Furthermore, attachment of large moieties, such as branched PEGs, to reactive zones of proteins can lead to decreased receptor affinity (Fishburn, 2008).

Other work by others has provided for a therapeutic protein to bind to albumin for increased circulation of the drug (Dennis, 2002; Walker, Dunlevy, et al., 2010). Considering the same general aforementioned rule on kidney filtration, Dennis and colleagues hypothesized that increasing the apparent size of the therapeutic by engineering it to bind another protein in the blood (such as serum albumin) would decrease the rate of drug clearance. In this manner, the drug attains its large molecular size only after administration into the blood stream. The addition of affinity-matured serum albumin-binding peptides to antibody fragments increased their circulation time 24 fold in mice (Dennis, 2002). Though effective, this method is complicated by the dynamics of albumin recycle by the neonatal Fc receptor (FcRn) and the use of cysteine-constrained cyclic peptides for functionality. Walker and colleagues corroborate the results contributed by Dennis in 2002, namely that imparting serum albumin affinity to a protein increases its half-life. The method described by Walker and colleagues involves recombinant addition of large antibody fragments to the protein drug, which may cause structural as well as manufacturing complications. Though elegant and effective, the methods of Dennis and Walker are complicated by use of complex cyclic or large domains for functionality. Though the peptides discovered by Dennis and colleagues displayed high affinity for albumin, they require the physical constraint of correctly forming a cyclic structure prior to use. A more bulky approach, Walker's method of fusing larger antibody fragments may not be amendable to proteins with an already complex folding structure or low expression yield.

*Single chain antibodies*

An embodiment of the invention is a molecular fusion of an scFv with a peptide that specifically binds to an erythrocyte. An scFv may be used a therapeutic agent, and its combination with an erythrocyte binding peptide may be used to extend its circulating half-life and provide access to body compartments. Recombinant antibodies and recombinant antibody fragments have potential as therapeutics in the biologics industry (Sheridan, 2010).

Single-chain variable fragment (scFv) antibody fragments comprise of the entire antigen-binding domain of a full-length IgG, but lack the hinge and constant fragment regions (Maynard and Georgiou, 2000). Recombinant construction of a scFv involves fusing the variable heavy ($V_H$) domain with the variable light ($V_L$) domain with a short polypeptide linker consisting of tandem repeats of glycine and serine (e.g. $(GGGGS)_4$) (SEQ ID NO:18). Though the simplicity of scFv's is attractive for therapeutic applications, their main drawback the short circulation half lives which they exhibit, by virtue of their relatively small molecular weight of 26 – 28 kDa (Weisser and Hall, 2009).

As the glycine-serine linker commonly used in scFv design is non-functional in nature, rather it exists as a physical bridge to ensure correct $V_H$-$V_L$ folding, linker domains were tested herein that exhibit a function of binding to erythrocytes in the blood. Thus, the engineered scFv may be multifunctional and bi-specific, displaying an affinity to its native antigen through the $V_H$-$V_L$ domains, and an affinity to erythrocytes in its linker domain. In binding to erythrocytes, the engineered scFv will exhibit a longer circulation half-life, as has been demonstrated for another model protein with this same functionality. An scFv antibody fragment may have a linker as described herein, or other linkers may be provided as is known to those of skill in these arts. An alternative embodiment provides for a free cysteine group engineered into the linker region of a scFv, and this cysteine thiol used to link by chemical conjugation an erythrocyte binding ligand.

Design of the engineered scFv antibodies focused on the importance of linker domain length, as well as spacing of the erythrocyte binding peptide. As the wild-type variant was designed and validated for antigen binding with a $(GGGGS)_4$ linker (SEQ ID NO:18), subsequent mutants were designed with a linker minimum linker length of 20 amino acids. As the linker domain can modulate correct folding of the scFv into its correct tertiary structure, two ERY1 containing mutants were designed. The REP mutant contains the ERY1 peptide centered in the linker domain, flanked by the correct number of Gly and Ser residues to maintain the parent 20 amino acid linker length. In the possible case where the hydrophobic nature of the ERY1 peptide does not linearly align, but clusters into a shorter assembled domain, the linker length of REP would be shorter and may thereby hinder correct folding. For such reasons, the INS mutant was designed to contain the ERY1 peptide added into the center of the parent linker domain, lengthening the linker to 32 amino acids. As the ERY1 peptide was discovered with a free N-terminus, it was unknown whether or not its presence in a constrained polypeptide conformation would effect erythrocyte binding. To address this potential behavior, a scFv variant was created by chemical conjugation with

synthetic ERY1 peptide, whereby the N-terminus of the peptide is free and the C-terminus is conjugated to the scFv .

In this manner, the number of erythrocyte binding peptides, and thus the erythrocyte-binding capacity of an scFv, may be tuned stoichiometrically during the conjugation reaction. Accordingly, ScFv can be engineered to comprise the erythrocyte-binding peptides as taught herein. Embodiments include an scFv comprising a number of ligands ranging from 1 to 20; artisans will immediately appreciate that all the ranges and values within the explicitly stated ranges are contemplated, e.g, between 2 and 6.

Embodiments include scFv conjugated to a tolerogenic antigen to make a molecular fusion that induces tolerance, e.g., as in Example 6 describing details for creating tolerance as exemplified with creation of tolerance against OVA that is attached to an scFv. Example 6 also details materials and methods for making scFv protein constructs recombinantly fused to an immune recognition epitope of an antigen. The scFv is directed to recognition of erythrocytes. The antigen is an antigen as described herein, e.g., a tolerogenic antigen. Working examples reported herein describe results using murine models, with the use of murine TER119 scFv used as the antibody domain in the constructs. TER119 is an antibody that binds to mouse erythrocytes. The TER119 antibody domain may be replaced by other antibody domains, for instance domains directed to an erythrocyte in human or other animals. For instance, the 10F7 antibody domain may be used to create antibody-antigen constructs capable of binding human erythrocytes. Additional fusions of scFv from Ter-119 were made with three different antigens, as reported in Example 6, including the immunodominant MHC-I epitope of OVA, the chromogranin-A mimotope 1040-p31, and proinsulin.

Example 13 describes other embodiments of scFvs that have been made, including embodiments for testing in animal models and embodiments reactive for humans. These include those relating to the protein level: TER119-SIINFEKL, TER119-ChrA, and TER119-proinsulin. These also include those relating to the genetic level: TER119-SIINFEKL, TER119-ChrA, TER119-proinsulin, TER119-uricase, TER119-InsB9-23, TER119-ISQAVHAAHAEINEAGR (SEQ ID NO:80), TER119-H-2kb, TER119-H-2kd, 10F7-SIINFEKL, 10F7-ChrA, 10F7-proinsulin, and 10F7-uricase.

*Binding of erythrocytes to particular sites, such as tumor vasculature*

In addition to increasing the half-life of a drug, the capacity of an engineered therapeutic to bind erythrocytes is useful in order to selectively bind and localize erythrocytes to a particular site in the body. In treatment of solid tumors, transarterial chemoembolization

(TACE) may be used to limit blood supply to the tumor, thereby hindering its access to nutrients required for growth. TACE treatment involves the surgical insertion of polymeric solid microparticles upstream of the tumor blood supply. As the microparticles reach the tumor vascular bed, they become physically trapped in the blood vessel network thereby creating a blockage for blood supply to the tumor (Vogl, Naguib, et al., 2009).

Pursuant to the TACE theme, an embodiment herein is to use autologous erythrocytes circulating in the blood as natural microparticles for tumor embolization by engineering a tumor-homing therapeutic to contain an erythrocyte-binding peptide. In this manner, the therapeutic localizes to the tumor vascular bed and recruits passing erythrocytes to bind to the vessel, thereby limiting and blocking blood flow to the tumor mass. Such a treatment is less invasive than classical TACE: the drug would be simply injected intravenously and use natural erythrocytes already present in the blood as the embolization particle. The term tumor-binding or tumor-homing refers to a peptide that binds to a component accessible from the blood compartment in tumor vasculature or on tumor cells.

Discovery of specific tumor-homing therapeutics is known in the cancer research field. The paradigm of bioactive targeting of tumors relies on binding to protein markers specifically expressed in the tumor environment. These include, but are not limited to: RGD-directed integrins, aminopeptidase-A and -N, endosialin, cell surface nucleolin, cell surface annexin-1, cell surface p32/gC1q receptor, cell surface plectin-1, fibronectin EDA and EDB, interleukin 11 receptor α, tenascin-C, endoglin/CD105, BST-2, galectin-1, VCAM-1, fibrin, and tissue factor receptor. (Fonsatti, Nicolay, et al., 2010; Dienst, Grunow, et al., 2005; Ruoslahti, Bhatia, et al., 2010; Thijssen, Postel, et al., 2006; Schliemann, Roesli, et al., 2010; Brack, Silacci, et al., 2006; Rybak, Roesli, et al., 2007). A therapeutic targeted towards any of these molecules may be a vector to carry an erythrocyte-binding peptide to the tumor vasculature to cause specific occlusion.

An embodiment is a first ligand that specifically binds erythrocytes conjugated with a second ligand that specifically binds to a cancerous cell or the tumor vasculature or a component of the tumor vasculature, such as a protein in the subendothelium (which is partially exposed to the blood in a tumor) or a protein on the surface of a tumor endothelial cell. The ligand may be part of a pharmaceutically acceptable composition that is introduced into a patient, e.g., into the bloodstream. The ligands bind to erythrocytes and the tumor-homing ligand binds to a site at or near the tumor or tumor vasculature, or to a cancerous cell. The erythrocytes collect at the targeted site and block access of the target site to nutrients, e.g., by embolizing a blood vessel. Given that the embolization is mechanical, being

determined by the physical size of the erythrocyte, embolization will be sudden.

Solid tumors depend heavily on their vascular supply, and biomolecular therapeutics as well as material therapeutics have been developed to either block growth of their vascular supply or to block flow to their vascular supply. An embodiment is a biomolecular formulation or a biomolecular-nanoparticulate formulation that is to be systemically injected to rapidly occlude the vasculature of solid tumors, in the primary tumor or in the metastases at known or unknown locations.

Tumor embolization has been approached in a number of ways, including the use of particle and biomolecular based methods. Biomaterial particles, including those made of polyvinyl alcohol, are of a diameter greater than the tumor microvasculature, e.g. 50-500 micrometers in diameter, and have been developed for use clinically in transcatheter arterial embolization, or TACE (Maluccio, Covey, et al., 2008). A parallel approach includes chemotherapeutics loaded inside the particles for slow release in transarterial chemoembolization (TACE) used mainly for the treatment for hepatocellular carcinoma (Gadaleta and Ranieri, 2010). In both cases, when particles are injected into the arterial circulation, usually by an interventional radiologist under radiographic guidance, these particles can flow into the tumor vasculature and occlude them, blocking flow (Maluccio, Covey, et al., 2008). With these local approaches, only the tumor that is directly targeted by the placement of the catheter is treated, and other tumors, such as metastases at known or unknown locations, go untreated since the particles are not easily targeted in the vessels. More recently, biomolecular approaches have been explored, for example using bispecific antibodies that recognize both a thrombosis factor and a tumor vascular endothelial marker not present in normal vasculature. After binding specifically to the tumor vasculature, the antibody accumulates and initiates the formation of blood clots within the tumor vessels to block them; this effect was only induced when the antibody was targeted to the tumor (Huang, Molema, et al., 1997). These biomolecular approaches have a benefit of targeting both primary and secondary tumors from intravenous infusions if specific tumor vascular signatures can be identified; yet they have a disadvantage of not providing sudden mechanical occlusion to the tumor.

Embodiments of the invention include a method of embolizing a tumor in a patient comprising administering a composition to a patient that comprises an erythrocyte-binding moiety coupled to a targeting moiety, wherein the targeting moiety is an antibody, antibody fragment, or peptide that is directed to a target chosen from the group consisting of a tumor and tumor microvasculature, and wherein the erythrocyte-binding moiety comprises a

peptide, an antibody, an antibody fragment, or an aptamer that specifically binds erythrocytes. The peptide may be, e.g., a sequence as set forth herein.

*Antigen-specific immunological tolerance*

In addition to improving the pharmacokinetic behavior of a therapeutic agent, it has been discovered that erythrocyte affinity may be used in methods of creating antigen-specific tolerance. Certain working and prophetic embodiments are set forth in the Examples. Tolerogenesis, as demonstrated herein, can result in elimination of circulating antibodies against an antigen, or reduction of circulating antibodies against an antigen by a factor of at least 10, i.e., at least 100, at least 1000, or more than 10,000. Tolerogenesis can prevent, cure, or slow disease progression, reduce or eliminate rejection of a transplanted tissue, and reduce or eliminate an allergic reaction to a substance.

Examples 3 and 10-12 detail how tolerance was created in mouse animal models predictive of human behavior. Example 3 showed the creation of tolerance using a test antigen (ovalbumin) connected to a peptide ligand that binds an erythrocyte surface. Examples 10-12 corroborate the results of Example 3 and provide further working examples showing creation of tolerance using ovalbumin attached to an scFv and asparaginase attached to a peptidic ligand. A working example showing creation of tolerance that reverses pre-existing immunorejection is provided in Example 12. Accordingly, embodiments include a fusion molecule for tolerogenesis comprising asparaginase, an antigenic asparaginase fragment, of an antigenic mimotope of asparaginase and an erythrocyte-binding moiety, with the erythrocyte-binding moiety specifically binding, for instance, glycophorin A or a target chosen from the group consisting of Band 3, glycophorin B, glycophorin C or other members of the Erythrocyte Target Group. The erythrocyte-binding moiety may be, for instance, chosen from the group consisting of antibodies, antibody fragments, scFvs, peptide ligands and aptamers and/or from the group consisting of ERY19, ERY59, ERY64, ERY123, ERY141, ERY162, ERY19', ERY59', ERY64', ERY123', ERY141', and ERY162' and conservative substitutions thereof. These fusion molecules may be applied prophylacticly before a first administration of asparaginase, during a course of treatment with asparaginase, and/or after a course of treatment with asparaginase. These fusion molecules may be applied before an immune response to asparaginase drug is observed, or after such a response is observed.

In Example 3, a peptide the binds mouse erythrocytes, ERY1, had been discovered. A molecular fusion of ERY1 was made with a test antigen, ovalbumin (OVA). The fusion

bound specifically bound to erythrocytes *in vivo* and did not bind other molecules, including those in blood or the vasculature. A lengthy circulating half-life was observed. Erythrocyte binding of ERY1-OVA was observed to lead to efficient cross-presentation of the OVA immunodominant MHC I epitope (SIINFEKL) by antigen-presenting cells (APCs) and corresponding cross-priming of reactive T cells. ERY1-OVA induced much higher numbers of annexin-$V^+$ proliferating OT-I $CD8^+$ T cells than OVA, suggesting an apoptotic fate that would eventually lead to clonal deletion. Using an established OT-I challenge-to-tolerance model (Liu, Iyoda, et al., 2002) (Fig. 2), ERY1-OVA was demonstrated to prevent subsequent immune responses to vaccine-mediated antigen challenge, even with a very strong bacterially-derived adjuvant. Intravenous administration of ERY1-OVA resulted in profound reductions in OT-I $CD8^+$ T cell populations in the draining lymph nodes (Fig. 2; gating in Fig. 12b) and spleens compared with mice administered unmodified OVA prior to antigen challenge with LPS (Fig. 2c), demonstrating deletional tolerance. This effective clonal deletion exhibited in mice administered ERY1-OVA supported earlier observations of enhanced OT-I $CD8^+$ T cell cross-priming and furthermore showed that cross-priming occurred in the absence of APC presentation of co-stimulatory molecules to lead to deletional tolerance. Intravenous administrations of ERY1-OVA caused a 39.8-fold lower OVA-specific serum IgG levels 19 d after the first antigen administration (Fig. 3) as compared to OVA-treated mice. To further validate the induction of antigen-specific immune tolerance, the OT-I challenge-to-tolerance model was combined with an OVA-expressing tumor graft model (Fig. 2), with favorable results. The results detailed in this Example demonstrate that erythrocyte binding by ERY1-OVA induces antigen-specific immune tolerance. This was shown in response to a strongly adjuvanted challenge as well as implanted cellular grafts expressing a xeno-antigen. Moreover, tolerance was acheived by functional inactivation and deletion of reactive $CD8^+$ T cells through interaction with antigen present on circulating erythrocytes, independent of direct $CD4^+$ T cell regulation. These detailed experiments with ERY1, a mouse erythrocyte binding peptide, are predictive of similar results in humans using human erythrocyte binding peptides, several of which are taught herein. Moreover, having shown that peptide ligands are effective, similar results may be made using conjugates with other erythrocyte binding ligands, e.g., antibodies, antibody fragments, or aptamers, with these moieties binding a surface molecule of an erythrocyte, for example, one of the surface proteins or antigens set forth herein.

Example 10 further demonstrated the creation of tolerance to a tolerogenic antigen by fusing the tolerogenic antigen to an scFv that binds an erythrocyte, and preparing the fusion

molecule as a medicament for administration to a patient. Example 10 demonstrates induction of tolerance to the MHC-I immunodominant domain of OVA, which is the peptide SIINFEKL (SEQ ID NO:3). This antigen was fused to the scFv TER-119, which is known to bind to mouse erythrocytes. OT-I $CD8^+$ T cell proliferation, determined by dilution of the fluor CFSE as measured by flow cytometry, was markedly enhanced in mice administered TER119-SIINFEKL compared to ERY1-OVA or OVA (Fig. 8), demonstrating that erythrocyte-binding increased antigen-specific $CD8^+$ T cell cross-priming compared to the soluble antigen SIINFEKL. Furthermore, these data demonstrate that antibody fragments designed to bind erythrocytes and display immune epitopes are efficiently processed by immune cells to cross-prime antigen specific T cells. These results on cross-presentation and cross-priming are consistent with other studies concerning tolerogenic antigen presentation on MHC I by APCs engulfing antigen from apoptotic cells (Albert 1998, Green 2009). TER119-SIINFEKL and ERY1-OVA induced much higher numbers of annexin-$V^+$ and PD-$1^+$ proliferating OT-I $CD8^+$ T cells than OVA (Fig. 13), indicating an exhausted and apoptotic fate that leads to clonal deletion. This phenotype of proliferating $CD8^+$ T cells is consistent with other reported OT-I adoptive transfer models in which regulated antigen-specific T cell receptor engagement by APCs fails to induce inflammatory responses (Bursch, Rich, et al., 2009).

Referring to Example 10, the ability of TER119-SIINFEKL and ERY1-OVA to prevent subsequent immune responses to a vaccine-mediated antigen challenge were demonstrated - even when challenged with a very strong bacterially-derived adjuvant. Fusion molecules with either an scFv or a peptide ligand for binding an erythrocyte were thus effective to produce tolerance.

Example 11 shows that it is possible to induce antigen-specific immune tolerance towards therapeutic proteins and proteins with therapeutic potential. Many therapeutic proteins induce immune reactions, rendering their clinical use dangerous or ineffective. Asparaginase is a microbially-derived therapeutic enzyme used to treat acute lymphoblastic leukemia, yet also induces dangerous immune responses that obviate its repeated use. A mouse model was used. A fusion molecule of asparaginase and an erythrocyte-binding moiety (the peptide ERY1) was administered to mice and compared to mice treated with the clinical drug asparaginase. The drug asparaginase produced antibodies but the fusion molecule did not; this test established that antibody levels could be used as a measure of tolerance. Tolerance was then proven by administering the fusion molecule to mice followed by repeated administration of the clinical drug asparaginase, and observing that there was no

potent antibody response, even after a very aggressive challenge by repeated dosing with the drug. In contrast, mice that received the drug but not the fusion molecule became immunoreactive and dangerously hypersensitive.

Example 12 shows that immunoreversal is possible. Mice that were immunoreactive to a protein were treated to become tolerant of the protein. The asparaginase mouse model of Example 11 was used. Mice that were immunoreactive to the drug asparaginase were treated with the fusion molecule (asparaginase-and-erythrocyte binder) and became tolerant of the drug asparaginase.

In contrast, prior reports teach that *immunorejection* is created by attaching an antigen to a surface of an erythrocyte to thereby make a vaccine, and other reports have used antigens encapsulated within erythrocytes to create vaccines. For instance when antigen is encapsulated within an erythrocyte, a vaccine is thereby made (Murray et al., Vaccine 24: 6129-6139 (2006)). Or antigens conjugated to an erythrocyte surface were immunogenic and proposed as vaccines (Chiarantini et al., Vaccine 15(3): 276-280 (1997)). These references show that the erythrocyte delivery approach an immune response as good as those obtained with normal vaccines with adjuvants. Others have reported that placement within an erythrocyte is needed for inducing tolerance, as in patent application WO 2011/051346, which also teaches several means by which to alter the erythrocyte surface to enhance clearance by Kupffer cells in the liver. This same application also teaches binding antibodies to erythrocyte surface proteins such as glycophorin A, but for the purpose of making immune complexes on the erythrocyte to enhance its clearance by Kupffer cells.

Embodiments set forth herein provide for a method of producing immunotolerance, the method comprising administering a composition comprising a molecular fusion that comprises a tolerogenic antigen and an erythrocyte-binding moiety that specifically binds an erythrocyte in the patient and thereby links the antigen to the erythrocyte, wherein the molecular fusion is administered in an amount effective to produce immunotolerance to a substance that comprises the tolerogenic antigen. The erythrocyte, and patient, may be free of treatments that cause other alterations to erythrocytes, and free of erythrocyte crosslinking, chemical covalent conjugations, coatings, and other alterations other than the specific binding of the peptide. The molecular fusion may comprise, or consist of, the erythrocyte-binding moiety directly covalently bonded to the antigen. The molecular fusion may comprise the erythrocyte-binding moiety attached to a particle that is attached to the antigen. The particle may comprise a microparticle, a nanoparticle, a liposome, a polymersome, or a micelle. The tolerogenic antigen may comprise a portion of a therapeutic protein, e.g., a blood factor

administered to a patient suffering from a lack of production of the factor. Embodiments include the instances wherein: the patient is a human and the tolerogenic antigen is a human protein of which the patient is genetically deficient; wherein the patient is a human and the tolerogenic antigen comprises a portion of a nonhuman protein; wherein the patient is a human and the tolerogenic antigen comprises a portion of an engineered therapeutic protein not naturally found in a human; wherein the patient is a human and the tolerogenic antigen comprises a portion of a protein that comprises nonhuman glycosylation; wherein the tolerogenic antigen comprises a portion of a human autoimmune disease protein; wherein the tolerogenic antigen is an antigen in allograft transplantation; wherein the tolerogenic antigen comprises a portion of a substance chosen from the group consisting of human food; and/or wherein the erythrocyte-binding moiety is chosen from the group consisting of a peptide, an antibody, and an antibody fragment. Embodiments include tolerization materials and methods wherein the erythrocyte-binding moiety comprises a peptide comprising at least 5 consecutive amino acid residues of a sequence chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:1, and conservative substitutions thereof, wherein said sequence specifically binds an erythrocyte.

The molecular fusion may be chosen to place the antigen on the inside or the outside of the erythrocyte. Without being bound to a particular mechanism of action, the following theory is presented. In man, approximately 1% of erythrocytes become apoptotic (eryptotic) and are cleared each day, a large number of cells, and their proteins are processed in a manner so as to maintain tolerance to the erythrocyte self-antigens. An antigen engineered to bind to erythrocytes through the use of the ERY1 peptide or a human erythrocyte binding peptide, an erythrocyte-binding single chain antibody or antibody, an erythrocyte-binding aptamer, or another erythrocyte-binding agent may also elicit the same tolerogenic response. Given that the current state-of-the-art method developed by Miller and colleagues (see above) is cumbersome, in that it requires harvesting and reacting donor splenocytes for re-administration, our non-covalent erythrocyte-binding method provides a simpler alternative. As the ERY1-erythrocyte or human erythrocyte binding peptide-erythrocyte or other affinity biomolecule (single chain antibody, antibody, or aptamer, for example) interaction occurs spontaneously after introduction of the antigen conjugate or fusion *in vivo*, the engineered antigen is simply administered by injection, and binding occurs *in situ*.

In some cases, the tolerogenic antigen is derived from a therapeutic agent protein to which tolerance is desired. Examples are protein drugs in their wild type, e.g., human factor

VIII or factor IX, to which patients did not establish central tolerance because they were deficient in those proteins; or nonhuman protein drugs, used in a human. Other examples are protein drugs that are glycosylated in nonhuman forms due to production, or engineered protein drugs, e.g., having non-native sequences that can provoke an unwanted immune response. Examples of tolerogenic antigens that are engineered therapeutic proteins not naturally found in humans include human proteins with engineered mutations, e.g., mutatuions to improve pharmacological characteristics. Examples of tolerogenic antigens that comprise nonhuman glycosylation include proteins produced in yeast or insect cells.

Embodiments include administering a protein at some frequency X or dose Y and also administering an antigen from that protein at a lesser frequency and/or dose, e.g., a frequency that is not more than 0.2X or a dose that is not more than 0.2Y; artisans will immediately appreciate that all the ranges and values within the explicitly stated ranges are contemplated, e.g., 0.01 or 005 X or some range therebetween.

Embodiments include choosing the tolerogenic antigen from proteins that are administered to humans that are deficient in the protein. Deficient means that the patient receiving the protein does not naturally produce enough of the protein. Moreover, the proteins may be proteins for which a patient is genetically deficient. Such proteins include, for example, antithrombin-III, protein C, factor VIII, factor IX, growth hormone, somatotropin, insulin, pramlintide acetate, mecasermin (IGF-1), β-gluco cerebrosidase, alglucosidase-α, laronidasc (α-L-iduronidase), idursuphase (iduronate-2-sulphatase), galsulphase, agalsidase-β (α-galactosidase), α-1 proteinase inhibitor, and albumin. Accordingly, embodiments include a fusion molecule for tolerogenesis comprising an erythrocyte-binding moiety and at least one antigen, antigenic fragment, or antigenic mimotope of one or more of these proteins, with the erythrocyte-binding moiety specifically binding, for instance, glycophorin A or a target chosen from the group consisting of Band 3, glycophorin B, glycophorin C or other members of the Erythrocyte Target Group. The erythrocyte-binding moiety may be, for instance, chosen from the group consisting of antibodies, antibody fragments, scFvs, peptide ligands and aptamers.

Embodiments include choosing the tolerogenic antigen from therapeutic antibodies and antibody-like molecules, including antibody fragments and fusion proteins with antibodies and antibody fragments. These include nonhuman (such as mouse) antibodies, chimeric antibodies, and humanized antibodies. Immune responses to even humanized antibodies have been observed in humans (Getts, 2010). Accordingly, embodiments include

a fusion molecule for tolerogenesis comprising an erythrocyte-binding moiety and at least one antigen, antigenic fragment, or antigenic mimotope of one or more of these proteins, with the erythrocyte-binding moiety specifically binding, for instance, glycophorin A or a target chosen from the group consisting of Band 3, glycophorin B, glycophorin C or other members of the Erythrocyte Target Group. The erythrocyte-binding moiety may be, for instance, chosen from the group consisting of antibodies, antibody fragments, scFvs, peptide ligands and aptamers.

Embodiments include choosing the tolerogenic antigen from proteins that are nonhuman. Examples of such proteins include adenosine deaminase, pancreatic lipase, pancreatic amylase, lactase, botulinum toxin type A, botulinum toxin type B, collagenase, hyaluronidase, papain, L-Asparaginase, rasburicase, lepirudin, streptokinase, anistreplase (anisoylated plasminogen streptokinase activator complex), antithymocyte globulin, crotalidae polyvalent immune Fab, digoxin immune serum Fab, L-arginase, and L-methionase.

Embodiments include choosing the tolerogenic antigen from human allograft transplantation antigens. Examples of these antigens are the subunits of the various MHC class I and MHC class II haplotype proteins, and single-amino-acid polymorphisms on minor blood group antigens including RhCE, Kell, Kidd, Duffy and Ss.

In some cases, the tolerogenic antigen is a self antigen against which a patient has developed an autoimmune response or may develop an autoimmune response. Examples are proinsulin (diabetes), collagens (rheumatoid arthritis), myelin basic protein (multiple sclerosis).

For instance, Type 1 diabetes mellitus (T1D) is an autoimmune disease whereby T cells that recognize islet proteins have broken free of immune regulation and signal the immune system to destroy pancreatic tissue. Numerous protein antigens that are targets of such diabetogenic T cells have been discovered, including insulin, GAD65, chromogranin-A, among others. In the treatment or prevention of T1D, it would be useful to induce antigen-specific immune tolerance towards defined diabetogenic antigens to functionally inactivate or delete the diabetogenic T cell clones. Example 14 details how a fusion molecule presenting the chromogranin-A (ChrA) mimotope as a tolerogenic antigen successfully created tolerogenesis to ChrA, based on T-cell data that was established as predictive in the other Examples. Accordingly, embodiments include a fusion molecule for tolerogenesis comprising chromogranin-A, an antigen or antigenic portion thereof, or a mimotope of the same with an erythrocyte-binding moiety, with the erythrocyte-binding moiety specifically

binding, for instance, glycophorin A or a target chosen from the group consisting of Band 3, glycophorin B, glycophorin C or other members of the Erythrocyte Target Group. The erythrocyte-binding moiety may be, for instance, chosen from the group consisting of antibodies, antibody fragments, scFvs, peptide ligands and aptamers and/or from the group consisting of ERY19, ERY59, ERY64, ERY123, ERY141, ERY162, ERY19', ERY59', ERY64', ERY123', ERY141', ERY162' and conservative substitutions thereof.

Accordingly, tolerance and/or delay of onset or progression of autoimmune diseases may be achieved for various of the many proteins that are human autoimmune proteins, a term referring to various autoimmune diseases wherein the protein or proteins causing the disease are known or can be established by routine testing.

Embodiments include testing a patient to identify an autoimmune protein and creating an antigen for use in a molecular fusion and creating immunotolerance to the protein. Embodiments include an antigen, or choosing an antigen from, one or more of the following proteins. In type 1 diabetes mellitus, several main antigens have been identified: insulin, proinsulin, preproinsulin, glutamic acid decarboxylase-65 (GAD-65), GAD-67, insulinoma-associated protein 2 (IA-2), and insulinoma-associated protein 2β (IA-2β); other antigens include ICA69, ICA12 (SOX-13), carboxypeptidase H, Imogen 38, GLIMA 38, chromogranin-A, HSP-60, caboxypeptidase E, peripherin, glucose transporter 2, hepatocarcinoma-intestine-pancreas/pancreatic associated protein, S100β, glial fibrillary acidic protein, regenerating gene II, pancreatic duodenal homeobox 1, dystrophia myotonica kinase, islet-specific glucose-6-phosphatase catalytic subunit-related protein, and SST G-protein coupled receptors 1-5. In autoimmune diseases of the thyroid, including Hashimoto's thyroiditis and Graves' disease, main antigens include thyroglobulin (TG), thyroid peroxidase (TPO) and thyrotropin receptor (TSHR); other antigens include sodium iodine symporter (NIS) and megalin. In thyroid-associated ophthalmopathy and dermopathy, in addition to thyroid autoantigens including TSHR, an antigen is insulin-like growth factor 1 receptor. In hypoparathyroidism, a main antigen is calcium sensitive receptor. In Addison's disease, main antigens include 21-hydroxylase, 17α-hydroxylase, and P450 side chain cleavage enzyme (P450scc); other antigens include ACTH receptor, P450c21 and P450c17. In premature ovarian failure, main antigens include FSH receptor and α-enolase. In autoimmune hypophysitis, or pituitary autoimmune disease, main antigens include pituitary gland-specific protein factor (PGSF) 1a and 2; another antigen is type 2 iodothyronine deiodinase. In multiple sclerosis, main antigens include myelin basic protein, myelin

oligodendrocyte glycoprotein and proteolipid protein. In rheumatoid arthritis, a main antigen is collagen II. In immunogastritis, a main antigen is $H^+$, $K^+$-ATPase. In pernicious angemis, a main antigen is intrinsic factor. In celiac disease, main antigens are tissue transglutaminase and gliadin. In vitiligo, a main antigen is tyrosinase, and tyrosinase related protein 1 and 2. In myasthenia gravis, a main antigen is acetylcholine receptor. In pemphigus vulgaris and variants, main antigens are desmoglein 3, 1 and 4; other antigens include pemphaxin, desmocollins, plakoglobin, perplakin, desmoplakins, and acetylcholine receptor. In bullous pemphigoid, main antigens include BP180 and BP230; other antigens include plectin and laminin 5. In dermatitis herpetiformis Duhring, main antigens include endomysium and tissue transglutaminase. In epidermolysis bullosa acquisita, a main antigen is collagen VII. In systemic sclerosis, main antigens include matrix metalloproteinase 1 and 3, the collagen-specific molecular chaperone heat-shock protein 47, fibrillin-1, and PDGF receptor; other antigens include Scl-70, U1 RNP, Th/To, Ku, Jo1, NAG-2, centromere proteins, topoisomerase I, nucleolar proteins, RNA polymerase I, II and III, PM-Slc, fibrillarin, and B23. In mixed connective tissue disease, a main antigen is U1snRNP. In Sjogren's syndrome, the main antigens are nuclear antigens SS-A and SS-B; other antigens include fodrin, poly(ADP-ribose) polymerase and topoisomerase. In systemic lupus erythematosus, main antigens include nuclear proteins including SS-A, high mobility group box 1 (HMGB1), nucleosomes, histone proteins and double-stranded DNA. In Goodpasture's syndrome, main antigens include glomerular basement membrane proteins including collagen IV. In rheumatic heart disease, a main antigen is cardiac myosin. Other autoantigens revealed in autoimmune polyglandular syndrome type 1 include aromatic L-amino acid decarboxylase, histidine decarboxylase, cysteine sulfinic acid decarboxylase, tryptophan hydroxylase, tyrosine hydroxylase, phenylalanine hydroxylase, hepatic P450 cytochromes P4501A2 and 2A6, SOX-9, SOX-10, calcium-sensing receptor protein, and the type 1 interferons interferon alpha, beta and omega. Example 15 provides detailed guidance for tolerogenesis to such proteins, and specifically describes a process with insulin as an example.

In some cases, the tolerogenic antigen is a foreign antigen against which a patient has developed an unwanted immune response. Examples are food antigens. Embodiments include testing a patient to identify foreign antigen and creating a molecular fusion that comprises the antigen and treating the patient to develop immunotolerance to the antigen or food. Examples of such foods and/or antigens are provided. Examples are from peanut: conarachin (Ara h 1), allergen II (Ara h 2), arachis agglutinin, conglutin (Ara h 6); from apple: 31 kda major allergen/disease resistance protein homolog (Mal d 2), lipid transfer

protein precursor (Mal d 3), major allergen Mal d 1.03D (Mal d 1); from milk: α-lactalbumin (ALA), lactotransferrin; from kiwi: actinidin (Act c 1, Act d 1), phytocystatin, thaumatin-like protein (Act d 2), kiwellin (Act d 5); from mustard: 2S albumin (Sin a 1), 11S globulin (Sin a 2), lipid transfer protein (Sin a 3), profilin (Sin a 4); from celery: profilin (Api g 4), high molecular weight glycoprotein (Api g 5); from shrimp: Pen a 1 allergen (Pen a 1), allergen Pen m 2 (Pen m 2), tropomyosin fast isoform; from wheat and/or other cerials: high molecular weight glutenin, low molecular weight glutenin, alpha- and gamma-gliadin, hordein, secalin, avenin; from strawberry: major strawberry allergy Fra a 1-E (Fra a 1), from banana: profilin (Mus xp 1).

Many protein drugs that are used in human and veterinary medicine induce immune responses, which creates risks for the patient and limits the efficacy of the drug. This can occur with human proteins that have been engineered, with human proteins used in patients with congenital deficiencies in production of that protein, and with nonhuman proteins. It would be advantageous to tolerize a recipient to these protein drugs prior to initial administration, and it would be advantageous to tolerize a recipient to these protein drugs after initial administration and development of immune response. In patients with autoimmunity, the self-antigen(s) to which autoimmunity is developed are known. In these cases, it would be advantageous to tolerize subjects at risk prior to development of autoimmunity, and it would be advantageous to tolerize subjects at the time of or after development of biomolecular indicators of incipient autoimmunity. For example, in Type 1 diabetes mellitus, immunological indicators of autoimmunity are present before broad destruction of beta cells in the pancreas and onset of clinical disease involved in glucose homeostasis. It would be advantageous to tolerize a subject after detection of these immunological indicators prior to onset of clinical disease.

Recent work by headed by Miller and colleagues has shown that covalently conjugating an antigen to allogenic splenocytes *ex vivo* creates antigen-specific immune tolerance when administered intravenously in mice (Godsel, Wang, et al., 2001; Luo, Pothoven, et al., 2008). The process involves harvesting donor splenic antigen-presenting cells and chemically reacting them in an amine-carboxylic acid crosslinking reaction scheme. The technique has proven effective in inducing antigen-specific tolerance for mouse models of multiple sclerosis (Godsel, Wang, et al., 2001), new onset diabetes type 1 (Fife, Guleria, et al., 2006), and allogenic islet transplants (Luo, Pothoven, et al., 2008). Though the exact mechanism responsible for the tolerogenic response is not known, it is proposed that a major

requirement involves antigen presentation without the expression of co-stimulatory molecules on apoptotic antigen-coupled cells (Miller, Turley, et al., 2007). It has also been contemplated to encapsulate antigens within erythrocyte ghosts, processing the erythrocytes *ex vivo* and re-injecting them, as in WO2011/051346.

*Administration*

Many embodiments of the invention set forth herein describe compositions that may be administered to a human or other animal patient. Embodiments of the invention include, for example, molecular fusions, fusion proteins, peptide ligands, antibodies, scFv, that recognize antigens on erythrocytes or tumors or tumor vasculature, as well as combinations thereof. These compositions may be prepared as pharmaceutically acceptable compositions and with suitable pharmaceutically acceptable carriers or excipients.

The compositions that bind erythrocytes may do so with specificity. This specificity provides for *in vivo* binding of the compositions with the erythrocytes, as well as alternative *ex vivo* processes. Accordingly, the compositions may be directly injected into a vasculature of the patient. An alternative is injection into a tissue, e.g., muscle, dermal, or subcutaneous, for subsequent erythrocyte contact and uptake. An embodiment of the invention is a method of inducing tolerogenesis in a patient comprising administering a tolerizing antigen to the patient in association with an erythrocyte-binding moiety to thereby attach the antigen to an erythrocyte *in vivo*; the *in vivo* attachment can thus be performed without *ex vivo* processing of erythrocytes. This process may be performed wherein the erythrocyte is not made to be apoptotic as a result of the process. This process may be performed so that it is free of (does not involve) exposing the erythrocytes to one or more of: a crosslinking agent; a crosslinking agent that crosslinks surface molecules of an erythrocyte; a crosslinking agent comprising at least two functional groups; a crosslinking agent that forms a covalent bind with an erythrocyte; functional groups that form a covalent bond with an erythrocyte; and an antigen and/or antigenic substance that covalently bonds to the erythrocyte. The process may be performed so that the erythrocytes and/or the antigens are free of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC; also EDAC or EDCI) and may also be free of any reactions with a carbodiimide. This process may be performed with autologous erythrocytes and may be free of allogeneic erythrocytes.

Pharmaceutically acceptable carriers or excipients may be used to deliver embodiments as described herein. Excipient refers to an inert substance used as a diluent or vehicle for a therapeutic agent. Pharmaceutically acceptable carriers are used, in general,

with a compound so as to make the compound useful for a therapy or as a product. In general, for any substance, a pharmaceutically acceptable carrier is a material that is combined with the substance for delivery to an animal. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. In some cases the carrier is essential for delivery, e.g., to solubilize an insoluble compound for liquid delivery; a buffer for control of the pH of the substance to preserve its activity; or a diluent to prevent loss of the substance in the storage vessel. In other cases, however, the carrier is for convenience, e.g., a liquid for more convenient administration. Pharmaceutically acceptable salts of the compounds described herein may be synthesized according to methods known to those skilled in the arts. Thus pharmaceutically acceptable compositions are highly purified to be free of contaminants, are biocompatible and not toxic, are suited to administration to a patient. In the case of water as the carrier, the water is highly purified and processed to be free of contaminants, e.g., endotoxins.

The compounds described herein are typically to be administered in admixture with suitable pharmaceutical diluents, excipients, extenders, or carriers (termed herein as a pharmaceutically acceptable carrier, or a carrier) suitably selected with respect to the intended form of administration and as consistent with conventional pharmaceutical practices. Thus the deliverable compound may be made in a form suitable for oral, rectal, topical, intravenous injection, intra-articular injection, or parenteral administration. Carriers include solids or liquids, and the type of carrier is chosen based on the type of administration being used. Suitable binders, lubricants, disintegrating agents, coloring agents, flavoring agents, flow-inducing agents, and melting agents may be included as carriers, e.g., for pills. For instance, an active component can be combined with an oral, non-toxic, pharmaceutically acceptable, inert carrier such as lactose, gelatin, agar, starch, sucrose, glucose, methyl cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, mannitol, sorbitol and the like. The compounds can be administered orally in solid dosage forms, such as capsules, tablets, and powders, or in liquid dosage forms, such as elixirs, syrups, and suspensions. The active compounds can also be administered parentally, in sterile liquid dosage forms. Buffers for achieving a physiological pH or osmolarity may also be used.

EXAMPLES

Example 1: Characterizing the Molecular Target of Binding to Mouse Erythrocytes

Result: To search for a molecular target for the ERY1 peptide, affinity pull-down

techniques using a biotinylated soluble peptide were employed; this method revealed glycophorin-A (GYPA) as the ERY1 ligand on the erythrocyte membrane. When whole erythrocytes were incubated with ERY1 peptide functionalized with biotin and a photoactivatable crosslinker, a single 28 kDa protein was conjugated with the peptide-biotin complex, as detected by a streptavidin Western blot. The reaction lysate was extensively washed and purified using streptavidin magnetic beads to ensure no unlabeled proteins from the erythrocyte lysate remained. As expected, the mismatch peptide failed to conjugate to any erythrocyte proteins. The mismatch peptide, PLLTVGMDLWPW (SEQ ID NO:2), was designed to contain the same amino acid residues as ERY1, and to match its hydrophobicity topography. Evidence of the apparent size of the interacting protein suggested several smaller, single pass membrane proteins as likely ligands, namely the glycophorins. Anti-GYPA Western blotting of the same purified samples from the crosslinking reaction confirmed that the candidate biotinlyated protein was GYPA.

Co-localization of ERY1 phage with GYPA was analyzed by high-resolution confocal microscopy. GYPA is naturally expressed and presented as part of a complex comprised of several membrane and cytoskeletal proteins (Mohandas and Gallagher, 2008). This is visually evident in GYPA staining, whereby non-uniform labeling was seen at the cell equatorial periphery. Labeling with ERY1 phage produced extremely similar staining topographies. A high overlap coefficient of 0.97 in co-localization analysis, corroborated the conclusion that ERY1 phage and anti-GYPA bind to the same protein. GYPA clustering was also witnessed in erythrocytes labeled with library phage, yet no phage binding thus no co-localization was evident.

Method: The ERY1 ($H_2N$-WMVLPWLPGTLDGGSGCRG-$CONH_2$) (SEQ ID NO:19) and mismatch ($H_2N$-PLLTVGMDLWPWGGSGCRG-$CONH_2$) (SEQ ID NO:20) peptides were synthesized using standard solid-phase f-moc chemistry on TGR resin. The peptide was cleaved from the resin in 95% tri-fluoroacetic acid, 2.5% ethanedithiol, 2.5% water, and precipitated in ice-cold diethyl ether. Purification was conducted on a Waters preparative HPLC-MS using a C18 reverse phase column.

The ERY1 and mismatch peptide were conjugated to Mts-Atf-biotin (Thermo Scientific) as suggested by the manufacturer. In brief, peptides were solubilized in PBS/DMF and reacted with 1.05 equivalents of Mts-atf-biotin overnight at 4°C. Following clarification of the reaction by centrifugation, biotinylated peptide was incubated with erythrocytes at in PBSA-50 for 1 h at 37°C, cells were washed twice in fresh PBS, and were UV irradiated at 365 nm for 8 min at room temperature. Cells were lysed by sonication and

the lysate was purified using streptavidin-coated magnetic beads (Invitrogen). The eluate was run on an SDS-PAGE and transferred to a PVDF membrane, and immunoblotted with streptavidin-HRP (R&D Systems) or anti-mouse GYPA.

Example 2: Characterizing of Binding to Human Erythrocytes

Result: To characterize the selected peptides that bound to human erythrocytes, bacteria displaying each individual peptide were subjected to binding assays with multiple cell types. Six (ERY19, ERY59, ERY64, ERY123, ERY141 and ERY162) of seven peptides bound specifically to human erythrocytes as compared to binding towards human epithelial 293T cells and human endothelial HUVECs. Additionally, peptides bound to human blood types A and B, but not to mouse blood indicating that these peptides were specific to human blood, but not dependent on the common blood group antigens. Peptides are synthesized using standard solid-phase f-moc chemistry, conjugated to nanoparticles, and analyzed for binding to individual cell types as above. Binding to erythrocyte surfaces was studied using both microscopy and flow cytometry.

Example 3: Inducing Antigen-specific Immunological Tolerance Through Non-covalent Erythrocyte-binding with ERY1 Peptide-conjugated Antigen or Human Erythrocyte Binding Peptide-Conjugated Antigen

To obtain strong and specific biophysical binding of an antigen to erythrocytes, we used a synthetic 12 amino acid peptide (ERY1) that we discovered by phage display to specifically bind to mouse glycophorin-A (Kontos and Hubbell, 2010). In this investigation, the model antigen OVA was used with a transgenic mouse strain (OT-I) whose $CD8^+$ T cell population expresses the T cell receptor specific for the MHC I immunodominant OVA peptide SIINFEKL (SEQ ID NO:3). The ERY1 peptide was chemically conjugated to OVA to create an OVA variant (ERY1-OVA) that binds mouse erythrocytes with high affinity and specificity (Fig. 1a). High-resolution confocal microscopy confirmed earlier observations concerning ERY1 binding (Kontos and Hubbell, 2010), namely localization to the cell membrane equatorial periphery, with no intracellular translocation of the ERY1-conjugated protein. ERY1-mediated binding to glycophorin-A was sequence-specific, as an OVA variant conjugated with a mismatch peptide (MIS-OVA), containing identical amino acids to ERY1 but scrambled in primary sequence, displayed negligible binding (Fig. 1b). OVA conjugated with only the crosslinking molecule used to conjugate the peptide did not display any measureable affinity towards erythrocytes, confirming that ERY1-OVA binding resulted

from non-covalent interaction between the ERY1 peptide and glycophorin-A on the erythrocyte surface. Furthermore, ERY1-OVA bound to erythrocytes with high affinity, exhibiting an antibody-like dissociation constant ($K_d$) of 6.2 ± 1.3 nM, as determined by equilibrium binding measurements (Fig. 1c).

ERY1-OVA binding was validated *in vivo* to circulating erythrocytes following intravenous administration in mice. Whole blood samples taken 30 min following injection of 150 μg of either OVA or ERY1-OVA confirmed the specific erythrocyte-binding capability of ERY1-OVA even amidst the complex heterogeneous milieu of blood and the vasculature. Consistent with glycophorin-A association, ERY1-OVA bound to erythrocytes ($CD45^-$) but not to leukocytes ($CD45^+$). ERY1-OVA binding was unbiased as to the apoptotic state of the erythrocytes, binding strongly to both annexin-$V^+$ and annexin-$V^-$ $CD45^-$ populations. Pharmacokinetic studies of the OVA conjugate demonstrated that ERY1-OVA erythrocyte binding was long-lived *in vivo*, exhibiting a cell-surface half-life of 17.2 h . ERY1-OVA remained bound to erythrocytes for as long as 72 h following administration; during this time frame, approximately 13% of erythrocytes are cleared in the mouse (Khandelwal and Saxena, 2006). Quantification of erythrocyte-bound ERY1-OVA *in vivo* showed a relatively high loading of 0.174 ± 0.005 ng of OVA per $10^6$ erythrocytes.

To exclude any potential physiological effects of OVA loading on erythrocyte function, hematological parameters were characterized at varying time points following intravenous administration of either ERY1-OVA or OVA. Erythrocyte binding by ERY1-OVA elicited no detectable differences in hematocrit, corpuscular volume, or erythrocyte hemoglobin content, as compared with administration of OVA. These results demonstrate that glycophorin-A-mediated erythrocyte binding with antigen did not alter their hematological parameters.

To reveal the cellular targets of erythrocyte-bound antigen upon administration, mice were intravenously injected with the highly fluorescent allophycocyanin protein, conjugated to either ERY1 (ERY1-allophycocyanin) or MIS peptide (MIS-allophycocyanin). Flow cytometric analysis of splenic DC populations 12 and 36 h following administration showed 9.4-fold enhanced uptake of ERY1-allophycocyanin by $MHCII^+$ $CD11b^-$ $CD11c^+$ DCs as compared with MIS-allophycocyanin, yet similar uptake of ERY1-allophycocyanin and MIS-allophycocyanin by $MHCII^+$ $CD11b^+$ $CD11c^+$ DCs. Additionally, $MHCII^+$ $CD8\alpha^+$ $CD11c^+$ $CD205^+$ splenic DCs were found to uptake ERY1-allophycocyanin to a 3.0-fold greater extent than MIS-allophycocyanin, though the absolute magnitude was markedly lower than for other DC populations in the spleen. Such targeting of antigen towards non-activated and

$CD8\alpha^+$ $CD205^+$ splenic DCs could strengthen the tolerogenic potential of erythrocyte binding, as these populations have been extensively implicated in apoptotic cell-driven tolerogenesis (Ferguson, Choi, et al., 2011; Yamazaki, Dudziak, et al., 2008). In the liver, ERY1-allophycocyanin also greatly enhanced uptake by hepatocytes ($CD45^-$ $MHCII^-$ $CD1d^-$; by 78.4-fold) and hepatic stellate cells ($CD45^-$ $MHCII^+$ $CD1d^+$; by 60.6-fold) as compared with MIS-allophycocyanin ; both populations have been reported as antigen-presenting cells that trigger $CD8^+$ T cell deletional tolerance (Holz, Warren, et al., 2010; Ichikawa, Mucida, et al., 2011; Thomson and Knolle, 2010). Interestingly, such uptake was not seen in liver DCs ($CD45^+$ $CD11c^+$) or Kupffer cells ($CD45^+$ $MHCII^+$ $F4/80^+$), which serve as members of the reticuloendothelial system that aid in clearance of erythrocytes and complement-coated particles. Increased uptake of erythrocyte-bound antigen by the tolerogenic splenic DC and liver cell populations suggests the potential for a complex interconnected mechanism of antigen-specific T cell deletion driven by non-lymphoid liver cell and canonical splenic cell cross-talk.

Erythrocyte binding of ERY1-OVA was observed to lead to efficient cross-presentation of the OVA immunodominant MHC I epitope (SIINFEKL) (SEQ ID NO:3) by APCs and corresponding cross-priming of reactive T cells. CFSE-labeled OT-I $CD8^+$ T cells ($CD45.2^+$) were adoptively transferred into $CD45.1^+$ mice. Measurements were made of the proliferation of the OT-I $CD8^+$ T cells over 5 d following intravenous administration of 10 μg of OVA, 10 μg ERY1-OVA, or 10 μg of an irrelevant erythrocyte-binding antigen, ERY1-glutathione-S-transferase (ERY1-GST). OT-I $CD8^+$ T cell proliferation, determined by dilution of the fluor CFSE as measured by flow cytometry, was markedly enhanced in mice administered ERY1-OVA compared to OVA, demonstrating that erythrocyte-binding increased antigen-specific $CD8^+$ T cell cross-priming compared to the soluble antigen. Similar results were also obtained by administration of a 10-fold lower antigen dose of 1 μg, demonstrating the wide dynamic range of efficacy of OT-I $CD8^+$ T cell proliferation induced by erythrocyte-bound antigen. The results on cross-presentation and cross-priming are consistent with other studies concerning tolerogenic antigen presentation on MHC I by APCs engulfing antigen from apoptotic cells (Albert, Pearce, et al., 1998; Green, Ferguson, et al., 2009).

To distinguish T cells being expanded into a functional effector phenotype from those being expanded and deleted, the proliferating OT-I $CD8^+$ T cells for annexin-V were analyzed as a hallmark of apoptosis and thus deletion. ERY1-OVA induced much higher numbers of annexin-$V^+$ proliferating OT-I $CD8^+$ T cells than OVA (Fig. 12d), suggesting an

apoptotic fate that would eventually lead to clonal deletion. The same proliferating OT-I $CD8^+$ T cells induced by ERY1-OVA administration exhibited an antigen-experienced phenotype at both 1 and 10 μg doses, displaying upregulated CD44 and downregulated CD62L. This phenotype of proliferating $CD8^+$ T cells is consistent with other reported OT-I adoptive transfer models in which regulated antigen-specific T cell receptor engagement by APCs fails to induce inflammatory responses (Bursch, Rich, et al., 2009).

Using an established OT-I challenge-to-tolerance model (Liu, Iyoda, et al., 2002) (Fig. 2a), ERY1-OVA was demonstrated to prevent subsequent immune responses to vaccine-mediated antigen challenge, even with a very strong bacterially-derived adjuvant. To tolerize, we intravenously administered 10 μg of either OVA or ERY1-OVA 1 and 6 d following adoptive transfer of OT-I $CD8^+$ ($CD45.2^+$) T cells to $CD45.1^+$ mice. After 9 additional days to allow potential deletion of the transferred T cells, we then challenged the recipient mice with OVA adjuvanted with lipopolysaccharide (LPS) by intradermal injection. Characterization of draining lymph node and spleen cells as well as their inflammatory responses 4 d after challenge allowed us to determine if deletion actually took place.

Intravenous administration of ERY1-OVA resulted in profound reductions in OT-I $CD8^+$ T cell populations in the draining lymph nodes (Fig. 2; gating in Fig. 2b) and spleens compared with mice administered unmodified OVA prior to antigen challenge with LPS (Fig. 2c), demonstrating deletional tolerance. Draining lymph nodes from ERY1-OVA-treated mice contained over 11-fold fewer OT-I $CD8^+$ T cells as compared to OVA-treated mice, and 39-fold fewer than challenge control mice that did not receive intravenous injections of antigen; responses in spleen cells were similar. This effective clonal deletion exhibited in mice administered ERY1-OVA supported earlier observations of enhanced OT-I $CD8^+$ T cell cross-priming and furthermore shows that cross-priming occurred in the absence of APC presentation of co-stimulatory molecules to lead to deletional tolerance.

To further evaluate the immune response following antigen challenge, the inflammatory nature of resident lymph node and spleen cells was characterized by expression of interferon-γ (IFNγ) by OT-I $CD8^+$ T cells (Fig. 2d). Following challenge with OVA and LPS, the lymph nodes of mice previously treated with ERY1-OVA harbored 53-fold fewer IFNγ-expressing cells compared to challenge control mice (previously receiving no antigen), and over 19-fold fewer IFNγ-expressing cells compared to mice previously treated with an equivalent dose of OVA (Fig. 2e), demonstrating the importance of erythrocyte binding in tolerogenic protection to challenge; responses in spleen cells were similar. In addition, of the small OT-I $CD8^+$ T cell population present in the lymph nodes and spleens of mice

previously treated with ERY1-OVA, a lower percentage expressed IFNγ, suggesting clonal inactivation. Furthermore, the magnitude of total IFNγ levels produced by cells isolated from the draining lymph nodes upon SIINFEKL restimulation was substantially reduced in mice previously treated with ERY1-OVA (Fig. 2f), erythrocyte binding reducing IFNγ levels 16-fold compared to OVA administration and over 115-fold compared to challenge controls. Of note, the suppressive phenomenon was also correlated with downregulated interleukin-10 (IL-10) expression, as lymph node cells from mice previously treated with ERY1-OVA expressed 38% and 50% less IL-10 as compared with previously OVA-treated and challenge control mice, respectively (Fig. 2g). Typically considered a regulatory $CD4^+$ T cell-expressed cytokine in the context of APC-T cell communication to dampen Th1 responses (Darrah, Hegde, et al., 2010; Lee and Kim, 2007), IL-10 expression was dispensible for desensitization to challenge. Similar IL-10 downregulation has been implicated in $CD8^+$ T cell mediated tolerogenesis (Fife, Guleria, et al., 2006; Arnaboldi, Roth-Walter, et al., 2009; Saint-Lu, Tourdot, et al., 2009). Erythrocyte-binding also substantially attenuated humoral immune responses against antigen, as mice treated with ERY1-OVA exhibited 100-fold lower antigen-specific serum IgG titers compared with mice treated with soluble OVA (Fig. 2h). A similar reduction in OVA-specific IgG titer reduction as a result of erythrocyte binding was seen in non-adoptively transferred C57BL/6 ($CD45.2^+$) mice. Following two intravenous administrations of 1 μg OVA or ERY1-OVA 7 d apart, ERY1-OVA treated mice exhibited 39.8-fold lower OVA-specific serum IgG levels 19 d after the first antigen administration (Fig 3). This apparent reduction in B cell activation, following erythrocyte ligation by the antigen, corroborates current hypotheses concerning non-inflammatory antigen presentation during tolerance induction (Miller, Turley, et al., 2007; Green, Ferguson, et al., 2009; Mueller, 2010).

To further validate the induction of antigen-specific immune tolerance, the OT-I challenge-to-tolerance model was combined with an OVA-expressing tumor graft model (Fig. 2i). Similar to the previous experimental design, mice were tolerized by two intravenous administrations of 10 μg ERY1-OVA or 10 μg OVA following adoptive transfer of OT-I $CD8^+$ T cells. Marked T cell deletion was detected 5 d following the first antigen administration, as ERY1-OVA injected mice harbored 2.9-fold fewer non-proliferating (generation 0) OT-I $CD8^+$ T cells in the blood (Fig. 2j). To determine the functional responsiveness of proliferating OT-I $CD8^+$ T cells in the absence of a strong exogenously administered adjuvant, OVA-expressing EL-4 thymoma cells (E.G7-OVA) were intradermally injected into the back skin of mice 9 d following adoptive transfer. To assess

the tolerogenic efficacy of erythrocyte-bound antigen, tumor-bearing mice were challenged with LPS-adjuvanted OVA 6 d following tumor grafting, analogous in dose and schedule to the challenge-to-tolerance model. Robust tumor growth was continuously observed in ERY1-OVA treated mice as compared to OVA-treated or non-treated control mice through to 8 d following tumor grafting (Fig. 2k), confirming that ERY1-OVA driven OT-I $CD8^+$ T cell proliferation induced functional immune non-responsiveness to OVA. That tumor size was arrested to a steady state 8 d following grafting may be indicative of residual OT-I $CD8^+$ T cells that had yet to undergo ERY1-OVA-driven deletional tolerance.

*Animals*

Swiss Veterinary authorities previously approved all animal procedures. 8-12 wk old female C57BL/6 mice (Charles River) were used for *in vivo* binding studies and as E.G7-OVA tumor hosts. C57BL/6-Tg(TcraTcrb) 1100Mjb (OT-I) mice (Jackson Labs) were bred at the EPFL Animal Facility, and females were used for splenocyte isolation at 6-12 wk old. 8-12 week old female B6.SJL-*Ptprc*$^a$*Pepc*$^b$/Boy (CD45.1) mice (Charles River) were used as recipient hosts for OT-I $CD8^+$ T cell adoptive transfer and tolerance induction studies.

*Peptide design and synthesis*

The ERY1 ($H_2N$-WMVLPWLPGTLDGGSGCRG-$CONH_2$) (SEQ ID NO:19) and mismatch ($H_2N$-PLLTVGMDLWPWGGSGCRG-$CONH_2$) (SEQ ID NO:20) peptides were synthesized using standard solid-phase f-moc chemistry using TGR resin (Nova Biochem) on an automated liquid handler (CHEMSPEED). The underlined sequence is the ERY1 12-mer sequence that we previously discovered by phage display as a mouse glycophorin-A binder (Kontos and Hubbell, 2010). The GGSG region served as a linker to the cysteine residue used for conjugation; the flanking arginine residue served to lower the pKa and thus increase the reactivity of the cysteine residue (Lutolf, Tirelli, et al., 2001). The peptide was cleaved from the resin for 3 h in 95% tri-fluoroacetic acid, 2.5% ethanedithiol, 2.5% water, and precipitated in ice-cold diethyl ether. Purification was conducted on a preparative HPLC-MS (Waters) using a C18 reverse phase column (PerSpective Biosystems).

*ERY1-antigen conjugation*

10 molar equivalents of succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, CAS# 64987-85-5, Thermo Scientific) dissolved in dimethylformamide were reacted with 5 mg/mL endotoxin-free (<1 EU/mg) OVA (Hyglos GmbH) in PBS for 1 h

at room temperature. Following desalting on a 2 mL ZEBA Desalt spin column (Thermo Scientific), 10 equivalents of ERY1 or MIS peptide dissolved in 3 M guanidine-HCl were added and allowed to react for 2 h at room temperature. The conjugate was desalted using 2 mL ZEBA Desalt spin columns, 0.2 μm sterile filtered, dispensed into working aliquots, and stored at -20 C. Protein concentration was determined via BCA Assay (Thermo Scientific). The scheme results in conjugation of the cysteine side chain on the peptide to lysine side-chains on the antigen. Glutathione-S-transferase (GST) was expressed in BL21 *Escherichia coli* and purified using standard glutathione affinity chromatography. On-column endotoxin-removal was performed by extensive Triton-X114 (Sigma Aldrich) washing, and endotoxin removal was confirmed with THP-1X Blue cells (InvivoGen). The same reaction procedure was used to conjugate ERY1 to GST. Maleimide-activated allophycocyanin (Innova Biosciences) was dissolved in PBS, and conjugated with ERY1 or MIS as described above.

*Microscopy of binding to erythrocytes*

$5x10^5$ freshly isolated mouse erythrocytes were exposed to 100 nM of ERY1-OVA or OVA in PBS containing 10 mg/mL BSA for 1 h at 37 C. Following centrifugation and washing, cells were labeled with 1:200 diluted goat anti-mouse glycophorin-A (Santa Cruz) and rabbit anti-OVA (AbD SEROTEC) for 20 min on ice. Following centrifugation and washing, cells were labeled with 1:200 ALEXAFLUOR488 anti-goat IgG (Invitrogen) and AlexaFluor546 anti-rabbit IgG (Invitrogen) for 20 min on ice. Following a final spin/wash cycle, cells were hard set mounted and imaged on a Zeiss LSM700 inverted confocal microscope with a 63x oil immersion objective. Image analysis was conducted in IMAGEJ (NIH), with identical processing to both images.

*In vivo binding and biodistribution*

150 μg of ERY1-OVA or OVA in 0.9 % saline (B. Braun) in a volume of 100 μL was injected intravenously into the tail of 8-12 week old female C57BL/6 mice while under anesthesia with isoflurane. Care was taken to ensure mice were kept at 37 C with a heating pad during experimentation. At predetermined time points, 5 μL of blood was taken from a small incision on the tail, diluted 100-fold into 10 mM EDTA in PBS, washed three times with PBS with 10 mg/mL BSA, and analyzed for OVA content by flow cytometry and ELISA. OVA was quantified by sandwich ELISA, using a mouse monoclonal anti-OVA antibody (Sigma) for capture, a polyclonal rabbit anti-OVA antibody (AbD SEROTEC) for detection, a goat anti-rabbit-IgG-HRP antibody (BioRad) for final detection, followed by

TMB substrate (GE Life Sciences). Hematological characterization was performed on an ADVIVA 2120 Hematology System (Siemens). Erythrocyte-bound ERY1-GST was detected by incubating labeled cells with goat anti-GST (GE Healthcare Life Sciences), followed by incubation with AlexaFluor488 donkey anti-goat (Invitrogen), and analyzed by flow cytometry. For biodistribution studies, 20 μg of ERY1-APC or MIS-APC was injected intravenously into the tail vein of 8-12 week old female C57BL/6 mice as described above. Mice were sacrificed at predetermined time points, and the spleen, blood, and liver were removed. Each organ was digested with collagenase D (Roche) and homogenized to obtain a single-cell suspension for flow cytometry staining.

*T cell adoptive transfer*

$CD8^+$ T cells from OT-I ($CD45.2^+$) mouse spleens were isolated using a CD8 magnetic bead negative selection kit (Miltenyi Biotec) as per the manufacturer's instructions. Freshly isolated $CD8^+$ OT-I cells were resuspended in PBS and labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 6 min at room temperature, and the reaction was quenched for 1 min with an equal volume of IMDM with 10% FBS (Gibco). Cells were washed, counted, and resuspended in pure IMDM prior to injection. $3x10^6$ CFSE-labeled $CD8^+$ OT-I cells were injected intravenously into the tail vein of recipient $CD45.1^+$ mice. For short-term proliferation studies, 10 μg of ERY1-OVA or OVA in 100 μL volume was injected 24 h following adoptive transfer. Splenocytes were harvested 5 d following antigen administration and stained for analysis by flow cytometry.

*OT-I tolerance and challenge model*

$3x10^5$ CFSE-labeled OT-I $CD8^+$ T cells were injected into $CD45.1^+$ recipient mice as described above. 1 and 6 d following adoptive transfer, mice were intravenously administered 10 μg of ERY1-OVA or OVA in 100 μL saline into the tail vein. 15 d following adoptive transfer, mice were challenged with 5 μg OVA and 25 ng ultra-pure *Escherichia coli* LPS (InvivoGen) in 25 μL intradermally into each rear leg pad (Hock method, total dose of 10 μg OVA and 50 ng LPS). Mice were sacrificed 4 d following challenge, and spleen and draining lymph node cells were isolated for restimulation. For flow cytometry analysis of intracellular cytokines, cells were restimulated in the presence of 1 mg/mL OVA or 1 μg/mL SIINFEKL (SEQ ID NO:3) peptide (Genscript) for 3 h. Brefeldin-A (Sigma, 5 μg/mL) was added and restimulation resumed for an additional 3 h prior to staining and flow cytometry analysis. For ELISA measurements of secreted factors,

cells were restimulated in the presence of 100 μg/mL OVA or 1 μg/mL SIINFEKL (SEQ ID NO:3) peptide for 4 d. Cells were spun and the media collected for ELISA analysis using IFNγ and IL-10 Ready-Set-Go kits (eBiosciences) as per the manufacturer's instructions. OVA-specific serum IgG was detected by incubating mouse serum at varying dilutions on OVA-coated plates, followed by a final incubation with goat anti-mouse IgG-HRP (Southern Biotech).

*OT-I E.G7-OVA tolerance model*

$1x10^6$ CFSE-labeled OT-I $CD8^+$ T cells were injected into 8-12 wk old female C57BL/6 mice as described above. 1 and 6 d following adoptive transfer, mice were intravenously administered 10 μg of ERY1-OVA or 10 μg OVA in 100 μL saline into the tail vein. Blood was drawn 5 d following adoptive transfer for characterization of OT-I $CD8^+$ T cell proliferation by flow cytometry. OVA-expressing EL-4 thymoma cells (E.G7-OVA, ATCC CRL-2113) were cultured as per ATCC guidelines. In brief, cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 1% puromycin/streptomycin (Invitrogen Gibco), and 0.4 mg/mL G418 (PAA Laboratories). Just prior to injection, cells were expanded in media without G418 and resuspended upon harvest in HBSS (Gibco). 9 d following adoptive transfer, mice were anesthetized with isoflurane, the back area was shaved, and $1x10^6$ E.G7-OVA cells were injected intradermally between the shoulder blades. 4 d following E.G7-OVA graft, tumor dimensions were measured every 24 h with a digital caliper, and tumor volume was calculated as an ellipsoid ($V=(\pi/6)\ l \cdot w \cdot h$), where $V$ is volume, $l$ is length, $w$ is width, and $h$ is the height of the tumor). 15 d following adoptive transfer, mice were challenged with 5 μg OVA and 25 ng ultra-pure *Escherichia coli* LPS (InvivoGen) in 25 μL intradermally into each front leg pad (total dose of 10 μg OVA and 50 ng LPS).

*Antibodies and flow cytometry*

The following anti-mouse antibodies were used for flow cytometry: CD1d Pacific Blue, CD3ε PerCP-Cy5.5, CD8α PE-Cy7, CD11b PE-Cy7, CD11c Pacific Blue, biotinylated CD45, CD45.2 Pacific Blue, CD45 Pacific Blue, IFNγ-APC, CD8α APC-eF780, CD44 PE-Cy5.5, CD62L PE, CD205 PE-Cy7, F4/80 PE, I-A/I-E MHCII FITC (all from eBioscience), in addition to fixable live/dead dye (Invitrogen), annexin-V-Cy5 labeling kit (BioVision),

streptavidin Pacific Orange (Invitrogen), and anti-OVA-FITC (Abcam). Samples were analyzed on a CyAn ADP flow cytometer (Beckman Coulter). Cells were washed first with PBS, stained for 20 min on ice with live/dead dye, blocked for 20 min on ice with 24G2 hybridoma medium, surface stained for 20 min on ice, fixed in 2% paraformaldehyde for 20 min ice, intracellularly stained in the presence of 0.5% saponin for 45 min on ice, followed by a final wash prior to analysis. For apoptosis staining, annexin-V-Cy5 was added 5 min prior to analysis. For CD45 staining, cells were stained with streptavidin Pacific Orange for 20 min on ice, washed, and analyzed.

*Implementation with particles*

The ERY1 peptide has also been implemented for tolerogenesis in the form of nanoparticles, to which the ERY1 peptide and the tolerogenic antigen are both conjugated.

To form conjugates of ERY1 with a polymer nanoparticle, which is also conjugated to the peptide or protein antigen, stoichiometric amounts of each component may be added consecutively to control conjugation conversions. To form a nanoparticle conjugated with both OVA and ERY1 or mismatch peptide, the peptides were first dissolved in aqueous 3M guanidine HCl, and 0.5 equivalents were added to nanoparticles containing a thiol-reactive pyridyldisulfide group. Absorbance measurements were taken at 343 nm to monitor the reaction conversion, as the reaction creates a non-reactive pyridine-2-thione species with a high absorbance at this wavelength. Following 2 h at room temperature, the absorbance at 343 nm had stabilized and OVA was dissolved in aqueous 3M guanidine HCl, and added to the nanoparticle solution at a 2-fold molar excess. Following 2 h at room temperature, the absorbance at 343 nm had once again stabilized to a higher value, and the concentrations of both the peptide and OVA in the solution were calculated. The bifunctionalized nanoparticles were purified from non-reacted components by gel filtration on a Sepharose CL6B packed column. Each 0.5 mL fraction was analyzed for the presence of protein and/or peptide by fluorescamine, and nanoparticle size was assessed by dynamic light scattering (DLS).

Should the antigen not contain any free thiol groups to perform such a reaction, they may be introduced by recombinant DNA technology to create a mutant that could then be expressed and purified recombinantly. Alternatively, amine-carboxylic acid crosslinking could be performed between the nanoparticle and antigen using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC).

To form conjugates of ERY1 with a polymer micelle, which is also conjugated to the

peptide or protein antigen, similar reactions would be used as described with polymeric nanoparticles. The micelle would be formed to contain functional groups desired for the appropriate conjugation scheme. Given that our nanoparticles and micelles may be synthesized to contain many different chemical group functionalizations, there exist numerous possibilities of conjugation schemes to employ in creating the nanoparticle/micelle-antigen-ERY1 complex.

Example 4: Development of antibodies and antibody-fragments with that bind mouse and/or human erythrocytes

As another method to non-covalently bind erythrocytes, an erythrocyte-binding antibody may also be used to induce antigen-specific immunological tolerance. Antibodies displaying high affinity towards erythrocyte surface proteins may be isolated by screening antibody libraries using state-of-the art display platforms, including but not limited to bacteriophage display, yeast and *E. coli* surface display. Upon discovery of the novel erythrocyte-binding antibody, similar biochemical characterization of binding may be assessed as was performed with the ERY1 peptide. In order to create higher-affinity mutants with improved binding characteristics, affinity maturation is conducted on the antibody fragments discovered to bind erythrocytes from the initial library screening. Using standard recombinant DNA techniques, such as error-prone PCR and site-directed mutagenesis, a new library is created from the parent binding sequence. The affinity maturation library is then displayed using state-of-the-art display platforms, as described above, for other antibody fragments with enhanced affinity for erythrocytes as compared with the parent binding sequence.

Affinity maturation is also performed on existing antibodies that bind either mouse erythrocytes or human erythrocytes. The rat monoclonal TER-119 clone antibody (Kina et al, Br J Haematol, 2000) binds mouse erythrocytes at a site yet to be fully determined, yet its specificity has led to its common use in removal of erythrocytes from heterogeneous cellular populations. Affinity maturation is performed on the TER-119 antibody, either as a full-length antibody or as an antibody fragment such as an scFv, to discover new antibodies with increased affinity towards mouse erythrocytes. The mouse monoclonal 10F7 clone antibody (Langlois et al, J Immunol 1984) binds to human glycophorin-A on the human erythrocyte cell surface. Affinity maturation is performed on the 10F7 antibody, either as a full-length antibody or as an antibody fragment such as an scFv, to discover new antibodies with increased affinity towards human erythrocytes.

To determine the primary sequence of the TER-119 antibody, we cloned the antibody-specific isolated cDNA from the TER-119 hybridoma into a plasmid allowing for facile sequencing of the gene fragments. A specific set of primers were used for the PCR amplification process of the antibody genes that allows for amplification of the multiple variable domains of the gene segments (Krebber et al., 1997; Reddy et al., 2010). The DNA sequence of the antibody domains allowed us to determine the variable regions of the heavy and light chains of the TER-119 IgG antibody. To construct an scFv version of the TER-119 IgG, we used assembly PCR to create a gene comprising of the variable heavy chain of TER-119, followed by a $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_4$ (SEQ ID NO:18) linker, followed by the variable light chain of TER-119.

Standard reverse transcriptase PCR (RT-PCR) was performed on mRNA from the TER-119 hybridoma clone using the Superscript III First Strand Synthesis System (Invitrogen) to create complimentary DNA (cDNA) of the clone. PCR was then conducted using the following set of primers to specifically amplify the DNA sequences of the variable heavy (VH) and variable light (VL) regions of the antibody:

| Primer name | Primer sequence (5' to 3') | SEQ ID NO |
|---|---|---|
| VL-FOR1 | AGC CGG CCA TGG CGG AYA TCC AGC TGA CTC AGC C | SEQ ID NO:21 |
| VL-FOR2 | AGC CGG CCA TGG CGG AYA TTG TTC TCW CCC AGT C | SEQ ID NO:22 |
| VL-FOR3 | AGC CGG CCA TGG CGG AYA TTG TGM TMA CTC AGT C | SEQ ID NO:23 |
| VL-FOR4 | AGC CGG CCA TGG CGG AYA TTG TGY TRA CAC AGT C | SEQ ID NO:24 |
| VL-FOR5 | AGC CGG CCA TGG CGG AYA TTG TRA TGA CMC AGT C | SEQ ID NO:25 |
| VL-FOR6 | AGC CGG CCA TGG CGG AYA TTM AGA TRA MCC AGT C | SEQ ID NO:26 |
| VL-FOR7 | AGC CGG CCA TGG CGG AYA TTC AGA TGA YDC AGT C | SEQ ID NO:27 |
| VL-FOR8 | AGC CGG CCA TGG CGG AYA TYC AGA TGA CAC AGA C | SEQ ID NO:28 |
| VL-FOR9 | AGC CGG CCA TGG CGG AYA TTG TTC TCA WCC AGT C | SEQ ID NO:29 |
| VL-FOR10 | AGC CGG CCA TGG CGG AYA TTG WGC TSA CCC AAT C | SEQ ID NO:30 |
| VL-FOR11 | AGC CGG CCA TGG CGG AYA TTS TRA TGA CCC ART C | SEQ ID NO:31 |
| VL-FOR12 | AGC CGG CCA TGG CGG AYR TTK TGA TGA CCC ARA C | SEQ ID NO:32 |

| | | |
|---|---|---|
| VL-FOR13 | AGC CGG CCA TGG CGG AYA TTG TGA TGA CBC AGK C | SEQ ID NO:33 |
| VL-FOR14 | AGC CGG CCA TGG CGG AYA TTG TGA TAA CYC AGG A | SEQ ID NO:34 |
| VL-FOR15 | AGC CGG CCA TGG CGG AYA TTG TGA TGA CCC AGW T | SEQ ID NO:35 |
| VL-FOR16 | AGC CGG CCA TGG CGG AYA TTG TGA TGA CAC AAC C | SEQ ID NO:36 |
| VL-FOR17 | AGC CGG CCA TGG CGG AYA TTT TGC TGA CTC AGT C | SEQ ID NO:37 |
| VL-FOR18 | AGC CGG CCA TGG CGG ARG CTG TTG TGA CTC AGG AAT C | SEQ ID NO:38 |
| VL-REV1 | GAT GGT GCG GCC GCA GTA CGT TTG ATT TCC AGC TTG G | SEQ ID NO:39 |
| VL-REV2 | GAT GGT GCG GCC GCA GTA CGT TTT ATT TCC AGC TTG G | SEQ ID NO:40 |
| VL-REV3 | GAT GGT GCG GCC GCA GTA CGT TTT ATT TCC AAC TTT G | SEQ ID NO:41 |
| VL-REV4 | GAT GGT GCG GCC GCA GTA CGT TTC AGC TCC AGC TTG G | SEQ ID NO:42 |
| VL-REV5 | GAT GGT GCG GCC GCA GTA CCT AGG ACA GTC AGT TTG G | SEQ ID NO:43 |
| VL-REV6 | GAT GGT GCG GCC GCA GTA CCT AGG ACA GTG ACC TTG G | SEQ ID NO:44 |
| VH-FOR1 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA KGT RMA GCT TCA GGA GTC | SEQ ID NO:45 |
| VH-FOR2 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT BCA GCT BCA GCA GTC | SEQ ID NO:46 |
| VH-FOR3 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT GCA GCT GAA GSA STC | SEQ ID NO:47 |
| VH-FOR4 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT CCA RCT GCA ACA RTC | SEQ ID NO:48 |
| VH-FOR5 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT YCA GCT BCA GCA RTC | SEQ ID NO:49 |
| VH-FOR6 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT YCA RCT GCA GCA GTC | SEQ ID NO:50 |
| VH-FOR7 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT CCA CGT GAA GCA GTC | SEQ ID NO:51 |
| VH-FOR8 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GAA SST GGT GGA ATC | SEQ ID NO:52 |
| VH-FOR9 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA VGT GAW GYT GGT GGA GTC | SEQ ID NO:53 |
| VH-FOR10 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GCA GSK GGT GGA GTC | SEQ ID NO:54 |
| VH-FOR11 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA KGT GCA MCT GGT GGA GTC | SEQ ID NO:55 |
| VH-FOR12 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GAA GCT GAT GGA RTC | SEQ ID NO:56 |

| | | |
|---|---|---|
| VH-FOR13 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GCA RCT TGT TGA GTC | SEQ ID NO:57 |
| VH-FOR14 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA RGT RAA GCT TCT CGA GTC | SEQ ID NO:58 |
| VH-FOR15 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA AGT GAA RST TGA GGA GTC | SEQ ID NO:59 |
| VH-FOR16 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT TAC TCT RAA AGW GTS TG | SEQ ID NO:60 |
| VH-FOR17 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT CCA ACT VCA GCA RCC | SEQ ID NO:61 |
| VH-FOR18 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA TGT GAA CTT GGA AGT GTC | SEQ ID NO:62 |
| VH-FOR19 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GAA GGT CAT CGA GTC | SEQ ID NO:63 |
| VH-REV1 | CCC TTG AAG CTT GCT GAG GAA ACG GTG ACC GTG GT | SEQ ID NO:64 |
| VH-REV2 | CCC TTG AAG CTT GCT GAG GAG ACT GTG AGA GTG GT | SEQ ID NO:65 |
| VH-REV3 | CCC TTG AAG CTT GCT GCA GAG ACA GTG ACC AGA GT | SEQ ID NO:66 |
| VH-REV4 | CCC TTG AAG CTT GCT GAG GAG ACG GTG ACT GAG GT | SEQ ID NO:67 |

The amplified VH and VL genes were then digested with restriction endonucleases (NcoI and NotI for VL, NdeI and HindIII for VH), the gene fragments were purified following agarose electrophoresis using a standard kit (Zymo Research, Orange, CA, USA), and ligated into a cloning plasmid pMAZ360. The plasmid containing either the VH or VL gene was sequenced, and a new gene was constructed using assembly PCR to create the TER-119 scFv sequence:

5'-GAGGTGAAGCTGCAGGAGTCTGGAGGAGGCTTGGTGCAACCTGGGGGGTCTC
TGAAACTCTCCTGTGTAGCCTCAGGATTCACTTTCAGGGACCACTGGATGAATTG
GGTCCGGCAGGCTCCCGGAAAGACCATGGAGTGGATTGGAGATATTAGACCTGA
TGGCAGTGACACAAACTATGCACCATCTGTGAGGAATAGATTCACAATCTCCAG
AGACAATGCCAGGAGCATCCTGTACCTGCAGATGAGCAATATGAGATCTGATTA
CACAGCCACTTATTACTGTGTTAGAGACTCACCTACCCGGGCTGGGCTTATGGAT
GCCTGGGGTCAAGGAACCTCAGTCACTGTCTCCTCAGCCGGTGGTGGTGGTTCTG
GTGGTGGTGGTTCTGGCGGCGGCGGCTCCGGTGGTGGTGGATCCGACATTCAGAT
GACGCAGTCTCCTTCAGTCCTGTCTGCATCTGTGGGAGACAGAGTCACTCTCAAC
TGCAAAGCAAGTCAGAATATTAACAAGTACTTAAACTGGTATCAGCAAAAGCTT
GGAGAAGCTCCCAAAGTCCTGATATATAATACAAACAATTTGCAAACGGGCATC
CCATCAAGGTTCAGTGGCAGTGGATCTGGTACAGATTTCACACTCACCATCAGTA

GCCTGCAGCCTGAAGATTTTGCCACATATTTCTGCTTTCAGCATTATACTTGGCCC ACGTTTGGAGGTGGGACCAAGCTGGAAATCAAACGTACT-3' (SEQ ID NO:69), which encodes for the VH region of the TER-119 clone at the N terminus of the translated protein, followed by a $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_4$ (SEQ ID NO:18) linker domain, followed by the VL region of the TER-119 clone at the C terminus of the translated protein. The TER-119 scFv gene was constructed by amplifying the TER-119 cDNA with primers SK07 and SK08, specific for the VH region, and SK09 and SK10, specific for the VL region:

| Primer name | Primer sequence (5' to 3') | SEQ ID NO |
|---|---|---|
| SK07 | ACT CGC GGC CCA GCC GGC CAT GGC GGA GGT GAA GCT GCA GGA GTC | SEQ ID NO:70 |
| SK08 | GGA GCC GCC GCC GCC AGA ACC ACC ACC ACC AGA ACC ACC ACC ACC GGC TGA GGA GAC AGT GAC TG | SEQ ID NO:71 |
| SK09 | GGC GGC GGC GGC TCC GGT GGT GGT GGA TCC GAC ATT CAG ATG ACG CAG TC | SEQ ID NO:7972 |
| SK10 | GAC TAC TAG GCC CCC GAG GCC AGT ACG TTT GAT TTC CAG CT | SEQ ID NO:73 |

Each final completed scFv gene product was digested with SfiI and XhoI (NEB, Ipswich, MA, USA), and ligated into the same sites on the pSecTagA mammalian expression plasmid (Invitrogen, Carlsbad, CA, USA).

To affinity mature the 10F7 scFv that binds to human glycophorin-A, the gene was commercially synthesized and obtained from DNA2.0 (Menlo Park, CA, USA) as the following sequence:

5'-GTTATTACTCGCGGCCCAGCCGGCCATGGCGGCGCAGGTGAAACTGCAGCAG AGCGGCGCGGAACTGGTGAAACCGGGCGCGAGCGTGAAACTGAGCTGCAAAGC GAGCGGCTATACCTTTAACAGCTATTTTATGCATTGGATGAAACAGCGCCCGGTG CAGGGCCTGGAATGGATTGGCATGATTCGCCCGAACGGCGGCACCACCGATTAT AACGAAAAATTTAAAAACAAAGCGACCCTGACCGTGGATAAAAGCAGCAACACC GCGTATATGCAGCTGAACAGCCTGACCAGCGGCGATAGCGCGGTGTATTATTGC GCGCGCTGGGAAGGCAGCTATTATGCGCTGGATTATTGGGGCCAGGGCACCACC GTGACCGTGAGCAGCGGCGGCGGCGGCAGCGGCGGCGGCGGCAGCGGCGGCGG

CGGCAGCGATATTGAACTGACCCAGAGCCCGGCGATTATGAGCGCGACCCTGGG
CGAAAAAGTGACCATGACCTGCCGCGCGAGCAGCAACGTGAAATATATGTATTG
GTATCAGCAGAAAAGCGGCGCGAGCCCGAAACTGTGGATTTATTATACCAGCAA
CCTGGCGAGCGGCGTGCCGGGCCGCTTTAGCGGCAGCGGCAGCGGCACCAGCTA
TAGCCTGACCATTAGCAGCGTGGAAGCGGAAGATGCGGCGACCTATTATTGCCA
GCAGTTTACCAGCAGCCCGTATACCTTTGGCGGCGGCACCAAACTGGAAATTAA
ACGCGCGGCGGCGGCCTCGGGGGCCGAGGGCGGCGGTTCT-3' (SEQ ID NO:74).

Similar affinity maturation using recombinant DNA techniques described above for TER-119 is performed on the 10F7 gene to obtain a library of mutants to enable screening for enhanced binding towards human erythrocytes.

Example 5: Inducing Antigen-specific Immunological Tolerance Through Non-covalent Erythrocyte-binding with Antibody-conjugated Antigen

The antibody may be conjugated with the antigen using standard crosslinking reactions as mentioned in Example 3 and elsewhere herein. The purified antibody-antigen conjugate will exhibit induction of tolerance towards the antigen in standard mouse models of type 1 diabetes, multiple sclerosis, islet transplantation, and OVA model antigen.

In order to demonstrate the induction of tolerance towards OVA, the OVA-antibody conjugate or OVA-nanoparticle-antibody conjugate may be administered either intravenously or extravascularly to mice. At a predetermined number of days following administration, mice are to be sacrificed and lymph nodes, spleen, and blood harvested for analysis. Splenocytes and lymph node derived cells are plated and re-stimulated for 3 days *ex vivo* with OVA and/or SIINFEKL peptide, and their down-regulation of IFNγ, IL-17a, IL-2, and IL-4 expression, and up-regulation of TGF-β1, which are established evidence of tolerance, are measured by ELISA. Intracellular staining of IFNγ, IL-17a, IL-2, and IL-4 are performed using flow cytometry on splenocytes and lymph node derived cells following 6 h of *ex vivo* re-stimulation with OVA and/or SIINFEKL peptide. Furthermore, flow cytometry is used to characterize the expression profiles of CD4, CD8, and regulatory T-cells from lymph node, spleen, and blood derived cells. Additionally, blood samples are taken from mice at varying time points to measure humoral antibody responses towards the OVA antigen. A variant experiment of the *ex vivo* re-stimulation is performed to determine if systemic tolerance has been established. Mice are administered with OVA-antibody conjugate or OVA-antibody-nanoparticle conjugate as described above, OVA is re-administered 9 days later with an adjuvant (lipopolysaccharide, complete Freud's adjuvant, alum, or other), and splenocyte

responsiveness to the OVA antigen is assessed by ELISA and/or flow cytometry as described above. The OVA-antibody conjugate and/or OVA-antibody-nanoparticle formulation will render splenocytes non-responsive to the second challenge with OVA and adjuvant, which is one method to demonstrate effective establishment of systemic tolerance. Following initial administration with OVA-antibody conjugate and/or OVA-antibody-nanoparticle formulations, similar *in vivo* challenge experiments may be conducted with transgenic cell lines as a further demonstration of tolerance, such as adoptive transfer with OT-I T cells, similar to studies described in detail in Example 3. To demonstrate immune tolerance in mouse models of autoimmunity or deimmunization of therapeutic molecules, analogous antibody conjugates may be made to the relevant antigens as was described herein with OVA.

Example 6: Inducing Antigen-specific Immunological Tolerance Through Non-covalent Erythrocyte-binding with Single Chain Antibody-fused Antigen

Single chain antibody fragments (scFv's) may be used as non-covalent binders to erythrocytes. ScFv's displaying high affinity towards erythrocyte surface proteins may be isolated by screening scFv libraries using state-of-the-art display platforms. Upon discovery of the novel erythrocyte-binding antibody fragment, similar biochemical characterization of binding are to be assessed as was performed with the ERY1 peptide. As the scFv has one polypeptide chain, it will be fused to the antigen in a site-specific recombinant manner using standard recombinant DNA techniques. Depending on the nature of the antigen fusion partner, the scFv is fused to the N- or C-terminus of the antigen to create the bifunctional protein species. In the case where the major histocompatibility complex (MHC) peptide recognition sequence is known for the antigen, the peptide is also inserted into the linker domain of the scFv, thus creating a new bifunctional antibody/antigen construct containing the native termini of the scFv.

In order to demonstrate the induction of tolerance towards OVA, an OVA-scFv or OVA-nanoparticle-scFv conjugate may be administered either intravenously or extravascularly to mice. At a predetermined number of days following administration, mice are to be sacrificed and lymph nodes, spleen, and blood are to be harvested for analysis. Splenocytes and lymph node derived cells are to be plated and re-stimulated for 3 days *ex vivo* with OVA and/or SIINFEKL peptide (SEQ ID NO:3), and their down-regulation of IFNγ, IL-17a, IL-2, and IL-4 expression, and up-regulation of TGF-β1, which are established evidence of tolerance, are to be measured, e.g., by ELISA. Intracellular staining of IFNγ, IL-

17a, IL-2, and IL-4 is performed using flow cytometry on splenocytes and lymph node derived cells following 6 h of *ex vivo* re-stimulation with OVA and/or SIINFEKL peptide(SEQ ID NO:3). Furthermore, flow cytometry may be used to characterize the expression profiles of CD4, CD8, and regulatory T-cells from lymph node, spleen, and blood derived cells. Additionally, blood samples are taken from mice at varying time points to measure humoral antibody responses towards the OVA antigen. A variant experiment of the *ex vivo* re-stimulation is performed to determine if systemic tolerance has been established. Mice are administered with OVA-scFv or OVA-nanoparticle-scFv conjugate as described above, OVA is re-administered 9 days later with an adjuvant (lipopolysaccharide, complete Freud's adjuvant, alum, or other), and splenocyte responsiveness to the OVA antigen is assessed by ELISA and/or flow cytometry as described above. The OVA-scFv and/or OVA-scFv-nanoparticle formulation will render splenocytes non-responsive to the second challenge with OVA and adjuvant, thereby illustrating effective establishment of systemic tolerance. Following initial administration with OVA-scFv and/or OVA-scFv-nanoparticle formulations, similar *in vivo* challenge experiments may be conducted with transgenic cell lines to demonstrate tolerance, such as adoptive transfer with OT-I T cells, similar to studies described in detail in Example 3. To demonstrate immune tolerance in mouse models of autoimmunity or deimmunization of therapeutic molecules, analogous scFv fusions may be made to the relevant antigens as was described here with OVA.

Standard recombinant DNA techniques were used to create an antibody construct that both binds mouse erythrocytes and displays the immunodominant MHC-I epitope of OVA (SGLEQLESIINFEKL) (SEQ ID NO:75). Using overlap extension PCR, we first created a DNA fragment that encoded for the terminal 3' domain, including the SGLEQLESIINFEKL(SEQ ID NO:75) peptide with an overlapping 5' domain that is complimentary to the 3' terminus of the TER119 sequence. This DNA fragment was used as a reverse primer, along with a complimentary forward 5' primer, in a standard PCR to create the entire DNA fragment encoding for TER119-SGLEQLESIINFEKL (SEQ ID NO:75):

5'-

GAGGTGAAGCTGCAGGAGTCTGGAGGAGGCTTGGTGCAACCTGGGGGGTCTCTG
AAACTCTCCTGTGTAGCCTCAGGATTCACTTTCAGGGACCACTGGATGAATTGGG
TCCGGCAGGCTCCCGGAAAGACCATGGAGTGGATTGGAGATATTAGACCTGATG
GCAGTGACACAAACTATGCACCATCTGTGAGGAATAGATTCACAATCTCCAGAG
ACAATGCCAGGAGCATCCTGTACCTGCAGATGAGCAATATGAGATCTGATTACA
CAGCCACTTATTACTGTGTTAGAGACTCACCTACCCGGGCTGGGCTTATGGATGC

CTGGGGTCAAGGAACCTCAGTCACTGTCTCCTCAGCCGGTGGTGGTGGTTCTGGT
GGTGGTGGTTCTGGCGGCGGCGGCTCCGGTGGTGGTGGATCCGACATTCAGATG
ACGCAGTCTCCTTCAGTCCTGTCTGCATCTGTGGGAGACAGAGTCACTCTCAACT
GCAAAGCAAGTCAGAATATTAACAAGTACTTAAACTGGTATCAGCAAAAGCTTG
GAGAAGCTCCCAAAGTCCTGATATATAATACAAACAATTTGCAAACGGGCATCC
CATCAAGGTTCAGTGGCAGTGGATCTGGTACAGATTTCACACTCACCATCAGTAG
CCTGCAGCCTGAAGATTTTGCCACATATTTCTGCTTTCAGCATTATACTTGGCCCA
CGTTTGGAGGTGGGACCAAGCTGGAAATCAAACGTACTCATCATCACCATCATCA
CGGTGGCGGTTCTGGCCTGGAGCAGCTGGAGTCTATTATTAATTTCGAAAAACTG
-3' (SEQ ID NO:76). The underlined sequence denotes the gene segment encoding for SGLEQLESIINFEKL. The DNA fragment was inserted into a mammalian and prokaryotic expression vector for recombinant expression.

Standard recombinant DNA techniques were used to create an antibody construct that both binds mouse erythrocytes and displays the chromogranin-A mimetope 1040-p31 (YVRPLWVRME) (SEQ ID NO:77). Using overlap extension PCR, a DNA fragment was created that encoded for the terminal 3' domain, including the YVRPLWVRME (SEQ ID NO:77) peptide with an overlapping 5' domain that is complimentary to the 3' terminus of the TER119 sequence. This DNA fragment was used as a primer, along with a complimentary forward 5' primer, in a standard PCR to create the entire DNA fragment encoding for TER119-YVRPLWVRME:

5'-
GAGGTGAAGCTGCAGGAGTCAGGAGGAGGCTTGGTGCAACCTGGGGGGTCTCTG
AAACTCTCCTGTGTAGCCTCAGGATTCACTTTCAGGGACCACTGGATGAATTGGG
TCCGGCAGGCTCCCGGAAAGACCATGGAGTGGATTGGGGATATTAGACCTGATG
GCAGTGACACAAACTATGCACCATCTGTGAGGAATAGATTCACAATCTCCAGAG
ACAATACCAGGAGCATCCTGTACCTGCAGATGGGCAATATGAGATCTGATTACA
CAGCCACTTATTACTGTGTTAGAGACTCACCTACCCGGGCTGGGCTTATGGATGC
CTGGGGTCAAGGAACCTCAGTCACTGTCTCCTCAGCCGGTGGTGGTGGTTCTGGT
GGTGGTGGTTCTGGCGGCGGCGGCTCCGGTGGTGGTGGATCCGACATTCAGATG
ACGCAGTCTCCTTCAGTCCTGTCTGCATCTGTGGGAGACAGAGTCACTCTCAACT
GCAAAGCAAGTCAGAATATTAACAAGTACTTAAACCGGTATCAGCAAAAGCTTG
GAGAAGCTCCCAAAGTCCTGGTATATAATACAAACAATTTGCAAACGGGCATCC
CATCAAGGTTCAGTGGCAGTGGATCTGGCACAGATTTCACACTCACCATCAGTAG
CCTGCAGCCTGAAGATTTTGCCACATATTTCTGCTTTCAGCATTATACTTGGCCCA

CGTTTGGAGGTGTGACCAAGCTGGAAATCAAACGTACTCATCATCACCATCATCA
CGGTGGCGGTTATGTCAGACCTCTGTGGGTCAGAATGGAA-3'(SEQ ID NO:78). The underlined sequence denotes the gene segment encoding for the chromogranin-A (1040-p31) mimetope (YVRPLWVRME) (SEQ ID NO:77). The DNA fragment was inserted into a mammalian and prokaryotic expression vector for recombinant expression.

Standard recombinant DNA techniques were used to create an antibody construct that both binds mouse erythrocytes and displays mouse proinsulin, a major diabetes autoantigen in the NOD mouse. Using overlap extension PCR, we first created a DNA fragment that encoded for the terminal 3' domain, including the entire proinsulin protein, with an overlapping 5' domain that is complimentary to the 3' terminus of the TER119 sequence. This DNA fragment was used as a primer, along with a complimentary forward 5' primer, in a standard PCR to create the entire DNA fragment encoding for TER119-proinsulin:

5'-
GAGGTGAAGCTGCAGGAGTCAGGAGGAGGCTTGGTGCAACCTGGGGGGTCTCTG
AAACTCTCCTGTGTAGCCTCAGGATTCACTTTCAGGGACCACTGGATGAATTGGG
TCCGGCAGGCTCCCGGAAAGACCATGGAGTGGATTGGAGATATTAGACCTGATG
GCAGTGACACAAACTATGCACCATCTGTGAGGAATAGATTCACAATCTCCAGAG
ACAATGCCAGGAGCATCCTGTACCTGCAGATGAGCAATATGAGATCTGATTACA
CAGCCACTTATTACTGTGTTAGAGACTCACCTACCCGGGCTGGGCTTATGGATGC
CTGGGGTCAAGGAACCTCAGTCACTGTCTCCTCAGCCGGTGGTGGTGGTTCTGGT
GGTGGTGGTTCTGGCGGCGGCGGCTCCGGTGGTGGTGGATCCGACATTCAGATG
ACGCAGTCTCCTTCAGTCCTGTCTGCATCTGTGGGAGACAGAGTCACTCTCAACT
GCAAAGCAAGTCAGAATATTAACAAGTACTTAAACTGGTATCAGCAAAAGCTTG
GAGAAGCTCCCAAAGTCCTGATATATAATACAAACAATTTGCAAACGGGCATCC
CATCAAGGTTCAGTGGCAGTGGATCTGGTACAGATTTCACACTCACCATCAGTAG
CCTGCAGCCTGAAGATTTTGCCACATATTTCTGCTCTCAGCATTATACTTGGCCCA
CGTTTGATGGTGGGACCAAGCTGGAAATCAAACGTACTCATCATCACCATCATCA
CGGTGGCGGTTTTGTGAAACAGCATCTGTGCGGTCCGCATCTGGTGGAAGCGCTG
TATCTGGTGTGCGGCGAACGTGGCTTTTTTTATACCCCGAAAAGCCGTCGTGAAG
TGGAAGATCCGCAGGTGGAACAGCTGGAACTGGGCGGCAGCCCGGGTGATCTGC
AGACCCTGGCCCTGGAAGTGGCGCGTCAGAAACGTGGCATTGTGGATCAGTGCT
GCACCAGCATTTGCAGCCTGTATCAGCTGGAAAACTATTACAAC-3' (SEQ ID NO:79). The underlined sequence denotes the proinsulin gene segment of the construct. The DNA fragment was inserted into a mammalian and prokaryotic expression vector for

recombinant expression.

Example 7: Synthesis of Branched Polymers Comprising Erythrocyte Binding Ligands and Other Functions

For the synthesis of 8-arm PEG-thioacetate, 8-arm PEG-OH (Nektar) was dissolved in toluene and reacted for 18 h with 10 equivalents of triethylamine (Sigma Aldrich, CAS# 121-44-8) and 10 equivalents of methanesulfonyl chloride (Sigma Aldrich, CAS# 124-63-0) at room temperature under argon. The residue was filtered and the filtrate concentrated under reduced pressure, dissolved in dimethylformamide (DMF), and 10 equivalents of potassium thioacetate (Sigma Aldrich, CAS# 10387-40-3) was added. After 18 h at room temperature, the residue was filtered, the filtrate was concentrated under reduced pressure and precipitated in diethyl ether. The precipitate was filtered and dried under reduced pressure to obtain the final product.

For the synthesis of 8-arm PEG-pyridyldisulfide, 8-arm PEG-thioacetate was dissolved in dimethylformamide (DMF) and deprotected with 1.05 equivalents of sodium methoxide (Sigma Aldrich, CAS# 124-41-4) for 1 h at room temperature under argon in a Schlenk tube. To reduce the deprotected thiols to thiolates, 2 equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Thermo Scientific, CAS# 51805-45-9) and 2 equivalents of distilled water were added to the solution. After 2 h at room temperature, 12 equivalents of 2,2'-dithiodipyridine (Aldrithiol-2, Sigma Aldrich, CAS# 2127-03-9) was added and the solution was stirred at room temperature for 24 h. The reaction mixture was then dialyzed against 5 L of distilled water in MWCO 3,500 Da dialysis tubing for 48 h, during which the distilled water was changed 4 times. Pyridyldisulfide loading onto the 8-arm PEG was quantified by reduction in 25 mM TCEP in 100 mM HEPES, pH 8.0, and UV-vis spectra were measured at 343 nm to monitor the presence of the pyridine-2-thione leaving group.

For the synthesis of 8-arm PEG-pyridyldisulfide-ALEXAFLUOR647, 8-arm PEG-thioacetate was dissolved in DMF and deprotected with 1.05 equivalents of sodium methoxide (Sigma Aldrich, CAS# 124-41-4) for 1 h at room temperature under argon in a Schlenk tube. To reduce the deprotected thiols to thiolates, 2 equivalents of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Thermo Scientific, CAS# 51805-45-9) and an equal volume of 100 mM HEPES pH 8.0 were added to the solution. After 2 h at room temperature, 0.125 equivalents (equivalent to 1 arm out of 8) of AlexaFluor647-C2-maleimide (Invitrogen) was added to the solution. After 2 h at room temperature, 12

equivalents of 2,2'-dithiodipyridine (Aldrithiol-2, Sigma Aldrich, CAS# 2127-03-9) was added and the solution was stirred at room temperature for 24 h. The reaction mixture was then dialyzed against 5 L of distilled water in MWCO 3,500 Da dialysis tubing for 48 h, during which the distilled water was changed 4 times. Pyridyldisulfide loading onto the 8-arm PEG was quantified by reduction in 25 mM TCEP in 100 mM HEPES, pH 8.0, and UV-vis spectra were measured at 343 nm to monitor the presence of the pyridine-2-thione leaving group.

Thiol-containing peptides were conjugated to the 8-arm PEG-pyridyldisulfide by adding stoichiometric quantities of the peptide, dissolved in aqueous 3 M guanidine-HCl (Sigma Aldrich, CAS# 50-01-10), to the aqueous solution of 8-arm PEG-pyridyldisulfide at room temperature. Reaction conversion was monitored by measuring UV-vis spectra at 343 nm to quantify the presence of the pyridine-2-thione leaving group. If more than one molecule was to be conjugated to the 8-arm PEG-pyridyldisulfide, the reaction procedure was repeated with the new molecule in the same pot. Once conjugation was completed, the reaction mixture was desalted on a ZEBASPIN desalting column (Thermo Scientific), and the purified product was stored under the appropriate sterile conditions.

The induction of tolerance towards OVA could be demonstrated for the 8-arm PEG-ERY1/MIS-SIINFEKL conjugate (SIINFEKL: SEQ ID NO:3) by administering it either intravenously or extravascularly to mice. This test would also indicate induction of tolerance in humans using human-specific ligands. In such a demonstration, a predetermined number of days following administration, mice would be sacrificed and lymph nodes, spleen, and blood harvested for analysis. Splenocytes and lymph node derived cells are plated and re-stimulated for 3 days *ex vivo* with OVA and/or SIINFEKL (SEQ ID NO:3) peptide, and their down-regulation of IFNγ, IL-17a, IL-2, and IL-4 expression, and up-regulation of TGF-β1, which are established evidence of tolerance, are measured by ELISA. Intracellular staining of IFNγ, IL-17a, IL-2, and IL-4 is performed using flow cytometry on splenocytes and lymph node derived cells following 6 h of *ex vivo* re-stimulation with OVA and/or SIINFEKL (SEQ ID NO:3) peptide. Furthermore, flow cytometry is used to characterize the expression profiles of CD4, CD8, and regulatory T-cells from lymph node, spleen, and blood derived cells. Additionally, blood samples are taken from mice at varying time points to measure humoral antibody responses towards the OVA antigen. A variant experiment of the *ex vivo* re-stimulation is performed to determine if systemic tolerance has been established. Mice are administered with 8-arm PEG-ERY1/MIS-SIINFEKL conjugate (SIINFEKL: SEQ ID NO:3)

as described above, OVA is re-administered 9 days later with an adjuvant (lipopolysaccharide, complete Freud's adjuvant, alum, or other), and splenocyte responsiveness to the OVA antigen is assessed by ELISA and/or flow cytometry as described above. The 8-arm PEG-ERY1-SIINFEKL conjugate (SIINFEKL: SEQ ID NO:3) formulation will render splenocytes non-responsive to the second challenge with OVA and adjuvant, which is a method of illustrating effective establishment of systemic tolerance. Following initial administration of the 8-arm PEG-ERY1/MIS-SIINFEKL conjugate formulations (SIINFEKL: SEQ ID NO:3), similar *in vivo* challenge experiments could be conducted with transgenic cell lines to further demonstrate tolerance, such as adoptive transfer with OT-I T cells, similar to studies described in detail in Example 3. To demonstrate immune tolerance in mouse models of autoimmunity or deimmunization of therapeutic molecules, analogous 8-arm PEG constructs may be made to the relevant antigens as was described here with SIINFEKL (SEQ ID NO:3).

Example 8: Inducing Antigen-specific Immunological Tolerance Through Non-covalent Erythrocyte-binding with Aptamer-conjugated Antigen

Methods may be performed using other non-antibody bioaffinity reagents to measure their ability to induce immunological tolerance through non-covalent erythrocyte binding. Other protein-based affinity moieties, such as designed ankyrin repeat proteins (DARPins) (Steiner, Forrer, et al., 2008), designed armadillo repeat proteins (Parmeggiani, Pellarin, et al., 2008), fibronectin domains (Hackel, Kapila, et al., 2008), and cysteine-knot (knottin) affinity scaffolds (Silverman, Levin, et al., 2009) are screened for those displaying binding affinity to erythrocytes.

Library screening to discover high-affinity DNA/RNA aptamers towards erythrocytes is conducted using the well-established Systematic Evolution of Ligands by Exponential Enrichment (SELEX) method (Archemix, Cambridge, MA, USA) (Sampson, 2003). Upon discovery of novel DNA/RNA sequences that binds erythrocytes with high affinity, they are chemically synthesized to include an additional chemical reactive group on either their 3' or 5' terminus for conjugation to an antigen and/or polymer micelle/nanoparticle. The chemically synthesized aptamer does, for example, harbor a reactive NH2 group, that is conjugated via EDC/NHS conjugation chemistry with the COOH groups present on either the nanoparticle or antigen or nanoparticle-antigen complex, to create a single bioconjugate comprising of the erythrocyte-binding aptamer and the antigen or antigen-nanoparticle. Various chemical conjugation techniques are attempted by altering orthogonal reactive

groups and conjugation schemes on both the aptamer, antigen, and/or antigen-nanoparticle.

In order to demonstrate the induction of tolerance towards OVA, the OVA-aptamer or OVA-nanoparticle-aptamer conjugate is administered either intravenously or extravascularly to mice. At a predetermined number of days following administration, mice are sacrificed and lymph nodes, spleen, and blood are harvested for analysis. Splenocytes and lymph node derived cells are plated and re-stimulated for 3 days *ex vivo* with OVA and/or SIINFEKL peptide (SEQ ID NO:3), and their down-regulation of IFNγ, IL-17a, IL-2, and IL-4 expression, and up-regulation of TGF-β1, which are established evidence of tolerance, is measured by ELISA. Intracellular staining of IFNγ, IL-17a, IL-2, and IL-4 is performed using flow cytometry on splenocytes and lymph node derived cells following 6 h of *ex vivo* re-stimulation with OVA and/or SIINFEKL (SEQ ID NO:3) peptide. Furthermore, flow cytometry is used to characterize the expression profiles of CD4, CD8, and regulatory T-cells from lymph node, spleen, and blood derived cells. Additionally, blood samples are taken from mice at varying time points to measure humoral antibody responses towards the OVA antigen. A variant experiment of the *ex vivo* re-stimulation is performed to determine if systemic tolerance has been established. Mice are administered with OVA-antibody or OVA-antibody-nanoparticle conjugate as described above, OVA is re-administered 9 days later with an adjuvant (lipopolysaccharide, complete Freud's adjuvant, alum, or other), and splenocyte responsiveness to the OVA antigen is assessed by ELISA and/or flow cytometry as described above. The OVA-antibody and/or OVA-antibody-nanoparticle formulations are expected to render splenocytes non-responsive to the second challenge with OVA and adjuvant, thereby illustrating effective establishment of systemic tolerance. Following initial administration with the OVA-aptamer and/or OVA-aptamer-nanoparticle formulations, similar *in vivo* challenge experiments will be conducted with transgenic cell lines to demonstrate tolerance, such as adoptive transfer with OT-I T cells, similar to studies described in detail in Example 3. To demonstrate immune tolerance in mouse models of autoimmunity or deimmunization of therapeutic molecules, analogous aptamer constructs are made to the relevant antigens as was described here with OVA.

Example 9: Characterizing of Binding to Human Erythrocytes

To characterize the selected bacterially displayed peptides that bound to human erythrocytes in a non-cell displayed context, the peptides were synthesized chemically and conjugated to the fluorescent protein allophycocyanin (APC). In this manner, the

erythrocyte-binding capacity of each peptide was characterized in a soluble context, i.e. as a protein conjugate. As demonstrated in Fig. 6, peptides ERY64, ERY123, and ERY141 bound to human erythrocytes as conjugates of APC

*APC-peptide conjugation method*

Peptides were ordered and custom-synthesized via standard fmoc solid-phase peptide synthesis from PolyPeptide Group (Strasbourg, France). 10 equivalents of peptide dissolved in 3 M guanidine-HCl were added to 2 mg/mL maleimide-activated APC (InnovaBiosciences, Cambridge, UK) in PBS. Following a 4 h incubation at 4°C, the reaction was desalted on a 2 mL ZEBA Desalting Column (Thermo Scientific) and stored at 4°C.

*Quantification of erythrocyte binding by flow cytometry method*

Freshly isolated human blood was dissolved 100-fold into PBS supplemented with 20 mg/mL BSA. $5x10^5$ erythrocytes were added to 150 μL of 1 μM APC-peptide conjugate and incubated at 37 C for 45 min. Cells were washed extensively in PBS + 20 mg/mL BSA, and analyzed for APC fluorescence on a CyAn ADP Flow cytometer (Beckman Coulter).

Example 10: Inducing Antigen-specific Immunological Tolerance Through Erythrocyte-binding with Single Chain Antibody-fused Antigen

A recombinant fusion of an scFv to the MHC-I immunodominant domain of OVA, SIINFEKL was created. The resultant scFv (herein termed TER119-SIINFEKL) bound mouse erythrocytes, as demonstrated by flow cytometry in Fig 7.

Since ERY1-OVA has already been shown herein to induce this tolerance, it is a useful system to characterize immunological events. It was determined that erythrocyte binding of ERY1-OVA led to efficient cross-presentation of the OVA immunodominant MHC I epitope (SIINFEKL) by antigen presenting cells (APCs) and corresponding cross-priming of reactive T cells. CFSE-labeled OT-I $CD8^+$ T cells ($CD45.2^+$) were adoptively transferred into $CD45.1^+$ mice, and then proliferation of the OT-I $CD8^+$ T cells over 5 days was measured following intravenous administration of 10 μg of OVA, 10 μg ERY1-OVA, an equimolar dose of TER119-SIINFEKL, or an equimolar dose of SIINFEKL. OT-I $CD8^+$ T cell proliferation, determined by dilution of the fluor CFSE as measured by flow cytometry, was markedly enhanced in mice administered TER119-SIINFEKL compared to ERY1-OVA or OVA (Fig. 8), demonstrating that erythrocyte-binding increased antigen-specific $CD8^+$ T cell cross-priming compared to the soluble antigen SIINFEKL.

To distinguish T cells being expanded into a functional effector phenotype from those being expanded and deleted, the proliferating OT-I $CD8^+$ T cells were analyzed for annexin-V, as a hallmark of apoptosis and thus deletion, as well as the exhaustion marker programmed death-1 (PD-1). TER119-SIINFEKL and ERY1-OVA induced much higher numbers of annexin-$V^+$ and PD-$1^+$ proliferating OT-I $CD8^+$ T cells than OVA (Fig. 9).

Using an established OT-I challenge-to-tolerance model (Liu, Iyoda, et al., 2002), the ability of TER119-SIINFEKL and ERY1-OVA to prevent subsequent immune responses to vaccine-mediated antigen challenge were demonstrated - even with a challenge involving a very strong bacterially-derived adjuvant. To tolerize, 10 μg of either OVA or ERY1-OVA, or an equimolar dose of TER119-SIINFEKL were intravenously administered at 1 and 6 days following adoptive transfer of OT-I $CD8^+$ ($CD45.2^+$) T cells to $CD45.1^+$ mice. After 9 additional days to allow potential deletion of the transferred T cells, the recipient mice were then challenged with OVA adjuvanted with lipopolysaccharide (LPS) by intradermal injection. Characterization of draining lymph node and spleen cells as well as their inflammatory responses 4 d after challenge allowed a determination as to whether or not deletion actually took place.

Intravenous administration of TER119-SIINFEKL or ERY1-OVA resulted in profound reductions in OT-I $CD8^+$ T cell populations in the draining lymph nodes and spleens compared with mice administered unmodified OVA prior to antigen challenge with LPS, demonstrating deletional tolerance. Draining lymph nodes from ERY1-OVA-treated mice contained over 11-fold fewer OT-I $CD8^+$ T cells as compared to OVA-treated mice, and 39-fold fewer than challenge control mice that did not receive intravenous injections of antigen; responses in spleen cells were similar. Draining lymph nodes from TER119-SIINFEKL-treated mice contained over 13-fold fewer OT-I $CD8^+$ T cells as compared to OVA-treated mice, and over 42-fold fewer than challenge control mice that did not receive intravenous injections of antigen; responses in spleen cells were similar. This effective clonal deletion exhibited in mice administered TER119-SIINFEKL or ERY1-OVA supported the earlier observations of enhanced OT-I $CD8^+$ T cell cross-priming (Fig. 8) and furthermore shows that cross-priming occurred in the absence of APC presentation of co-stimulatory molecules to lead to deletional tolerance.

To further evaluate the immune response following antigen challenge, the inflammatory nature of resident lymph node and spleen cells was characterized by expression of interferon-γ (IFNγ) by OT-I $CD8^+$ T cells (Fig. 10). Following challenge with OVA and LPS, the lymph nodes of mice previously treated with ERY1-OVA harbored 10-fold fewer

IFNγ-expressing cells compared to challenge control mice (previously receiving no antigen), and over 4-fold fewer IFNγ-expressing cells compared to mice previously treated with an equivalent dose of OVA. Following challenge with OVA and LPS, the lymph nodes of mice previously treated with TER119-SIINFEKL harbored 33-fold fewer IFNγ-expressing cells compared to challenge control mice (previously receiving no antigen), and over 14-fold fewer IFNγ-expressing cells compared to mice previously treated with an equivalent dose of OVA, demonstrating the importance of erythrocyte binding in tolerogenic protection to challenge; responses in spleen cells were similar.

*Animal methods*

Swiss Veterinary authorities previously approved all animal procedures. C57BL/6-Tg(TcraTcrb) 1100Mjb (OT-I) mice (Jackson Labs) were bred at the EPFL Animal Facility, and females were used for splenocyte isolation at 6-12 wk old. 8-12 week old female B6.SJL-*Ptprc*$^{a}$*Pepc*$^{b}$/Boy (CD45.1) mice (Charles River) were used as recipient hosts for OT-I CD8$^{+}$ T cell adoptive transfer and tolerance induction studies.

*Peptide design and synthesis methods*

The ERY1 ($H_2N$-WMVLPWLPGTLDGGSGCRG-$CONH_2$) peptide (SEQ ID NO:128) was synthesized using standard solid-phase f-moc chemistry using TGR resin (Nova Biochem) on an automated liquid handler (Chemspeed). The underlined sequence is the ERY1 12-mer sequence that we previously discovered by phage display as a mouse glycophorin-A binder (Kontos and Hubbell, 2010). The GGSG region served as a linker to the cysteine residue used for conjugation; the flanking arginine residue served to lower the pKa and thus increase the reactivity of the cysteine residue (Lutolf, Tirelli, et al., 2001). The peptide was cleaved from the resin for 3 h in 95% tri-fluoroacetic acid, 2.5% ethanedithiol, 2.5% water, and precipitated in ice-cold diethyl ether. Purification was conducted on a preparative HPLC-MS (Waters) using a C18 reverse phase column (PerSpective Biosystems).

*ERY1-antigen conjugation methods*

10 molar equivalents of succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, CAS# 64987-85-5, Thermo Scientific) dissolved in dimethylformamide were reacted with 5 mg/mL endotoxin-free (<1 EU/mg) OVA (Hyglos GmbH) in PBS for 1 h at room temperature. Following desalting on a 2 mL ZEBA Desalt spin column (Thermo Scientific), 10 equivalents of ERY1 peptide dissolved in 3 M guanidine-HCl were added and

allowed to react for 2 h at room temperature. The conjugate was desalted using 2 mL Zeba Desalt spin columns, 0.2 μm sterile filtered, dispensed into working aliquots, and stored at -20 C. Protein concentration was determined via BCA Assay (Thermo Scientific). The scheme results in conjugation of the cysteine side chain on the peptide to lysine side-chains on the antigen.

*T cell adoptive transfer methods*

$CD8^+$ T cells from OT-I ($CD45.2^+$) mouse spleens were isolated using a CD8 magnetic bead negative selection kit (Miltenyi Biotec) as per the manufacturer's instructions. Freshly isolated $CD8^+$ OT-I cells were resuspended in PBS and labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 6 min at room temperature, and the reaction was quenched for 1 min with an equal volume of IMDM with 10% FBS (Gibco). Cells were washed, counted, and resuspended in pure IMDM prior to injection. $3x10^6$ CFSE-labeled $CD8^+$ OT-I cells were injected intravenously into the tail vein of recipient $CD45.1^+$ mice. For short-term proliferation studies, 10 μg of ERY1-OVA or OVA, or an equimolar dose of TER119-SIINFEKL in 100 μL volume was injected 24 h following adoptive transfer. Splenocytes were harvested 5 d following antigen administration and stained for analysis by flow cytometry.

*OT-I tolerance and challenge model methods*

$3x10^5$ CFSE-labeled OT-I $CD8^+$ T cells were injected into $CD45.1^+$ recipient mice as described above. 1 and 6 d following adoptive transfer, mice were intravenously administered 10 μg of ERY1-OVA or OVA, or an equimolar dose of TER119-SIINFEKL in 100 μL saline into the tail vein. 15 d following adoptive transfer, mice were challenged with 5 μg OVA and 25 ng ultra-pure *Escherichia coli* LPS (InvivoGen) in 25 μL intradermally into each rear leg pad (Hock method, total dose of 10 μg OVA and 50 ng LPS). Mice were sacrificed 4 d following challenge, and spleen and draining lymph node cells were isolated for restimulation. For flow cytometry analysis of intracellular cytokines, cells were restimulated in the presence of 1 mg/mL OVA or 1 μg/mL SIINFEKL peptide (Genscript) for 3 h. Brefeldin-A (Sigma, 5 μg/mL) was added and restimulation resumed for an additional 3 h prior to staining and flow cytometry analysis.

*Antibodies and flow cytometry methods*

The following anti-mouse antibodies were used for flow cytometry: CD1d Pacific

Blue, CD3ε PerCP-Cy5.5, CD8α PE-Cy7, CD45.2 Pacific Blue, IFNγ-APC, CD8α APC-eF780, (all from eBioscience), in addition to fixable live/dead dye (Invitrogen), and annexin-V-Cy5 labeling kit (BioVision). Samples were analyzed on a CyAn ADP flow cytometer (Beckman Coulter). Cells were washed first with PBS, stained for 20 min on ice with live/dead dye, blocked for 20 min on ice with 24G2 hybridoma medium, surface stained for 20 min on ice, fixed in 2% paraformaldehyde for 20 min ice, intracellularly stained in the presence of 0.5% saponin for 45 min on ice, followed by a final wash prior to analysis. For apoptosis staining, annexin-V-Cy5 was added 5 min prior to analysis.

Example 11: Creation of Antigen-specific Immunological Tolerance Towards Therapeutic Proteins Through Fusion Molecule of Erythrocyte Binding Peptide-and-Conjugated Antigen.

A mouse erythrocyte-binding variant of asparaginase (ERY1-ASNase) was created by conjugating the mouse erythrocyte-specific peptide ERY1 (Kontos and Hubbell, 2010). To determine the immunogenicity of erythrocyte-bound versus wild-type asparaginase, a study was performed to evaluate to evaluate two regimens, namely exposure to a 2-dose and a 6-dose regimen of the ERY conjugate (ERY1-ASNase) or the native form (ASPARAGINASE MEDAC, Medac GmbH; which was also used as the raw material for the ERY1-ASNase conjugate). In each regimen, 15 μg of ERY1-ASNase or ASNase was administered intravenously every 2 days, and blood was drawn every 7 days.

Following the exposures at therapeutic doses, antibody titers were measured at various time points, up to 21 days following the final injection. The result is clear in showing vast reduction in immunogenicity through erythrocyte conjugation, with no antibodies observable at all in the 2-dose regimen and the 6-dose regimen series (Fig 11). By contrast, the wild type, native asparaginase (as currently used clinically, in the product ASPARAGINASE MEDAC), induced vigorous immunity even following the 2 dose regimen.

A prophylaxis study was performed to determine if erythrocyte-binding asparaginase induced *bona-fide* immune tolerance towards wild-type asparaginase. Mice previously administered with wild-type clinical asparaginase were challenged with an additional dose following the onset of humoral immunity. Antibody levels increased or remained high following re-administration of the protein; this is representative of a dangerous clinical situation of hypersensitivity and shock reactions towards the therapeutic. Mice previously treated with ERY1-conjugated asparaginase failed to induce potent antibody responses even after six challenges with wild-type asparaginase. On average, mice tolerized with

erythrocyte-binding asparaginase developed approx. 6000-fold fewer antibodies towards clinical wild-type asparaginase.

*ERY1-ASNase conjugation method*

10 molar equivalents of succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, CAS# 64987-85-5, Thermo Scientific) dissolved in dimethylformamide were reacted with 5 mg/mL Asparaginase Medac in PBS for 2 h at room temperature. Following desalting on a 2 mL ZEBA Desalt spin column (Thermo Scientific), 2.5 equivalents of ERY1 peptide dissolved in 3 M guanidine-HCl were added and allowed to react for 2 h at room temperature. The conjugate was desalted using 2 mL Zeba Desalt spin columns, 0.2 μm sterile filtered, dispensed into working aliquots, and stored at -20 C. Protein concentration was determined via BCA Assay (Thermo Scientific). The scheme results in conjugation of the cysteine side chain on the peptide to lysine side-chains on the protein.

*Flow cytometry detection of binding method*

Freshly isolated human blood was diluted 100-fold into PBS supplemented with 20 mg/mL BSA. Approximately 500,000 erythrocytes were added to 100 nM of asparaginase and incubated at 37 C for 1 h. Following washing, cells were incubated with goat anti-asparaginase (Abnova) for 30 min on ice. Following a second round of washing, cells were incubated with ALEXAFLUOR488-conjugated anti-goat IgG antibody (Invitrogen) for 20 min on ice. After a final wash, cells were analyzed on a flow cytometer to detect for erythrocyte-bound asparaginase.

*Asparaginase administration method*

Desired dosages of ERY1-ASNase or ASNase were prepared in sterile 0.9% saline, and 100 μL of the solution was injected into the tail vein of anesthetized C57BL/6 mice. Blood was drawn at pre-determined time points either by cheek puncture or tail incisions.

*Anti-asparaginase antibody detection from serum method* Serum from experimental groups was serially diluted into PBS and incubated for 2 h at RT on ASNase-coated ELISA plates. HRP-conjugated anti-mouse IgG (Southern Biotech) was used as the detection antibody.

Example 12: Immune Reversal to Create Tolerance after Immunoreaction

In the clinical case where immunity (exemplified by the presence of anti-drug-antibodies) towards a therapeutic protein or protein of interest has already been induced in the

patient, it is desirable to reverse the immune response towards the drug, thus enabling further use of the therapeutic. Again, the clinically available microbial enzyme asparaginase was used in this example to demonstrate the induction of tolerance towards a therapeutic protein in mice with pre-existing immunity towards asparaginase. A mouse erythrocyte-binding variant of asparaginase (ERY1-ASNase) was created by conjugating the mouse erythrocyte-specific peptide ERY1.

A cross-over study was performed to determine if erythrocyte-binding asparaginase could reverse immunity towards clinical asparaginase. Mice were intravenously administered multiple doses of wild-type asparaginase two days apart. 21 days following administration, high levels of anti-asparaginase antibodies were detected in serum. Immunized mice were then therapeutically treated with erythrocyte-binding asparaginase regimens at varying doses. In both dosing regimens, ERY-conjugated asparaginase reduced antibody levels approx. 10-fold, thus reversing pre-existing humoral immunity

*ERY1-ASNase conjugation method*

10 molar equivalents of succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, CAS# 64987-85-5, Thermo Scientific) dissolved in dimethylformamide were reacted with 5 mg/mL ASPARAGINASE MEDAC in PBS for 2 h at room temperature. Following desalting on a 2 mL ZEBA Desalt spin column (Thermo Scientific), 2.5 equivalents of ERY1 peptide dissolved in 3 M guanidine-HCl were added and allowed to react for 2 h at room temperature. The conjugate was desalted using 2 mL ZEBA Desalt spin columns, 0.2 μm sterile filtered, dispensed into working aliquots, and stored at -20 C. Protein concentration was determined via BCA Assay (Thermo Scientific). The scheme results in conjugation of the cysteine side chain on the peptide to lysine side-chains on the protein.

*Flow cytometry detection of binding method*

Freshly isolated human blood was diluted 100-fold into PBS supplemented with 20 mg/mL BSA. Approximately 500,000 erythrocytes were added to 100 nM of asparaginase and incubated at 37 C for 1 h. Following washing, cells were incubated with goat anti-asparaginase (Abnova) for 30 min on ice. Following a second round of washing, cells were incubated with ALEXAFLUOR488-conjugated anti-goat IgG antibody (Invitrogen) for 20 min on ice. After a final wash, cells were analyzed on a flow cytometer to detect for erythrocyte-bound asparaginase.

*Asparaginase administration method*

Desired dosages of ERY1-ASNase or ASNase were prepared in sterile 0.9% saline, and 100 μL of the solution was injected into the tail vein of anesthetized C57BL/6 mice. Blood was drawn at pre-determined time points either by cheek puncture or tail incisions.

*Anti-asparaginase antibody detection from serum method*

Serum from experimental groups was serially diluted into PBS and incubated for 2 h at RT on ASNase-coated ELISA plates. HRP-conjugated anti-mouse IgG (Southern Biotech) was used as the detection antibody.

Example 13: Development of antibodies and antibody-fragments that bind mouse and/or human erythrocytes

Several erythrocyte binding antigen constructs have been created. These include those relating to the protein level: TER119-SIINFEKL, TER119-ChrA, and TER119-proinsulin. These also include those relating to the genetic level: TER119-SIINFEKL, TER119-ChrA TER119-proinsulin, TER119-uricase, TER119-InsB9-23, TER119-ISQAVHAAHAEINEAGR (SEQ ID NO:80), TER119-H-2kb, TER119-H-2kd, 10F7-SIINFEKL, 10F7-ChrA, 10F7-proinsulin, and 10F7-uricase.

*Methods*

mRNA from the TER-119 hybridoma clone was obtained as a gift from Prof. Shozo Izui at the University of Geneva, Switzerland. Standard reverse transcriptase PCR (RT-PCR) was performed using the SUPERSCRIPT III FIRST STRAND SYNTHESIS SYSTEM (Invitrogen) to create complimentary DNA (cDNA) of the clone. PCR was then conducted using the following set of primers to specifically amplify the DNA sequences of the variable heavy (VH) and variable light (VL) regions of the antibody:

| Primer name | Primer sequence (5' to 3') | SEQ ID NO |
|---|---|---|
| VL-FOR1 | AGC CGG CCA TGG CGG AYA TCC AGC TGA CTC AGC C | SEQ ID NO:81 |
| VL-FOR2 | AGC CGG CCA TGG CGG AYA TTG TTC TCW CCC AGT C | SEQ ID NO:82 |
| VL-FOR3 | AGC CGG CCA TGG CGG AYA TTG TGM TMA CTC AGT C | SEQ ID NO:83 |
| VL-FOR4 | AGC CGG CCA TGG CGG AYA TTG TGY TRA CAC AGT C | SEQ ID NO:84 |

| | | |
|---|---|---|
| VL-FOR5 | AGC CGG CCA TGG CGG AYA TTG TRA TGA CMC AGT C | SEQ ID NO:85 |
| VL-FOR6 | AGC CGG CCA TGG CGG AYA TTM AGA TRA MCC AGT C | SEQ ID NO:86 |
| VL-FOR7 | AGC CGG CCA TGG CGG AYA TTC AGA TGA YDC AGT C | SEQ ID NO:87 |
| VL-FOR8 | AGC CGG CCA TGG CGG AYA TYC AGA TGA CAC AGA C | SEQ ID NO:88 |
| VL-FOR9 | AGC CGG CCA TGG CGG AYA TTG TTC TCA WCC AGT C | SEQ ID NO:89 |
| VL-FOR10 | AGC CGG CCA TGG CGG AYA TTG WGC TSA CCC AAT C | SEQ ID NO:90 |
| VL-FOR11 | AGC CGG CCA TGG CGG AYA TTS TRA TGA CCC ART C | SEQ ID NO:91 |
| VL-FOR12 | AGC CGG CCA TGG CGG AYR TTK TGA TGA CCC ARA C | SEQ ID NO:92 |
| VL-FOR13 | AGC CGG CCA TGG CGG AYA TTG TGA TGA CBC AGK C | SEQ ID NO:93 |
| VL-FOR14 | AGC CGG CCA TGG CGG AYA TTG TGA TAA CYC AGG A | SEQ ID NO:94 |
| VL-FOR15 | AGC CGG CCA TGG CGG AYA TTG TGA TGA CCC AGW T | SEQ ID NO:95 |
| VL-FOR16 | AGC CGG CCA TGG CGG AYA TTG TGA TGA CAC AAC C | SEQ ID NO:96 |
| VL-FOR17 | AGC CGG CCA TGG CGG AYA TTT TGC TGA CTC AGT C | SEQ ID NO:97 |
| VL-FOR18 | AGC CGG CCA TGG CGG ARG CTG TTG TGA CTC AGG AAT C | SEQ ID NO:98 |
| VL-REV1 | GAT GGT GCG GCC GCA GTA CGT TTG ATT TCC AGC TTG G | SEQ ID NO:99 |
| VL-REV2 | GAT GGT GCG GCC GCA GTA CGT TTT ATT TCC AGC TTG G | SEQ ID NO:100 |
| VL-REV3 | GAT GGT GCG GCC GCA GTA CGT TTT ATT TCC AAC TTT G | SEQ ID NO:101 |
| VL-REV4 | GAT GGT GCG GCC GCA GTA CGT TTC AGC TCC AGC TTG G | SEQ ID NO:102 |
| VL-REV5 | GAT GGT GCG GCC GCA GTA CCT AGG ACA GTC AGT TTG G | SEQ ID NO:103 |
| VL-REV6 | GAT GGT GCG GCC GCA GTA CCT AGG ACA GTG ACC TTG G | SEQ ID NO:104 |
| VH-FOR1 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA KGT RMA GCT TCA GGA GTC | SEQ ID NO:105 |
| VH-FOR2 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT BCA GCT BCA GCA GTC | SEQ ID NO:106 |
| VH-FOR3 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT GCA GCT GAA GSA STC | SEQ ID NO:107 |
| VH-FOR4 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT CCA RCT GCA ACA RTC | SEQ ID NO:108 |

| | | |
|---|---|---|
| VH-FOR5 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT YCA GCT BCA GCA RTC | SEQ ID NO:109 |
| VH-FOR6 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT YCA RCT GCA GCA GTC | SEQ ID NO:110 |
| VH-FOR7 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT CCA CGT GAA GCA GTC | SEQ ID NO:111 |
| VH-FOR8 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GAA SST GGT GGA ATC | SEQ ID NO:112 |
| VH-FOR9 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA VGT GAW GYT GGT GGA GTC | SEQ ID NO:113 |
| VH-FOR10 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GCA GSK GGT GGA GTC | SEQ ID NO:114 |
| VH-FOR11 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA KGT GCA MCT GGT GGA GTC | SEQ ID NO:115 |
| VH-FOR12 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GAA GCT GAT GGA RTC | SEQ ID NO:116 |
| VH-FOR13 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GCA RCT TGT TGA GTC | SEQ ID NO:117 |
| VH-FOR14 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA RGT RAA GCT TCT CGA GTC | SEQ ID NO:118 |
| VH-FOR15 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA AGT GAA RST TGA GGA GTC | SEQ ID NO:119 |
| VH-FOR16 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT TAC TCT RAA AGW GTS TG | SEQ ID NO:120 |
| VH-FOR17 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GCA GGT CCA ACT VCA GCA RCC | SEQ ID NO:121 |
| VH-FOR18 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA TGT GAA CTT GGA AGT GTC | SEQ ID NO:122 |
| VH-FOR19 | GTT ATT GCT AGC GGC TCA GCC GGC AAT GGC GGA GGT GAA GGT CAT CGA GTC | SEQ ID NO:123 |
| VH-REV1 | CCC TTG AAG CTT GCT GAG GAA ACG GTG ACC GTG GT | SEQ ID NO:124 |
| VH-REV2 | CCC TTG AAG CTT GCT GAG GAG ACT GTG AGA GTG GT | SEQ ID NO:125 |
| VH-REV3 | CCC TTG AAG CTT GCT GCA GAG ACA GTG ACC AGA GT | SEQ ID NO:126 |
| VH-REV4 | CCC TTG AAG CTT GCT GAG GAG ACG GTG ACT GAG GT | SEQ ID NO:127 |

The amplified VH and VL genes were then digested with restriction endonucleases (NcoI and NotI for VL, NdeI and HindIII for VH), the gene fragments were purified following agarose electrophoresis using a standard kit (Zymo Research, Orange, CA, USA), and ligated into a cloning plasmid pMAZ360. The cloning plasmid was then sequenced to determine the DNA sequence of the VH and VL genes of TER119.

TER119 and 10F7-antigen DNA constructs were designed and commercially synthesized and obtained from DNA2.0 (Menlo Park, CA, USA).

Each final completed scFv gene was digested with SfiI and XhoI (NEB, Ipswich, MA, USA), and ligated into the same sites on the pSecTagA mammalian expression plasmid (Invitrogen, Carlsbad, CA, USA).

Example 14: Induction of Tolerance Towards Diabetogenic Antigens using Erythrocyte-binding Diabetes Antigens

Chromogranin-A mimotope peptide (termed 1040-p31 or ChrA) was engineered to bind to mouse erythrocytes by recombinantly fusing the peptide to the mouse erythrocyte-specific scFv TER119. The resultant scFv, herein termed TER119-ChrA, tightly bound mouse erythrocytes (data not shown). To study T cell behavior towards the chromogranin-A antigen variants, the NODBDC2.5 transgenic mouse was used, which harbors T cells specific for the recognition of the chromogranin-A antigen. To determine if TER119-ChrA was processed and displayed on major histocompatibility-II (MHC-II) complexes in a manner sufficient for BDC2.5 T cell priming, the antigen was added to a co-culture system with BDC2.5 splenic dendritic cells and CD4 T cells. As demonstrated in Figure 12, TER119-ChrA was efficiently processed and the correct antigenic domain displayed on MHC-II to prime antigen-specific proliferation of the transgenic BDC2.5 CD4 T cells *in vitro*, which is a requisite for T cell tolerance *in vivo*.

To determine the consequences of erythrocyte binding of the autoantigen on CD4 proliferation *in vivo*, a similar proliferation study was conducted using BDC2.5 CD4 T cells labeled with the fluorescent molecule CFSE. Following adoptive transfer of the labeled BDC2.5 CD4 T cells into naïve NOD mice, either 10 μg of TER119-ChrA or an equimolar dose of free soluble 1040-p31 peptide was administered intravenously. As is demonstrated in Fig. 13, mice treated with TER119-ChrA harbored far fewer non-proliferating BDC2.5 CD4 T cells in both the spleen and pancreatic lymph nodes (pLN) compared with mice treated with the soluble 1040-p31 peptide. These data demonstrate that erythrocyte-binding antigen drives potent antigen-specific CD4 T cell signaling and priming in a manner functionally distinct from soluble antigen, similar to the inventors thorough investigations of CD8 T cell deletional tolerance. Such marked reduction in systemic (spleen) and local (pancreatic lymph node) diabetogenic T cells is clear positive indication of a favorable outcome for control of disease onset.

Example 15: Induction of Tolerance Towards Insulin

As already demonstrated herein (for instance, see Example 14), tolerance can be

created towards various antigens. This example describes how tolerance to insulin can be created. In order to induce tolerance towards insulin, which is another islet antigen recognized by diabetogenic T cells, insulin is engineered to bind mouse erythrocytes by recombinant fusion to the mouse erythrocyte-specific scFv TER119. The resultant scFv, herein termed TER119-proinsulin, is implemented in the conventional mouse model of spontaneous onset T1D, namely the NOD/ShiLt mouse. The NOD/ShiLt mouse spontaneously undergoes immune cell mediated destruction of insulin-producing pancreatic tissue, thus inducing hyperglycemia and clinical onset of disease. Similarly, human insulin and human erythrocyte binding factors may be used to create tolerance to insulin in humans.

To demonstrate tolerance in this robust spontaneously autoimmune mouse model, TER119-proinsulin is to be administered to young (~3 week old) mice at varying time intervals and doses in order to functionally inactivate and/or delete insulin-reactive T cells. Glucose levels of the treated mice are monitored to assess hyperglycemia and clinical onset of disease. TER119-proinsulin is also used in a therapeutic mode to induce remission of disease. In such a study, TER119-proinsulin is administered to new-onset hyperglycemic mice at varying doses and time intervals, and glucose levels are monitored to assess reduction of hyperglycemia and return to homeostasis, thus demonstrating remission of clinical onset of T1D.

Example 16: Creating Tolerance Towards cellular grafts and transplants by using erythrocyte-binding MHC molecules

A process of creating tolerance of grafts is provided by way of example. Erythrocyte-binding MHC molecules are engineered by chemically conjugating erythrocyte-binding peptides to soluble MHC domains. Alternatively, the MHC molecule may be recombinantly expressed as a fusion with an erythrocyte-specific scFv.

In order to demonstrate tolerance towards MHC molecules by erythrocyte binding, mouse models of cellular grafting, such as tumor grafts, skin grafts, islet transplants, and hematopoietic cell transplants are conducted. In each study, the host mouse is tolerized towards the donor MHC class by administration of erythrocyte-binding MHC prior to or during the graft procedure. Graft acceptance is monitored using methods appropriate for each study; i.e., measurement of tumor growth in tumor grafts, monitoring tissue necrosis or growth in skin transplant, monitoring islet mass and glycemia in islet transplants, and characterizing chimerism in hematopoietic cell transplants.

FURTHER DISCLOSURE

An embodiment is a method of producing immunotolerance, the method comprising administering a composition comprising a molecular fusion that comprises a tolerogenic antigen and an erythrocyte-binding moiety that specifically binds an erythrocyte in the patient and thereby links the antigen to the erythrocyte, wherein the molecular fusion is administered in an amount effective to produce immunotolerance to a substance that comprises the tolerogenic antigen. An embodiment is the method wherein the molecular fusion consists of at least one erythrocyte-binding moiety directly covalently bonded to the antigen: for instance, a fusion protein comprising the moiety and the antigen. An embodiment is the method wherein the molecular fusion comprises at least one erythrocyte-binding moiety attached to a particle that is attached to or contains the antigen, e.g., wherein the particle is chosen from the group consisting of a microparticle, a nanoparticle, a liposome, a polymersome, and a micelle. An embodiment is the case wherein the tolerogenic antigen comprises a portion of a therapeutic protein, e.g., the protein comprises factor VIII or factor IX. An embodiment is the case wherein the tolerogenic antigen comprises a portion of a nonhuman protein. An embodiment is the case wherein the protein comprises adenosine deaminase, L-asparaginase, rasburicase, antithymocyte globulin, L-arginase, and L-methionase. An embodiment is the method wherein the patient is a human and the tolerogenic antigen comprises a portion of a protein not found in nature. An embodiment is the case wherein the patient is a human and the tolerogenic antigen comprises a glycan of a protein that comprises nonhuman glycosylation. An embodiment is the case wherein the tolerogenic antigen comprises at least a portion of a human transplantation antigen. An embodiment is the case wherein the tolerogenic antigen comprises a portion of a human autoimmune disease protein, e.g., chosen from the group consisting of preproinsulin, proinsulin, insulin, GAD65, GAD67, IA-2, IA-2β, thyroglobulin, thyroid peroxidase, thyrotropin receptor, myelin basic protein, myelin oligodendrocyte glycoprotein, proteolipid protein, collagen II, collagen IV, acetylcholine receptor, matrix metalloprotein 1 and 3, molecular chaperone heat-shock protein 47, fibrillin-1, PDGF receptor α, PDGF receptor β, and nuclear protein SS-A. An embodiment is the case wherein the tolerogenic antigen comprises a portion of a human food, e.g., is chosen from the group consisting of conarachin (Ara h 1), allergen II (Ara h 2), arachis agglutinin (Ara h 6), α-lactalbumin (ALA), lactotransferrin, glutein, low molecular weight glutein, α- and γ-gliadin, hordein, secalin, and avenin. An embodiment is the case wherein the erythrocyte-binding moiety is chosen from

the group consisting of a peptide ligand, an antibody, an antibody fragment, and a single chain antigen binding domain (ScFv). An embodiment is the case wherein the erythrocyte-binding moiety comprises an antibody, an antibody fragment or an ScFv derived from a hybridoma that produces an antibody against an erythrocyte, with the hybridoma being chosen from the group consisting of BRIC 4, BRIC 5, BRIC 6, BRIC 10, BRIC 14, BRIC 18, BRIC 39, BRIC 66, BRIC 68, BRIC 69, BRIC 87, BRIC 108, BRIC 110, BRIC 111, BRIC 125, BRIC 126, BRIC 128, BRIC 145, BRIC 155, BRIC 157, BRIC 163, BRIC 170, BRIC 198, BRIC 203, BRIC 216, BRIC 220, BRIC 221, BRIC 222, BRIC 229, BRIC 230, BRIC 231, BRIC 235, BRIC 256, BRAC 17, BRAC 18, BGRL 1, BGRL 2, BGRL 11, BGRL 100, BRAD 3, BIRMA D6, BIRMA D10, BIRMA K3, BIRMA K3, 84B; 6A7; COE; or KZ1. An embodiment is the case wherein the erythrocyte-binding moiety specifically binds to a biomolecule chosen from the group consisting of Band 3 (CD233), aquaporin-1, Glut-1, Kidd antigen, RhAg/Rh50 (CD241), Rh (CD240), Rh30CE (CD240CE), Rh30D (CD240D), Kx, glycophorin A, glycophorin B (CD235b), glycophorin C (CD235c), glycophorin D (CD235d), Kell (CD238), Duffy/DARCi (CD234), CR1 (CD35), DAF (CD55), Globoside, CD44, ICAM-4 (CD242), Lu/B-CAM (CD239), XG1/XG2 (CD99), EMMPRIN/neurothelin (CD147), JMH, Glycosyltransferase, Cartwright, Dombrock, C4A/CAB, Scianna, MER2, stomatin, BA-1 (CD24), GPIV (CD36), CD108, CD139, and H antigen (CD173). The tolerogenic antigen may comprise a mimotope. An embodiment is the case wherein the scFv comprises some or all of 10F7, e.g., onc or more of a light chain of 10F7 and/or a heavy chain of 10F7 and/or a higher affinity variant of a light chain of 10F7 and/or a heavy chain of 10F7. An embodiment is the method wherein the erythrocyte-binding moiety comprises a peptide ligand comprising at least 5 consecutive amino acid residues of a sequence chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:1, and conservative substitutions thereof, wherein said sequence specifically binds an erythrocyte.

An embodiment is a composition comprising a molecular fusion that comprises a tolerogenic antigen and an erythrocyte-binding moiety that specifically binds an erythrocyte in the patient and thereby links the antigen to the erythrocyte. An instance is the case wherein the erythrocyte-binding moiety is covalently bonded to the antigen. Another instance is the case wherein the molecular fusion comprises the erythrocyte-binding moiety attached to a particle that is attached to the antigen, .e.g, a microparticle, a nanoparticle, a liposome, a polymersome, or a micelle. Examples of a tolerogenic antigen are: a portion of a therapeutic protein, s a portion of a nonhuman protein, a portion (including the whole portion,

i.e., all) of a protein not naturally found in a human, a glycan of a protein that comprises nonhuman glycosylation, a portion of a human autoimmune antigen, a portion of a human food. An embodiment is the composition wherein the erythrocyte-binding moiety is chosen from the group consisting of a peptide ligand, an antibody, an antibody fragment, and a single chain antigen binding domain (ScFv), e.g., all or a portion of 10F7. The erythrocyte-binding moiety may comprises a peptide ligand comprising at least 5 consecutive amino acid residues of a sequence chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:1, and conservative substitutions thereof, wherein said sequence specifically binds an erythrocyte. The erythrocyte-binding moiety may be one that comprises a peptide ligand that has a dissociation constant of between about 10 μM and 0.1 nM as determined by equilibrium binding measurements between the peptide and erythrocytes. An embodiment is the case wherein the erythrocyte-binding moiety comprises an antibody, an antibody fragment or an ScFv derived from a hybridoma that produces an antibody against an erythrocyte, with the hybridoma being chosen from the group consisting of BRIC 4, BRIC 5, BRIC 6, BRIC 10, BRIC 14, BRIC 18, BRIC 39, BRIC 66, BRIC 68, BRIC 69, BRIC 87, BRIC 108, BRIC 110, BRIC 111, BRIC 125, BRIC 126, BRIC 128, BRIC 145, BRIC 155, BRIC 157, BRIC 163, BRIC 170, BRIC 198, BRIC 203, BRIC 216, BRIC 220, BRIC 221, BRIC 222, BRIC 229, BRIC 230, BRIC 231, BRIC 235, BRIC 256, BRAC 17, BRAC 18, BGRL 1, BGRL 2, BGRL 11, BGRL 100, BRAD 3, BIRMA D6, BIRMA D10, BIRMA K3, BIRMA K3, 84B; 6A7; COE; or KZ1. An embodiment is the case wherein the erythrocyte-binding moiety specifically binds to a biomolecule chosen from the group consisting of Band 3 (CD233), aquaporin-1, Glut-1, Kidd antigen, RhAg/Rh50 (CD241), Rh (CD240), Rh30CE (CD240CE), Rh30D (CD240D), Kx, glycophorin A, glycophorin B (CD235b), glycophorin C (CD235c), glycophorin D (CD235d), Kell (CD238), Duffy/DARCi (CD234), CR1 (CD35), DAF (CD55), Globoside, CD44, ICAM-4 (CD242), Lu/B-CAM (CD239), XG1/XG2 (CD99), EMMPRIN/neurothelin (CD147), JMH, Glycosyltransferase, Cartwright, Dombrock, C4A/CAB, Scianna, MER2, stomatin, BA-1 (CD24), GPIV (CD36), CD108, CD139, and H antigen (CD173). The tolerogenic antigen may comprise a mimotope.

The tolerogenic compositions may be used in treating a pathologic condition, for instance a pathologic condition chosen from the group consisting of transplant rejection, autoimmune disease, food allergy, and immune response against a therapeutic agent.

An embodiment is a pharmaceutically acceptable composition for use in immunoreversal of an immune response against a substance comprising a fusion molecule

that comprises an erythrocyte-binding moiety and an antigen of the substance. The composition may have the tolerogenic antigen is chosen from, for example, the group consisting of a protein, a portion of a protein, a human protein or portion thereof, a nonhuman protein or portion thereof, a glycan, a glycan of a protein that comprises nonhuman glycosylation, a human autoimmune antigen, a protein therapeutic for a human or a portion thereof, and a portion of a human food. The composition may have the tolerogenic antigen is chosen from, for example, the group consisting of proteins deficient by genetic disease, proteins with nonhuman glycosylation, nonhuman proteins, synthetic proteins not naturally found in humans, human food antigens, human transplantation antigens, and human autoimmune antigens. The erythrocyte-binding moiety may be provides that specifically binds to a biomolecule chosen from the group consisting of Band 3 (CD233), aquaporin-1, Glut-1, Kidd antigen, RhAg/Rh50 (CD241), Rh (CD240), Rh30CE (CD240CE), Rh30D (CD240D), Kx, glycophorin A, glycophorin B (CD235b), glycophorin C (CD235c), glycophorin D (CD235d), Kell (CD238), Duffy/DARCi (CD234), CR1 (CD35), DAF (CD55), Globoside, CD44, ICAM-4 (CD242), Lu/B-CAM (CD239), XG1/XG2 (CD99), EMMPRIN/neurothelin (CD147), JMH, Glycosyltransferase, Cartwright, Dombrock, C4A/CAB, Scianna, MER2, stomatin, BA-1 (CD24), GPIV (CD36), CD108, CD139, and H antigen (CD173). The erythrocyte-binding moiety may be chosen from the group consisting of a peptide ligand, an antibody, an antibody fragment, and a single chain antigen binding domain (ScFv). The erythrocyte-binding moiety may be prepared so that it comprises an antibody, an antibody fragment or an ScFv derived from a hybridoma that produces an antibody against an erythrocyte, with the hybridoma being chosen from the group consisting of BRIC 4, BRIC 5, BRIC 6, BRIC 10, BRIC 14, BRIC 18, BRIC 39, BRIC 66, BRIC 68, BRIC 69, BRIC 87, BRIC 108, BRIC 110, BRIC 111, BRIC 125, BRIC 126, BRIC 128, BRIC 145, BRIC 155, BRIC 157, BRIC 163, BRIC 170, BRIC 198, BRIC 203, BRIC 216, BRIC 220, BRIC 221, BRIC 222, BRIC 229, BRIC 230, BRIC 231, BRIC 235, BRIC 256, BRAC 17, BRAC 18, BGRL 1, BGRL 2, BGRL 11, BGRL 100, BRAD 3, BIRMA D6, BIRMA D10, BIRMA K3, BIRMA K3, 84B; 6A7; COE; or KZ1.

Embodiments include a fusion molecule comprising an erythrocyte-binding moiety and an antigen of asparaginase. As is evident from the multiple disclosures of erythrocyte-binding moieties, there are many options for the same, including: an scFv that binds a human erythrocyte or a peptidic binding ligand chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, and conservative substitutions thereof, wherein said sequence specifically binds an

erythrocyte. Such a composition comprising the fusion molecule may be used for inducing tolerance to asparaginase.

Embodiments include a fusion molecule comprising and an erythrocyte-binding moiety and an antigen of chromogranin-A. The antigen may be a mimotope or some or all of a chromogranin-A. As is evident from the multiple disclosures of erythrocyte-binding moieties, there are many options for the same, including: an scFv that binds a human erythrocyte or a peptidic binding ligand chosen from the group consisting of SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, and conservative substitutions thereof, wherein said sequence specifically binds an erythrocyte. Such a composition comprising the fusion molecule may be used for inducing tolerance to chromogranin-A and/or for preventing diabetes or reducing progression a diabetes disease.

Another instance is a composition comprising an erythrocyte-binding moiety that specifically binds an erythrocyte joined to an entity chosen from the group consisting of a synthetic polymer, a branched synthetic polymer, and a particle. The particle may be, e.g., a microparticle, a nanoparticle, a liposome, a polymersome, and a micelle. The composition may further comprise a tolerogenic antigen, a therapeutic agent, or a tumor homing ligand.

An embodiment is a single chain antigen binding domain (scFv) comprising a peptide ligand that specifically binds an erythrocyte. The peptide may be attached to the scFv or disposed in a linker portion. One or more of the peptide ligands may be included.

* * *

All patent applications, patents, and publications mentioned herein are hereby incorporated by reference herein for all purposes; in the case of conflict, the instant specification controls.

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**CLAIMS**

1. A pharmaceutically acceptable composition comprising an erythrocyte-binding moiety, a linker, and a tolerogenic antigen, which are recombinantly fused or chemically conjugated, wherein:

said erythrocyte-binding moiety is an antibody, an antibody fragment or a single chain antigen binding domain (scFv) moiety that specifically binds to Band3, glycophorin A, glycophorin B, glycophorin C, or glycophorin D on a human erythrocyte *in situ* in blood,

said linker is a peptide, a linear or branched polymer, a covalent bond, or an ionic bond;

said tolerogenic antigen is an antigen to which subjects exposed to the antigen develop an unwanted immune response,

wherein the tolerogenic antigen is a food antigen, a therapeutic agent, a self-antigen, a fragment of any of such antigens, or a mimotope of any such antigens; and

wherein upon administration to a subject, the composition is able to induce one or more of (i) proliferation of CD8+ T cells having an apoptotic- or exhausted-fate phenotype, (ii) deletion of CD4 and/or CD8+ T cells specific for the tolerogenic antigen, and (iii) the ability to induce regulatory cell phenotypes.

2. The pharmaceutically acceptable composition of claim 1 wherein said erythrocyte-binding moiety comprises an antibody fragment or scFv moiety that specifically binds glycophorin A.

3. The pharmaceutically acceptable composition of claim 1 wherein said erythrocyte-binding moiety is derived from a 10F7 clone.

4. The pharmaceutically acceptable composition of claim 1 wherein the tolerogenic antigen comprises:

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: insulin, proinsulin, preproinsulin, glutamic acid decarboxylase-65 (GAD-65), GAD-67, insulinoma-associated protein 2 (IA-2), insulinoma-associated protein 2β (IA-2β), ICA69, ICA12 (SOX-13), carboxypeptidase H, Imogen 38, GLIMA 38, chromogranin-A, HSP-60, caboxypeptidase E, peripherin, glucose transporter 2, hepatocarcinoma-intestine-pancreas/pancreatic associated protein, S100β, glial fibrillary acidic protein, regenerating gene II, pancreatic duodenal homeobox 1, dystrophia myotonica kinase, islet-specific

glucose-6-phosphatase catalytic subunit-related protein, and SST G-protein coupled receptors 1-5;

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: myelin basic protein, myelin oligodendrocyte glycoprotein and proteolipid protein; and

a food antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: conarachin (Ara h 1), allergen II (Ara h 2), arachis agglutinin, conglutin (Ara h 6), a-lactalbumin (ALA), lactotransferrin, Pen a 1 allergen (Pen a 1), allergen Pen m 2 (Pen m 2), tropomyosin fast isoform, gliadin, high molecular weight glutenin, low molecular weight glutenin, alpha- and gamma-gliadin, hordein, seclain, and avenin.

5. A pharmaceutically acceptable composition according to any one of Claims 1 to 4, wherein the tolerogenic antigen comprises a mimotope of the food antigen, the therapeutic agent, the self-antigen, or of fragments of any such antigens.

6. A pharmaceutically acceptable composition according to any one of claims 1 to 5, wherein said linker is a branched polymer that increases the affinity of erythrocyte binding by avidity effects.

7. The pharmaceutically acceptable composition of claim 6 wherein said branched polymer is conjugated to more than one erythrocyte-binding moiety.

8. A pharmaceutically acceptable composition according to any one of claims 1 to 3, wherein the tolerogenic antigen is a therapeutic agent selected from the group consisting of Factor VIII, Factor IX, asparaginase and uricase, a fragment of any such antigens, or a mimotope of any such antigens.

9. Use of a therapeutically effective amount of a pharmaceutically acceptable composition for the manufacture of a medicament for treating an unwanted immune response, the composition comprising an erythrocyte-binding moiety, a linker, and a tolerogenic antigen wherein the erythrocyte-binding moiety, the linker and the tolerogenic antigen are recombinantly fused or chemically conjugated, wherein:

said erythrocyte-binding moiety is an antibody, an antibody fragment or a single chain antigen binding domain (scFv) moiety that specifically binds to Band3, glycophorin A, glycophorin B, glycophorin C or glycophorin D on a human erythrocyte *in situ* in blood,

said linker is a peptide, a linear or branched polymer, a covalent bond, an ionic bond, an aptamer, a nucleic acid or a particle;

said tolerogenic antigen is an antigen to which patients develop subjects develop an unwanted immune response;

wherein the tolerogenic antigen is a food antigen, a therapeutic agent, a self-antigen, a fragment of any such antigens, or a mimotope of any such antigens; and

wherein, upon administration to a patient, the composition is able to induce one or more of (i) proliferation of CD8+ T cells having an apoptotic- or exhausted-fate phenotype, (ii) deletion of CD4 and/or CD8+ T cells specific for the tolerogenic antigen, and (iii) the ability to induce regulatory cell phenotypes.

10. The use of claim 9 wherein said erythrocyte-binding moiety comprises an antibody fragment or an scFv moiety that specifically binds glycophorin A.

11. The use of claim 9 wherein said erythrocyte-binding moiety is derived from a 10F7 clone.

12. A use according to any one of claims 9 to 11, wherein said composition is administered to a subject prior to an unwanted immune response to the antigen, wherein said composition prevents or reduces the unwanted immune response.

13. A use according to any one of claims 9 to 11, wherein said composition is administered to a subject having an unwanted immune response to the antigen, wherein said composition prevents or reduces the unwanted immune response.

14. A use according to any one of claims 9 to 13, where the unwanted immune response is associated with a disease selected from the group: type 1 diabetes mellitus; multiple sclerosis; rheumatoid arthritis; celiac disease; vitiligo; pemphigus vulgaris and variants; bullous pemphigoid; hemophilia and cancer.

15. A use according to any one of claims 9 to 13, where the tolerogenic antigen comprises:

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: insulin, proinsulin, preproinsulin, glutamic acid decarboxylase-65 (GAD-65), GAD-67, insulinoma-associated protein 2 (IA-2), insulinoma-associated protein 2β (IA-2β), ICA69,

ICA12 (SOX-13), carboxypeptidase H, Imogen 38, GLIMA 38, chromogranin-A, HSP-60, caboxypeptidase E, peripherin, glucose transporter 2, hepatocarcinoma-intestine-pancreas/pancreatic associated protein, S100β, glial fibrillary acidic protein, regenerating gene II, pancreatic duodenal homeobox 1, dystrophia myotonica kinase, islet-specific glucose-6-phosphatase catalytic subunit-related protein, and SST G-protein coupled receptors 1-5;

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: myelin basic protein, myelin oligodendrocyte glycoprotein and proteolipid protein;

collagen II, a fragment thereof, or a mimotope thereof;

tissue transglutaminase, a fragment thereof, or a mimotope thereof ~~and gliadin~~;

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: tyrosinase, and tyrosinase related protein 1 and 2;

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: desmoglein 3, desmoglein 1, desmoglein 4, pemphaxin, desmocollins, plakoglobin, perplakin, desmoplakins, and acetylcholine receptor;

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: BP180, BP230, plectin and laminin 5;

a self-antigen, a fragment thereof, or a mimotope thereof selected from the group consisting of: conarachin (Ara h 1), allergen II (Ara h 2), arachis agglutinin, conglutin (Ara h 6), a-lactalbumin (ALA), lactotransferrin, Pen a 1 allergen (Pen a 1), allergen Pen m 2 (Pen m 2), tropomyosin fast isoform, gliadin, high molecular weight glutenin, low molecular weight glutenin, alpha- and gamma-gliadin, hordein, seclain, and avenin; or

a therapeutic agent, a fragment thereof, or a mimotope thereof selected from the group consisting of Factor VIII, Factor IX, asparaginase and uricase.

16. The use of claim 9 wherein the unwanted immune response is associated with celiac disease and wherein the tolerogenic antigen is tissue trans-glutaminase, high molecular weight glutenin, low molecular weight glutenin, alpha- or gamma-gliadin.

17. Use of a therapeutically effective amount of a pharmaceutically acceptable composition for the manufacture of a medicament for treating an unwanted immune response, the composition comprising:

an erythrocyte-binding moiety,

a linker, and

a tolerogenic antigen, wherein:

the erythrocyte-binding moiety, the linker and the tolerogenic antigen are recombinantly fused,

the erythrocyte-binding moiety is an antibody fragment or a single chain antigen binding domain (scFv) moiety that non-covalently and specifically binds to Band 3, glycophorin A, glycophorin B, glycophorin C or glycophorin D on a human erythrocyte *in situ* in blood,

said linker is a peptide, a linear or branched polymer, a covalent bond, an ionic bond, an aptamer, a nucleic acid or a particle; and

said tolerogenic antigen is a foreign antigen to which patients develop an unwanted immune response.

18. A pharmaceutically acceptable composition comprising an erythrocyte-binding moiety and a tolerogenic antigen, which are recombinantly fused or chemically conjugated, wherein:

said erythrocyte-binding moiety is an antibody, an antibody fragment or scFv moiety that specifically binds to glycophorin A on a human erythrocyte,

said tolerogenic antigen is an antigen to which patients develop an unwanted immune response; and

wherein the tolerogenic antigen is a food antigen, a therapeutic agent, a self-antigen, or a fragment of any of such antigens.

19. The pharmaceutically acceptable composition of claim 18, wherein said erythrocyte-binding moiety is a scFv.

20. The pharmaceutically acceptable composition of claim 18, wherein said erythrocyte-binding moiety is an antibody fragment.

21. A pharmaceutically acceptable composition according to any one of claims 18 to 20, wherein the food antigen is selected from the group of gliadin, alpha-gliadin, gamma-gliadin, a fragment of such antigens and a mimotope of any of such antigens.

22. A pharmaceutically acceptable composition according to any one of claims 18 to 20, wherein the self-antigen is selected from the group of insulin, proinsulin, preproinsulin, glutamic acid decarboxylase-65 (GAD-65), GAD-67, islet-specific glucose-6-phosphatase catalytic subunit-related protein, a fragment of such antigens and a mimotope of any of such antigens.

23. Use of the pharmaceutically acceptable composition according to any one of claims 18 to 22 for the manufacture of a medicament for treating an unwanted immune response.

24. Use of the pharmaceutically acceptable composition of claim 21 for the manufacture of a medicament for treating Celiac disease.

25. Use of the pharmaceutically acceptable composition of claim 22 for the manufacture of a medicament for treating type 1 diabetes mellitus.

26. A pharmaceutically acceptable composition according to any one of claims 18 to 20, wherein the self-antigen is selected from the group of myelin basic protein, myelin oligodendrocyte glycoprotein and proteolipid protein, a fragment of such antigens and a mimotope of any of such antigens.

27. Use of the pharmaceutically acceptable composition of claim 26 for the manufacture of a medicament for treating multiple sclerosis.

# ABSTRACT

## ERYTHROCYTE-BINDING THERAPEUTICS

Peptides that specifically bind erythrocytes are described. These are provided as peptidic ligands having sequences that specifically bind, or as antibodies or fragments thereof that provide specific binding, to erythrocytes. The peptides may be prepared as molecular fusions with therapeutic agents, tolerizing antigens, or targeting peptides. Immimotolerance may be created by use of the fusions and choice of an antigen on a substance for which tolerance is desired.

***The Most Illustrative Drawing : Fig 1***

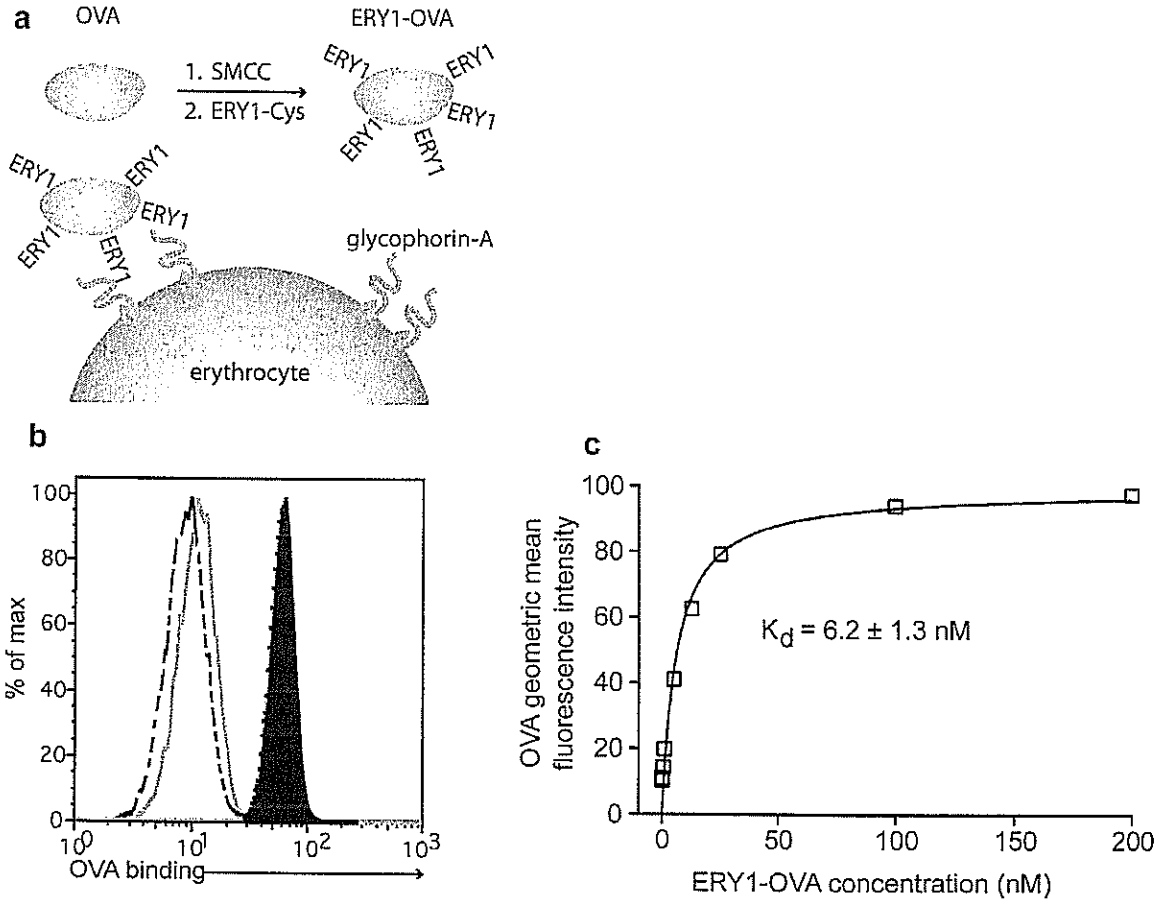

a
OVA
ERY1-OVA
1. SMCC
2. ERY1-Cys
ERY1
glycophorin-A
erythrocyte
b
% of max
OVA binding
c
OVA geometric mean fluorescence intensity
$K_d$ = 6.2 ± 1.3 nM
ERY1-OVA concentration (nM)

Fig. 1

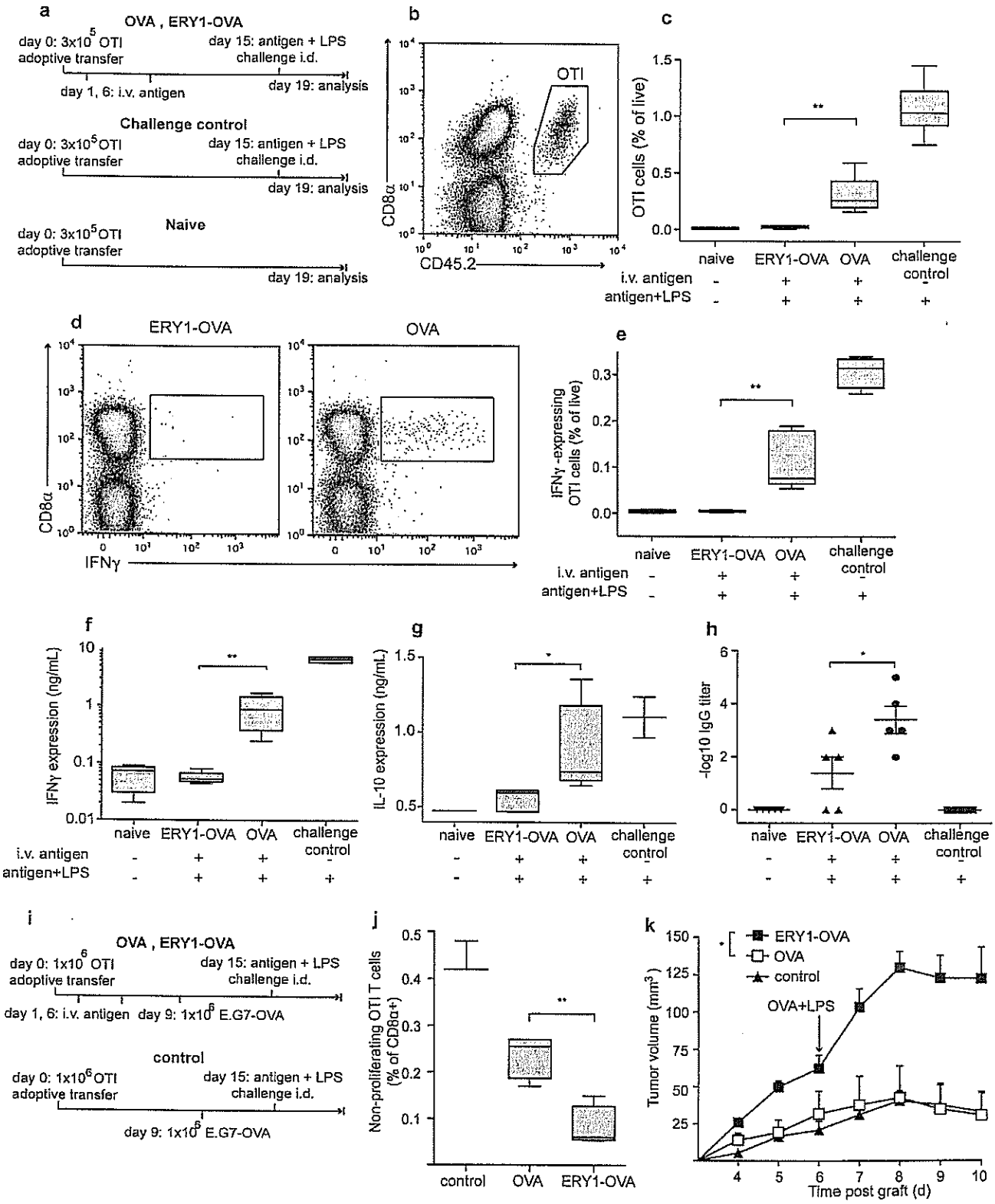

a
OVA , ERY1-OVA
day 0: 3x10^5 OTI adoptive transfer
day 15: antigen + LPS challenge i.d.
day 1, 6: i.v. antigen
day 19: analysis
Challenge control
day 0: 3x10^5 OTI adoptive transfer
day 15: antigen + LPS challenge i.d.
day 19: analysis
Naive
day 0: 3x10^5 OTI adoptive transfer
day 19: analysis
b
OTI
CD8α
CD45.2
c
OTI cells (% of live)
naive
ERY1-OVA
OVA
challenge control
i.v. antigen
antigen+LPS
d
ERY1-OVA
OVA
CD8α
IFNγ
e
IFNγ -expressing OTI cells (% of live)
f
IFNγ expression (ng/mL)
g
IL-10 expression (ng/mL)
h
-log10 IgG titer
i
OVA , ERY1-OVA
day 0: 1x10^6 OTI adoptive transfer
day 15: antigen + LPS challenge i.d.
day 1, 6: i.v. antigen
day 9: 1x10^6 E.G7-OVA
control
day 0: 1x10^6 OTI adoptive transfer
day 15: antigen + LPS challenge i.d.
day 9: 1x10^6 E.G7-OVA
j
Non-proliferating OTI T cells (% of CD8α+)
control
OVA
ERY1-OVA
k
ERY1-OVA
OVA
control
OVA+LPS
Tumor volume (mm^3)
Time post graft (d)

Fig. 2

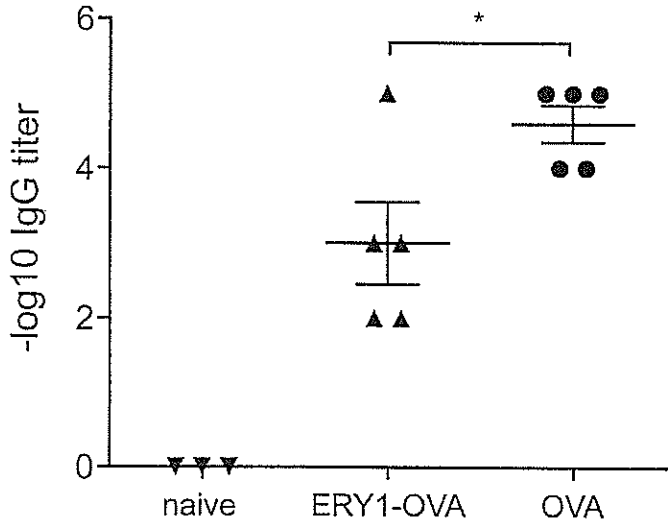

6
4
2
0
-log10 IgG titer
*
naive
ERY1-OVA
OVA

Fig. 3

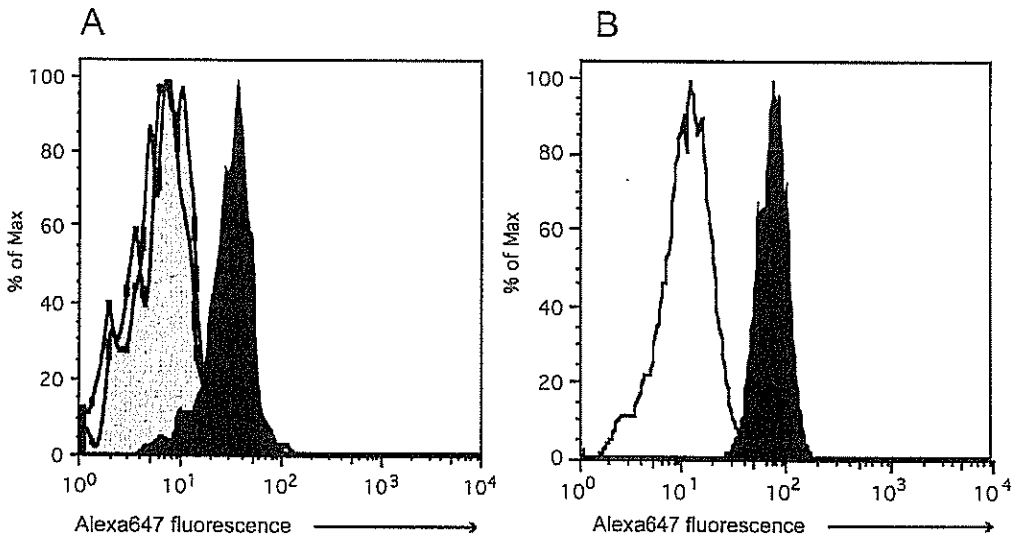

A
B
100
80
60
40
20
0
% of Max
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
Alexa647 fluorescence

Fig. 4

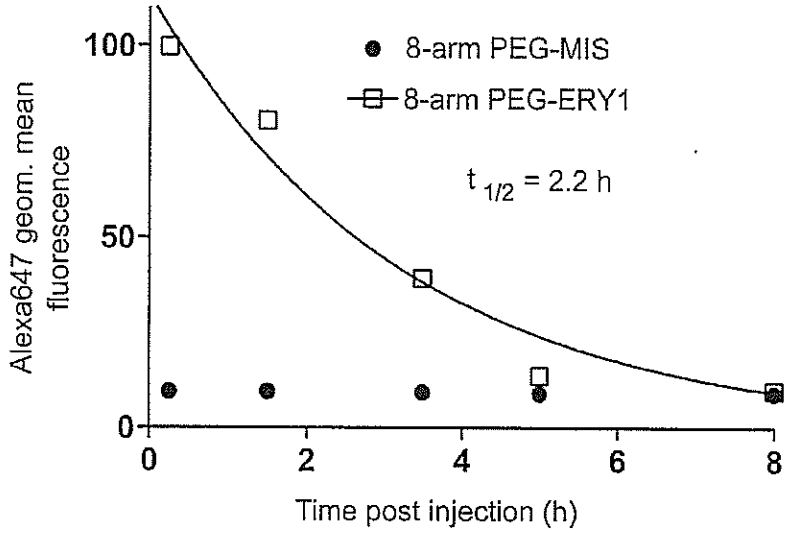

100
50
0
0
2
4
6
8
Alexa647 geom. mean fluorescence
Time post injection (h)
8-arm PEG-MIS
8-arm PEG-ERY1
t 1/2 = 2.2 h

Fig. 5

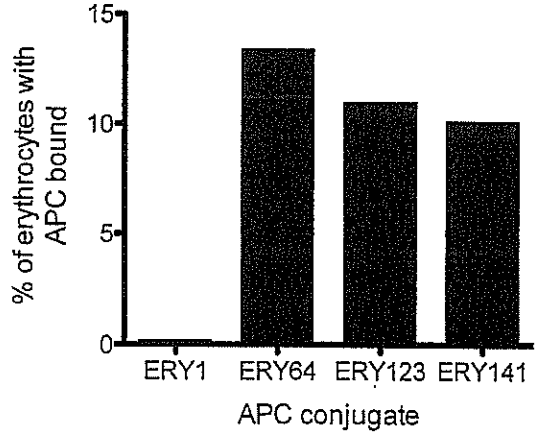

15
10
5
0
% of erythrocytes with APC bound
ERY1
ERY64
ERY123
ERY141
APC conjugate

Fig. 6

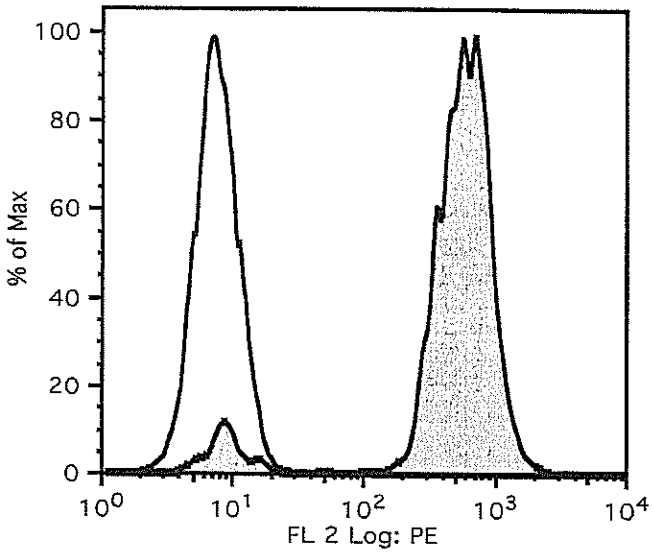

100
80
60
40
20
0
% of Max
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
FL 2 Log: PE

Fig. 7

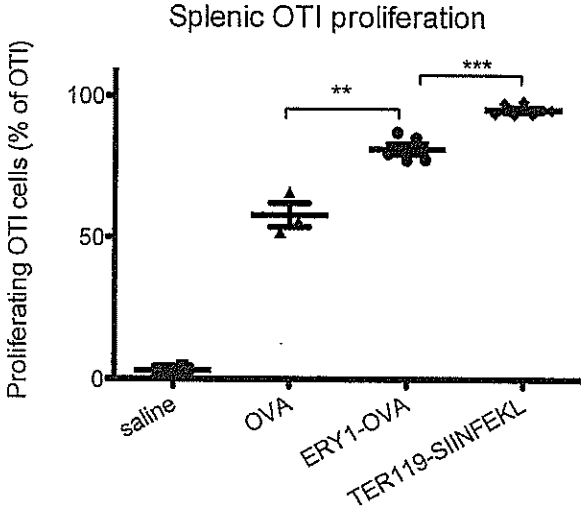

Splenic OTI proliferation
Proliferating OTI cells (% of OTI)
100
50
0
**
***
saline
OVA
ERY1-OVA
TER119-SIINFEKL

Fig. 8

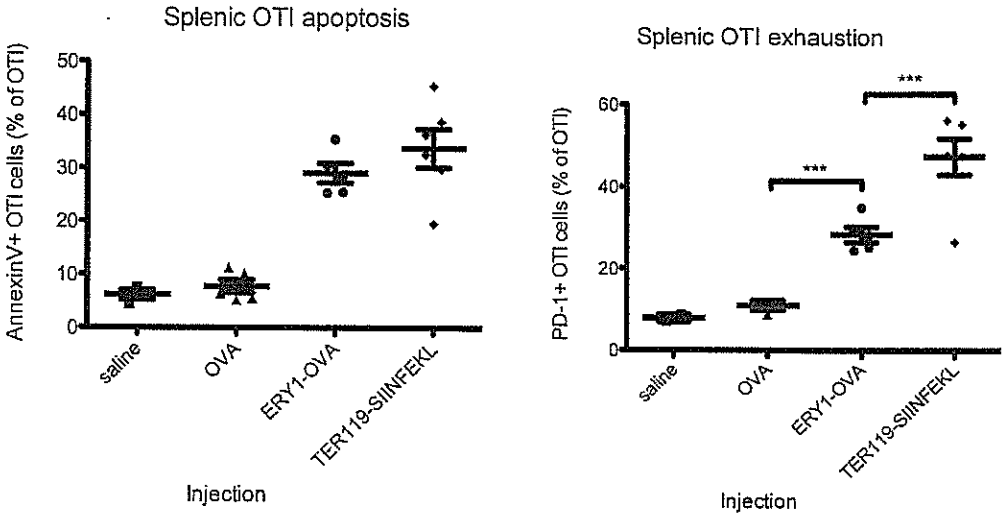

Splenic OTI apoptosis
AnnexinV+ OTI cells (% of OTI)
50
40
30
20
10
0
saline
OVA
ERY1-OVA
TER119-SIINFEKL
Injection
Splenic OTI exhaustion
PD-1+ OTI cells (% of OTI)
60
40
20
0
***
***
saline
OVA
ERY1-OVA
TER119-SIINFEKL
Injection

Fig. 9

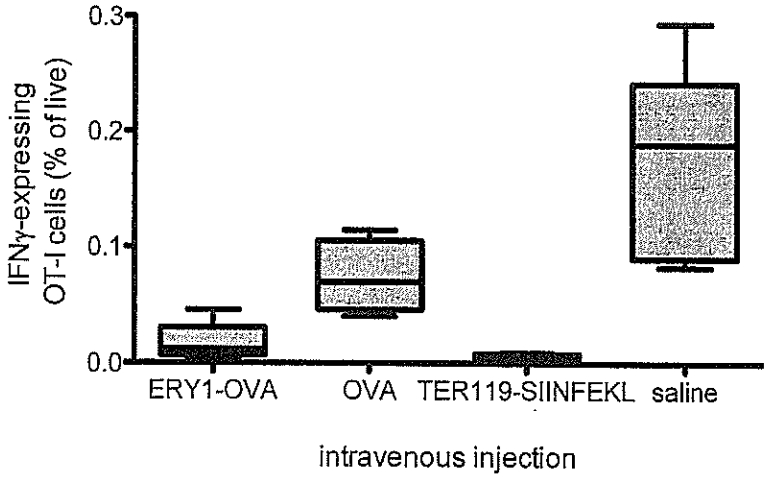

IFNγ-expressing
OT-I cells (% of live)
0.3
0.2
0.1
0.0
ERY1-OVA
OVA
TER119-SIINFEKL
saline
intravenous injection

Fig. 10

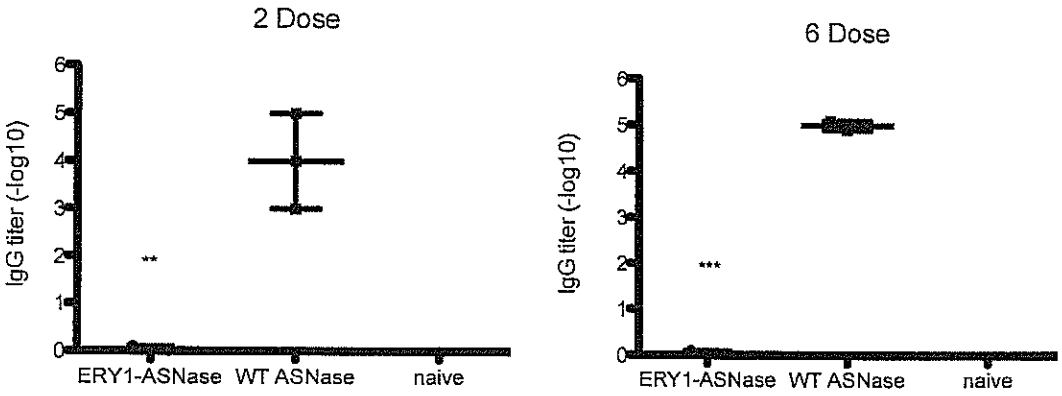

2 Dose
6 Dose
IgG titer (-log10)
**
***
ERY1-ASNase
WT ASNase
naive

Fig. 11

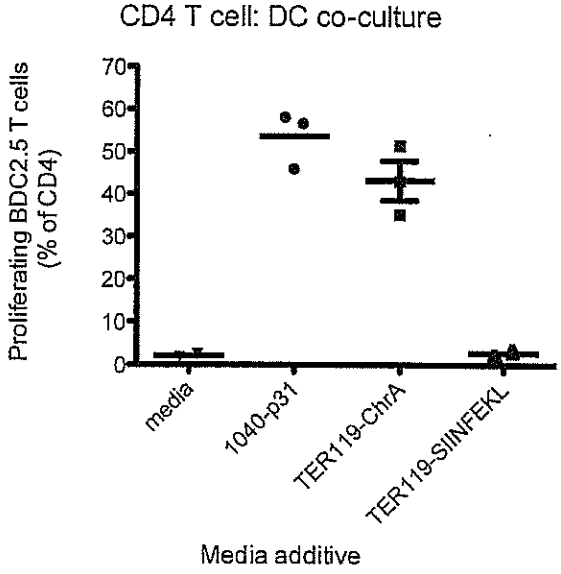

CD4 T cell: DC co-culture
Proliferating BDC2.5 T cells (% of CD4)
media
1040-p31
TER119-ChrA
TER119-SIINFEKL
Media additive

Fig. 12

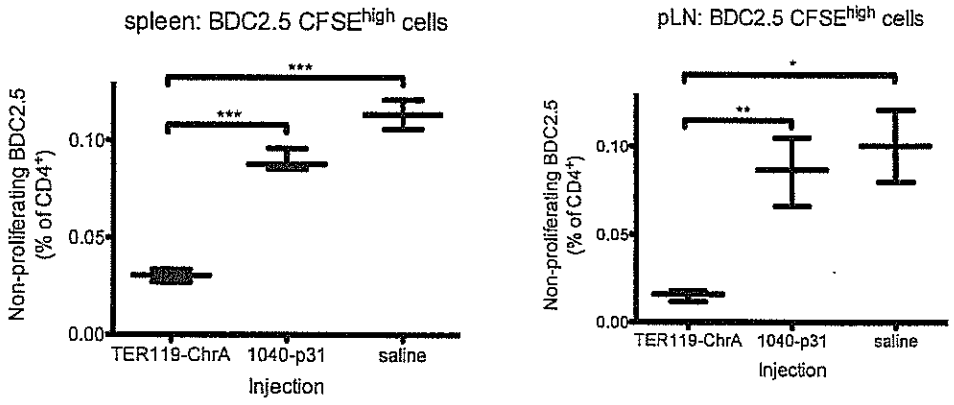

spleen: BDC2.5 CFSE^high cells
pLN: BDC2.5 CFSE^high cells
Non-proliferating BDC2.5 (% of CD4+)
0.00
0.05
0.10
***
**
*
TER119-ChrA
1040-p31
saline
Injection

Fig. 13