The invention generally relates to methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72. The invention further relates to methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors preferentially expressed on B lymphocytes and containing an immunoreceptor tyrosine inhibitory motif (ITIM).
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METHODS FOR MODULATING ANTIGEN-SPECIFIC B CELL RESPONSES

STATEMENT OF GOVERNMENT SUPPORT

[0001] This invention was made by government support by Grant Nos. DE16381, GM37905, and RR00166 from the National Institutes of Health. The Government has certain rights in this invention.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims benefit under 35 U.S.C. § 119(c) to U.S. Provisional Application No. 60/888,746, filed February 7, 2007, hereby incorporated by reference in its entirety.

FIELD

[0003] The invention generally relates to methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72. The invention further relates to methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors preferentially expressed on B lymphocytes and containing an immunoreceptor tyrosine inhibitory motif (ITIM).
BACKGROUND

Dendritic cells (DCs) are widely considered the sentinels of the immune system, which recognize foreign antigens (Ags) efficiently and effectively present them to T lymphocytes (Steinman and Hemmi, 2006). Studies comparing the ability of DCs vs. B cells into activate naive T cells led to a widely held conclusion that DCs but not B cells can activate naive T cells (e.g., Fuchs and Matzinger, 1992) and that B cells are not needed to initiate T cell immune responses (Epstein et al, 1995). However, both B cells and DCs have been implicated in immune responses to autoantigens (e.g., see Yan et al, 2006). Yan et al (2006) showed that recognition of auto-Ag by Ag-specific B cells was required to initiate a T cell autoimmune response.

MAbs to a range of different surface molecules have been used to deliver Ags to DCs in vivo and/or in vitro including MHC class II, CD1 Ic, DEC205/CD205, DC-SIGN/CD209, F4/80-like receptor (FIRE), CIRE, Dectin-1, Dectin-2, 33D1/DCIR2, and the mannose receptor (Finkelman et al, 1996, Bonifaz et al, 2002, 2004, Engering et al, 2002, Chieppa et al, 2003, Ramakrishna et al, 2004, Demangel et al, 2005, Tacken et al, 2005, Corbett et al, 2005, Boscardin et al, 2006, Trumpfheller et al, 2006, Carter et al, 2006a,b, Dudziak et al, 2006). The mAb-based Ag delivery system can be two orders of magnitude more effective than non-targeted Ags in activating protective immunity. Simultaneous injection of Ag-anti-DC conjugates with a anti-CD40 mAb significantly enhanced host immune responses more than the classic Alum+ Ag vaccine (Bonifaz et al, 2004, Boscardin et al, 2006). This important finding is highly pertinent to the field of DNA vaccination, where many studies have found that DNA alone can induce Ab responses comparable with unadjuvanted protein immunogens, but ‘for sheer magnitude of Ab titers DNA alone could not equal a potent protein plus adjuvant’ (Donnelly et al, 2005). Also, Ag delivered via anti-FIRE, anti-CIRE or anti-Dectin-1 mAbs triggers specific antibody responses without an adjuvant, while Ag with anti-DEC205 does not (Corbett et al, 2005, Carter et al, 2006b). Furthermore, several recent studies suggest that different immune responses are induced depending on which DC subset is targeted to receive the antigen. Targeting Ags to 33D1 or Dectin-1 using mAbs promotes CD4 T cell and antibody responses better than CD8 responses while targeting Ags to DEC205 promotes better CD8 than CD4/antibody responses (Boscardin et al, 2006, Dudziak et al, 2006, Carter et al, 2006).
None of these studies have directly compared the effect of targeting Ags with mAb to DC vs. B cell subsets. Also, none of these studies have considered that there can be a different type of immune response induced depending on which cell surface molecule on a B cell is targeted.

**SUMMARY**

The invention generally relates to methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72. The antigen can be from an infectious agent, is a tumor-associated antigen, or an autoantigen. In some aspects, modulating the immune response generates protective immunity to a pathogen. In other aspects, modulating an immune response suppresses autoantibody production.

The invention further relates to methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors preferentially expressed on B lymphocytes and containing an immunoreceptor tyrosine inhibitory motif (ITIM). The antigen can be from an infectious agent, is a tumor-associated antigen, or an autoantigen. In other aspects, modulating an immune response suppresses autoantibody production. A method for modulating an immune response in a subject is provided which comprises targeting antigens to B cell surface receptors using an scFvIg derived from an antibody which binds to the receptor, and which genetically fuses the antigen to the carboxyl end of the scFvIg moiety.

A method for creating an scFvIg-Ag fusion protein is provided which comprises genetically fusing the scFvIg directly to the antigen specificity of interest, without any intervening sequence. A method for creating an scFvIg-Ag fusion protein is provided which comprises genetically fusing the scFvIg to the antigen through use of a spacer linker to physically separate functional domains. A method for creating an scFvIg-Ag fusion protein is provided which comprises genetically fusing the scFvIg to the antigen by a hydrophilic linker to improve solubility and decrease steric hindrance between protein domains. A composition is provided which comprises a CD22 binding molecule that does not fix complement, or mediate ADCC, or lead to destruction of the cells to which it binds.

A method for modulating an immune response in a subject is provided which comprises targeting antigens into the immune system through surface
receptors on dendritic cells together with an inhibitor of CD22 function or binding. The antigen can be from an infectious agent, a tumor-associated antigen, or an autoantigen. In one aspect, modulating the immune response can generate protective immunity to a pathogen. In a further aspect, modulating the immune response can suppress autoantibody production.

[0011] A method for modulating an immune response in a subject is provided which comprises targeting antigens into the immune system through surface receptors on dendritic cells together with an inhibitor of CD22 function or binding. The antigen can be from an infectious agent, a tumor-associated antigen, or an autoantigen. In one aspect, modulating the immune response can generate protective immunity to a pathogen. In a further aspect, modulating the immune response can suppress autoantibody production.

[0012] A method for modulating an immune response in a subject is provided which comprises targeting antigens into the immune system through CD22 receptors on B lymphocytes whereby CD22 binding to dendritic cells is inhibited. In one aspect, the immune system is altered or suppressed.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1. Immature DCs, but not mature DCs, inhibit BCR-induced proliferation of B cells. Purified CFSE-labeled B cells from WT mice were co-cultured with either WT bone marrow-derived iDCs (A) or mDCs (B) and the indicated graded doses of anti-IgM. CFSE dilution of the B cells was examined 72 hours post-incubation by flow cytometry. Two different B cell:DC ratios were examined (4:1 and 16:1). The histograms shown are from cultures where the B cell:DC ratio was 4:1. Results from both ratios are shown in graph format. These results are representative of at least 8 independent experiments.

[0014] Figure 2. iDC-mediated inhibition of WT B cells is contact dependent. Purified CFSE-labeled B cells from WT mice were co-cultured with either WT bone marrow-derived iDCs (A) or mDCs (B) and the indicated concentrations of anti-IgM ("contact"). In some of the co-cultures, the DCs and B cells were physically separated using transwells with a pore size of 0.4µm ("transwell"). CFSE dilution of the B cells was examined 72 hours post-incubation by
Two different ratios of B cell:DC were examined (4:1 and 16:1). These results are representative of 6 independent experiments.

**Figure 3.** (A) WT iDCs do not inhibit BCR-induced proliferation of CD22-deficient B cells. (B) ST6Gal-I-deficient iDCs inhibit BCR-induced proliferation of WT B cells in a contact-dependent manner. (A) Purified CFSE-labeled B cells from CD22-deficient mice were co-cultured together ("contact") or in transwells ("transwell") with WT bone marrow-derived iDCs and graded doses of anti-IgM ("contact"). (B) Purified CFSE-labeled B cells from WT mice were co-cultured with ST6Gal-I-deficient bone marrow-derived iDCs and graded doses of anti-IgM. The same method described in Figure 2 was used. These results are representative of at least 3 independent experiments.

**Figure 4. Soluble CD22 binds to WT and SToGalI KO DCs.** The binding of purified human IgG (Fc fragment), CD22 Rgil-7 or CD22 Rg3-7 to the surface of WT and ST6Gal-I-deficient iDCs and B cells was quantified by flow cytometry. (A) iDCs and (B) B cells were examined for their ability to bind to the purified chimeric CD22 fusion proteins. The cells were stained with purified fusion proteins along with anti-human IgG-PE secondary, CD1 lC-FITC (iDCs) and CD19-FITC (B cells). In the histograms shown, the cells were gated on CD1 lC+ or CD19+ cells. The results shown are representative of 3 independent experiments.

**Figure 5. Cleavage of sialic acids does not disrupt CD22 binding on DCs.** The binding of the purified chimeric CD22 fusion proteins to the surface of WT DCs and B cells following cleavage of sialic acids was quantified by flow cytometry. *Arthrobacter ureafaciens* neuraminidase treated cells (A) and cells treated by mild sodium metaperiodate oxidation for 30 minutes (B) were stained with the indicated fusion proteins or control human IgG (Fc fragment) along with anti-human IgG-PE secondary and CD19-FITC (for the splenocytes) or CD1 lC-FITC (for the iDCs). In the FACS histograms shown, the cells were gated on CD19+ (B cells) or CD1 lC+ (iDCs). The neuraminidase and sodium metaperiodate-treated human IgG control was similar to the untreated sample shown. The results shown are representative of 3 experiments.

**Figure 6.** CD22 and STöGal-I KO mice have fewer MZ B cells and T2 B cell precursors compared to WT. (A) Development of splenic B cell subsets and expression of various surface markers. (B) Total splenocytes were isolated from WT and STöGal-I KO mice. The splenocytes were analyzed for surface
expression of IgM, IgD and CD21 by flow cytometric analysis. The percent of each B cell subset is shown in the table. Data represent mean ± standard deviation. MZ, marginal zone; FO, follicular mature; *, P < 0.05; and **, P < 0.01. n>3 mice per genotype. (C) CD22 expression in the B cell subsets. WT splenocytes were stained with IgM, IgD, CD21 and CD22 and analyzed by flow cytometry. The graph shows the average mean fluorescence intensity of CD22 expression for each B cell subset. n=9 mice.

[0019] Figure 7. Absolute numbers of B cell subsets in spleen. Total splenocytes were isolated from WT and CD22 KO mice. The splenocytes were analyzed for surface expression of IgM, IgD and CD21 by flow cytometric analysis. The absolute number of each B cell subset is shown in the table. Data represent mean ± standard deviation. MZ, marginal zone; FO, follicular mature; *, P < 0.05; and **, P < 0.01. n>10 mice.

[0020] Figure 8. CD22-deficient iDCs do not inhibit BCR-induced proliferation of CD22-deficient B cells. Purified CFSE-labeled B cells from CD22-deficient mice were co-cultured with CD22-deficient bone marrow-derived iDCs and the indicated concentrations of anti-IgM. CFSE dilution of the B cells was examined 72 hours post-incubation by flow cytometry. Two different ratios of B cell:DC were examined (4:1 and 16:1).

[0021] Figure 9. ST6Gall-deficient DCs do not express α2-6-linked sialic acids. The binding of SNA on iDCs from WT and ST6Gal-I-deficient mice was quantified by flow cytometry. The figure displays the histogram overlays of the staining pattern on immature dendritic cells.

[0022] Figure 10. Schematic structure of planned scFvIg-Ag targeting fusion genes. The scFv domain of the desired monoclonal antibody is cloned from the expressing hybridoma. The VL and VH domains are linked by one of several linker domains, including (gly4ser)3, (gly4ser)4, and a hydrophilic linker containing gly and ser residues. The scFv is expressed as part of a fusion gene that contains the mouse, human, or macaque IgG2a or IgGl domains, mutated at a series of residues implicated in mediation of effector function. The fusion gene also contains an antigen fragment or the intact antigen which is to be targeted to the lymphocyte subset of interest.
[0023] **Figure 11.** Alignment of murine IgG2a, human IgG1, and macaque IgG1- [hinge-CH2-CH3]- amino acid sequences. Sequence matches between all three sequences are highlighted in yellow, homology matches between two sequences are highlighted in light blue, and conservative sequence changes are highlighted in green. The consensus sequence is listed at the bottom. Residues enclosed in boxes are a subset of the residues identified as important in ADCC, CDC, or FcR binding. These residues are P238 (ADCC), N297 (FcR binding, ADCC, CDC, and glycosylation), K322 (Clq binding, CDC), and P331 (Clq binding, CDC).

[0024] **Figure 12.** Structure of a prototype scFv-Ag fusion protein. cDNA cassettes encoding anti CD22 V_L and V_H segments were cloned from the hybridoma, fused by one of three potential (glyser) based linkers, and attached to a mutated murine IgG2a (hiIgG1 or macaque IgG1) Fc domain, including an SCC hinge and (P238S/K322S/P331S) Fc. The single chain cDNA is expressed from a DNA vaccine or mammalian expression vector designed to express soluble protein. The single chain fusion protein also encodes a linker-Ag domain attached to the carboxyl end of the CH3.

[0025] **Figure 13.** niDCAL-2 expression. mDCAL-2 (red) is expressed on mouse CD8- & CD8+ DCs, pDCs, and B cells in spleen but not on NK cells or CD4+ or CD8+ T cells. Mouse spleen cells were isolated from 6 week old wild type mice and fractionated into different subsets using magnetic beads (Miltenyi). Cell subsets were stained with various diagnostic marker antibodies and cells gated based on their expression pattern. Staining is shown with rat P4G2 anti-mDCAL-2 mAb, with rat isotype control staining in purple.

[0026] **Figure 14.** mDCAL-2 is internalized upon crosslinking by P4G2 + anti-rIgG2a. JAWS-II cells (immature bone marrow derived cell line) were stained with anti- mDCAL-2 (P4G2), followed by crosslinking by anti-ratIgG2a-Biotin. Then cells were cultured at 37C for indicated time length and stained with streptavidin-PE. Intracellular staining was performed to demonstrate that fluorescence decrease is due to internalization but not to Ab stripping or fluorescence decay.

[0027] **Figure 15.** Table of scFv Targeting Constructs. Each fusion protein is indicated by the target receptor and the hybridoma used as the source of V regions. The Fc domain to which the constructs are fused are indicated. Each of these constructs includes an open reading frame at the end of the Fc domain in order to attach the desired antigen domains for targeting.
Figure 16. CD22 KO mice, unlike WT mice, do not produce IgG antibodies to antigen targeted to dendritic cells. Groups of three wildtype (WT) or CD22 deficient (CD22 KO) mice were inoculated by the intravenous (i.v.) route with different doses of a rat IgG2b monoclonal antibody (mAb) 33D1, which binds to mouse 33D1 (DCIR2) molecules on the surface of mouse DCs (Dudziak et al., Science, 5:315, 2007). Sera were obtained from one group of WT (black circles) or CD22 KO (open circles) mice 10 days after immunization. ELISA was performed to measure serum levels of mouse IgGl anti-rat IgG2b. After inoculation of 100 ng of 33D1 mAb, WT mice unlike CD22 KO mice produced high levels of IgGl anti-rat IgG2b antibodies, but at 1 µg inoculation, both WT and CD22 KO mice produced only low levels of IgGl antibodies. WT mice made more IgGl when inoculated with even higher levels of antigen (10-100 µg, i.v.), but CD22 KO mice did not. Both WT and CD22 KO mice could produce IgGl antibodies to rat IgG2b antigen not targeted to DCs (MI/70).

Figure 17. P4G2scFvIg, P4G2scFvIg-OVA, and P4G2scFvIg-HEL fusion proteins bind specifically to A20 murine B cell lymphoma cells. Culture supernatants from COS transient transfections or from CHO master wells were incubated with 10E6 A20 murine B cell lymphoma cells in PBS/2%FBS (staining media) for 45 minutes on ice. Cells were washed and incubated with either biotinylated anti-mouse IgG2a antibody or with biotinylated anti-HEL antibody at 1:100, 45 minutes on ice. Cells were washed and incubated with PE-SA (phycoerythrin conjugated to streptavidin) at 1:400, 45 minutes on ice. Cells were washed and binding assayed by flow cytometry using a FACSCalibur instrument and Cell Quest software. Only the P4G2 scFvIg-HEL fusion proteins were detected with the anti-HEL conjugated antibody, demonstrating that the functional epitope for HEL was only present on these fusion proteins and not the P4G2 scFvIg or the P4G2 scFvIg-OVA fusion proteins.

Figure 18. Serial dilutions of culture supernatants from CHO cells transfected with P4G2 scFvIg, P4G2 scFvIg-OVA and P4G2 scFvIg-HEL2X demonstrate dose dependent binding to A20 B cell lymphoma cells. Culture supernatants from individual master wells transfected with the anti-mouse DCAL2 scFvIg and scFvIg-Ag fusion genes were harvested from 96 well plates and incubated at varying dilutions with A20 B cell lymphoma cells (10E6). Supernatants were
incubated at one of three dilutions: undiluted, 1:5, and 1:10, in staining media (PBS/2%FBS) for 45 minutes on ice. Cells were washed and incubated with biotinylated anti-mouse IgG2a (5.7) diluted 1:100 for 45 minutes on ice. Cells were washed and incubated with PE-SA at 1:400 in staining media for 45 minutes on ice, washed, and analyzed by flow cytometry. Each culture supernatant shows a dose dependent binding to A20 cells.

[0031] Figure 19. Nucleotide and predicted amino acid sequence of P4G2 scFvIg-OVA fusion gene. The P4G2 scFvIg-OVA fusion gene was constructed as described in the examples. Each cassette or functional segment is indicated in the boxed regions, with the single letter amino acid sequence indicated above each codon.

DETAILED DESCRIPTION

GENERAL INTRODUCTION AND OVERVIEW

[0032] We have found that the B cell surface receptor CD22 is required for DC dependent inhibition of antigen-receptor induced B cell proliferation. This has led us to propose in this invention that CD22 is a special target for delivering antigen to B cells and into the immune system. Further, we propose that receptors on B cells with immunoreceptor tyrosine inhibitory motifs (ITIMs) are especially well suited for delivering antigens into the immune system. The B cell restricted receptors with ITIMs include CD22 and CD72, but any ITIM-bearing receptor, which is relatively restricted to B cells would also fall under this invention.

[0033] It is to be understood that this invention is not limited to particular methods, reagents, compounds compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a cell" includes a combination of two or more cells, and the like.

[0034] "About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, more preferably ±5%, even more preferably ±1%, and still more
preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

[0035] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein. In describing and claiming the present invention, the following terminology will be used.

[0036] "Patient", "subject" or "mammal" are used interchangeably and refer to mammals such as human patients and non-human primates, as well as experimental animals such as rabbits, rats, and mice, and other animals. Animals include all vertebrates, e.g., mammals and non-mammals, such as sheep, dogs, cows, chickens, amphibians, and reptiles.

[0037] "Treating" or "treatment" includes the administration of the compositions, compounds or agents of the present invention to prevent or delay the onset of the symptoms, complications, or biochemical indicia of a disease, alleviating or ameliorating the symptoms or arresting or inhibiting further development of the disease, condition, or disorder. "Treating" further refers to any indicia of success in the treatment or amelioration or prevention of the disease, condition, or disorder, including any objective or subjective parameter such as abatement; remission; diminishing of symptoms or making the disease condition more tolerable to the patient; slowing in the rate of degeneration or decline; or making the final point of degeneration less debilitating. The treatment or amelioration of symptoms can be based on objective or subjective parameters; including the results of an examination by a physician. Accordingly, the term "treating" includes the administration of the compounds or agents of the present invention to prevent or delay, to alleviate, or to arrest or inhibit development of the symptoms or conditions associated with a disease or disorder. The term "therapeutic effect" refers to the reduction, elimination, or prevention of the disease or disorder, symptoms of the disease or disorder, or side effects of the disease or disorder in the subject. "Treating" or "treatment" using the methods of the present invention includes preventing the onset of symptoms in a subject that can be at increased risk of a disease or disorder or but does not yet experience or exhibit symptoms, inhibiting the symptoms of a disease or disorder (slowing or arresting its development), providing relief from the symptoms or side-
effects of a disease or disorder (including palliative treatment), and relieving the symptoms of a disease or disorder (causing regression). Treatment can be prophylactic (to prevent or delay the onset of the disease, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease or condition.

[0038] A chronic disease or condition is a disease or condition that is long-lasting or recurrent. The term chronic describes the course of the disease, or its rate of onset and development. A chronic course is distinguished from a recurrent course; recurrent diseases or conditions relapse repeatedly, with periods of remission in between. Treatment of recurrent diseases and conditions with the proteins disclosed herein are also contemplated. A chronic disease or condition can have one or more of the following characteristics: a chronic disease or condition is permanent, leaves residual disability, can be caused by nonreversible pathological alteration, requires special training of the patient for rehabilitation, or can be expected to require a long period of supervision, observation, or care.

[0039] "Tumor associated antigen" denotes a protein or peptide or other molecule capable of inducing an immune response to a tumor.

[0040] An "autoantigen" denotes an endogenous antigen that stimulates the production of autoantibodies, as in an autoimmune reaction.

[0041] An "autoantibody" is an antibody that reacts with the cells, tissues, or native proteins of the individual in which it is produced.

[0042] An intact "antibody" comprises at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxyl-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant
regions of the antibodies can mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (Clq) of the classical complement system. The term antibody includes antigen-binding portions of an intact antibody that retain capacity to bind. Examples of binding include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CH1 domains; (ii) a F(ab')_2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward et al, Nature 341: 544-546, 1989), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR).

[0043] "Single chain antibodies" or "single chain Fv (scFv)" refers to an antibody fusion molecule of the two domains of the Fv fragment, V_L and V_H. Although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); see, e.g., Bird et al., Science 242: 423-426, 1988; and Huston et al, Proc. Natl. Acad. Sci. USA, 85: 5879-5883, 1988). Such single chain antibodies are included by reference to the term "antibody" fragments can be prepared by recombinant techniques or enzymatic or chemical cleavage of intact antibodies.

[0044] "Human sequence antibody" includes antibodies having variable and constant regions (if present) derived from human germline immunoglobulin sequences. The human sequence antibodies of the invention can include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis in vitro or by somatic mutation in vivo). Such antibodies can be generated in non-human transgenic animals, e.g., as described in PCT Publication Nos. WO 01/14424 and WO 00/37504. However, the term "human sequence antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences (e.g., humanized antibodies).
Also, recombinant immunoglobulins can be produced. See, Cabilly, U.S. Pat. No. 4,816,567, incorporated herein by reference in its entirety and for all purposes; and Queen et al., Proc. Nat'l Acad. Sci. USA 86: 10029-10033, 1989.

"Monoclonal antibody" refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions (if present) derived from human germline immunoglobulin sequences. In one aspect, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, e.g., a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

"Immune cell response" refers to the response of immune system cells to external or internal stimuli (e.g., antigen, cell surface receptors, cytokines, chemokines, and other cells) producing biochemical changes in the immune cells that result in immune cell migration, killing of target cells, phagocytosis, production of antibodies, other soluble effectors of the immune response, and the like.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in a protein is preferably replaced with another amino acid residue from the same side chain family.

Alternatively, in another aspect, mutations can be introduced randomly along all or part of a coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis of the protein sequences, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.
"Genetic fusion" and variations thereof, refers to the creation of a single gene construct that results in production of a single fusion protein with multiple functional domains, one of which is target antigen binding (the scFv) and the other is the antigen being targeted, for example, OVA, HEL, HA(flu), CSP (malaria) or some other antigen or antigen fragment and the like.

This is different from chemical conjugation techniques which often result in multiple sites of conjugation of an antigen to a molecule such as an antibody. It fixes the ratio of the antigen:targeting motif at 1:1, whether the molecule oligomerizes or not, there will still be a single targeting binding site for each antigen attached.

A "biologically active portion" of a protein includes a fragment of a protein which participates in an interaction between a molecule and an effector molecule. Biologically active portions of a protein include peptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the protein of interest.

A biologically active portion of a protein can be a polypeptide which is, for example, 10, 25, 50, 100, 200, or more, amino acids in length. Biologically active portions of a protein can be used as targets for developing agents which modulate a various activities as described herein.

Calculations of homology or sequence identity (the terms are used interchangeably herein) between sequences are performed as follows. To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred aspect, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid
or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology").
The percent identity between the two sequences is a function of the number of
identical positions shared by the sequences, taking into account the number of gaps,
and the length of each gap, which need to be introduced for optimal alignment of the
two sequences.

[0054] The comparison of sequences and determination of percent identity
between two sequences can be accomplished using a mathematical algorithm. In a
preferred aspect, the percent identity between two amino acid sequences is determined
using the (Needleman and Wunsch, J. Mol. Biol. 48: 444-453, 1970) algorithm which
has been incorporated into the GAP program in the GCG software package (available
at http://www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix, and a
gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet
another preferred aspect, the percent identity between two nucleotide sequences is
determined using the GAP program in the GCG software package (available at
http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50,
60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A particularly preferred set of
parameters (and the one that should be used if the practitioner is uncertain about what
parameters should be applied to determine if a molecule is within a sequence identity
or homology limitation of the invention) are a Blossum 62 scoring matrix with a gap
penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[0055] The percent identity between two amino acid or nucleotide sequences
can be determined using the algorithm of (Meyers and Miller, CABIOS 4: 11-17,
1989) which has been incorporated into the ALIGN program (version 2.0), using a
PAM 120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

[0056] The nucleic acid and protein sequences described herein can be used
as a "query sequence" to perform a search against public databases to, for example,
identify other family members or related sequences. Such searches can be performed
using the NBLAST and XBLAST programs (version 2.0) of (Altschul et al., J. Mol.
Biol 215: 403-10, 1990). BLAST nucleotide searches can be performed with the
NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences
homologous to nucleic acid molecules of the invention. BLAST protein searches can
be performed with the XBLAST program, score=50, wordlength=3 to obtain amino
acid sequences homologous to protein molecules of the invention. To obtain gapped
alignments for comparison purposes, Gapped BLAST can be utilized as described in
(Altschul et al, Nucleic Acids Res 25: 3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

[0057] Particular polypeptides of the present invention have an amino acid sequence sufficiently identical or substantially identical to the amino acid sequence of the protein sequences. "Sufficiently identical" or "substantially identical" is used herein to refer to a first amino acid or nucleotide sequence that contains a sufficient or minimum number of identical or equivalent (e.g., with a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have a common structural domain or common functional activity. For example, amino acid or nucleotide sequences that contain a common structural domain having at least about 60%, or 65% identity, likely 75% identity, more likely 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity are defined herein as sufficiently or substantially identical.

[0058] For convenience, immune responses are often described as being either "primary" or "secondary" immune responses. A primary immune response, which is also described as a "protective" immune response, refers to an immune response produced in an individual as a result of some initial exposure (e.g., the initial "immunization") to a particular antigen. Such an immunization can occur, for example, as the result of some natural exposure to the antigen (for example, from initial infection by some pathogen that exhibits or presents the antigen) or from antigen presented by cancer cells of some tumor in the individual. Alternatively, the immunization can occur as a result of vaccinating the individual with a vaccine containing the antigen.

[0059] A primary immune response can become weakened or attenuated over time and can even disappear or at least become so attenuated that it cannot be detected. Accordingly, the present invention also relates to a "secondary" immune response, which is also described here as a "memory immune response." The term secondary immune response refers to an immune response elicited in an individual after a primary immune response has already been produced. Thus, a secondary or immune response can be elicited, e.g., to enhance an existing immune response that has become weakened or attenuated, or to recreate a previous immune response that has either disappeared or can no longer be detected. An agent that can be
administrated to elicit a secondary immune response is after referred to as a "booster" since the agent can be said to "boost" the primary immune response.

[0060] "Immunologically cross-reactive" or "immunologically reactive" refers to an antigen which is specifically reactive with an antibody which was generated using the same ("immunologically reactive") or different ("immunologically cross-reactive") antigen.

[0061] "Immunologically reactive conditions" refers to conditions which allow an antibody, generated to a particular epitope of an antigen, to bind to that epitope to a detectably greater degree than the antibody binds to substantially all other epitopes, generally at least two times above background binding, preferably at least five times above background. Immunologically reactive conditions are dependent upon the format of the antibody binding reaction and typically are those utilized in immunoassay protocols. See, Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, 1988 for a description of immunoassay formats and conditions.

[0062] "Inhibitors," "activators," and "modulators" of B-lymphocyte receptors in cells are used to refer to inhibitory, activating, or modulating molecules, respectively, identified using in vitro and in vivo assays for receptor binding or signaling on B-lymphocyte through surface receptors CD22 and CD72, e.g., ligands, agonists, antagonists, and their homologs and mimetics.

[0063] "Modulator" includes inhibitors and activators. Inhibitors are agents that, e.g., bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate the activity of B-lymphocytes through surface receptors CD22 and CD72, e.g., antagonists. Activators are agents that, e.g., bind to, stimulate, increase, open, activate, facilitate, enhance activation, sensitize or up regulate the activity of B-lymphocytes through surface receptors CD22 and CD72, e.g., agonists. Modulators include agents that, e.g., alter the interaction of B-lymphocyte receptor with: proteins that bind activators or inhibitors, receptors, including proteins, peptides, lipids, carbohydrates, polysaccharides, or combinations of the above, e.g., lipoproteins, glycoproteins, and the like. Modulators include genetically modified versions of naturally-occurring B-lymphocyte receptor ligands, e.g., with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, small chemical molecules and the like. "Cell-based assays" for inhibitors and activators include, e.g., applying putative modulator compounds to a
cell expressing a B-lymphocyte receptor, e.g., surface receptors CD22 and CD72, and then determining the functional effects on B-lymphocyte receptor signaling, as described herein. "Cell based assays" include, but are not limited to, in vivo tissue or cell samples from a mammalian subject or in vitro cell-based assays comprising B-lymphocyte receptor that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) can be assigned a relative B-lymphocyte receptor activity value of 100%. Inhibition of B-lymphocyte receptor is achieved when the B-lymphocyte receptor activity value relative to the control is about 80%, optionally 50% or 25-0%. Activation of B-lymphocyte receptor is achieved when the B-lymphocyte receptor activity value relative to the control is 110%, optionally 150%, optionally 200-500%, or 1000-3000% higher.

[0064] The ability of a molecule to bind to B-lymphocyte receptor can be determined, for example, by the ability of the putative ligand to bind to B-lymphocyte receptor immunoadhesin coated on an assay plate. Specificity of binding can be determined by comparing binding to non-B-lymphocyte receptors.

[0065] "Test compound" refers to any compound tested as a modulator of an immune response via B-lymphocyte receptor, e.g., surface receptors CD22 and CD72. The test compound can be a biological entity, such as a protein, e.g., an antibody or peptide, or scFv fusion to an antigen to B-lymphocytes through surface receptors CD22 and CD72 surface receptor. Alternatively, test compound can be modulators that are genetically altered versions of scFv fusion to an antigen to B cell surface receptor or scFv fusion to the antigen. Typically, test compounds will be peptides or peptidemimetics.

[0066] In one aspect, antibody binding to B-lymphocyte receptor can be assayed by either immobilizing the ligand or the receptor. For example, the assay can include immobilizing B-lymphocyte receptor fused to a His tag onto Ni-activated NTA resin beads. Antibody can be added in an appropriate buffer and the beads incubated for a period of time at a given temperature. After washes to remove unbound material, the bound protein can be released with, for example, SDS, buffers with a high pH, and the like and analyzed.

[0067] "Signaling responsiveness" refers to signaling via a B-lymphocyte receptor, e.g., surface receptors CD22 and CD72. Signaling responsiveness can refer
to, for example, an B-lymphocyte receptor, e.g., surface receptors CD22 and CD72, response dependent on the binding of a scFvIG-antigen fusion, through which a signal is propagated. The B-lymphocyte receptor signaling can occur, for example, in B cells or dendritic cells. Signal generating compounds for measurement in cell-based assays can be generated, e.g., by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol.

[0068] "Detecting an effect of a test compound on B-lymphocyte receptor signaling" can refer to a therapeutic or prophylactic effect in a mammalian subject, such as the reduction, elimination, or prevention of the disease, symptoms of the disease, or side effects of the disease in the subject. "Detecting an effect of a test compound on B-lymphocyte receptor signaling" can refer to a compound having an effect in a cell-based assay, e.g., a diagnostic assay, as measured by scFv antigen fusion protein binding to a B-lymphocyte through surface receptors CD22 or CD72.


[0070] While much is known about how DCs process and present Ags to T cells (Trombetta and Mellman, 2005), less is known about how Ag-specific responses of B cells occur and are regulated. Given our results in the manuscript attached herein, we propose that ITIM bearing receptors on B cells play an important role in regulating Ag entry into the immune system and to B cells in particular. Thus, they are ideal targets for delivering Ag into the immune system.

[0071] Others and we reported that human DCs activated through CD40 could drive B cells to proliferate and mature (Pinchuk et al, 1996, Clark, 1997, Dubois et al, 1997, 1998, Fayette et al, 1998, Wykes et al, 1998). Since then a number of studies have suggested that DCs can play an important role in Ag
preservation and presentation to B cells (Carrasco and Batista, 2006). Balazs et al. (2002) found that a rapid antibody response to intravenous injection of intact, particulate Strepococcus pneumoniae involves both dendritic cells (DCs) and B cells. Blood DCs efficiently capture bacteria and deliver them to the spleen, where DCs encounter and trigger marginal zone B cells to mature and produce protective IgM antibodies. As with Ag-specific activation of T cells by DCs (Clark and Ledbetter, 1994), this B cell maturation requires 'co-stimulation': the bacterial Ag and a second signal provided by BAFF/BLys. The DCs are a likely source of BAFF since bacterial products stimulate DCs to produce BAFF (e.g., Craxton et al., 2003, Boule et al., 2004). More recently Qi et al. (2006) showed that B cells entering lymph nodes (LN) interact with DCs bearing antigen and are activated through a mechanism apparently requiring cell-cell contact. We propose that ITIM bearing receptors on B cells regulate these processes and thus are important for Ag delivery into the immune system through scFv technology.

[0072] B cells clearly can acquire Ags from DCs and other target cells and recognize membrane-associated Ags (Batista et al., 2001, Bergtold et al., 2005, Carrasco and Batista, 2006). The fact that DCs can activate Ag-specific B cells suggests that DCs have a means of retaining Ags in an intact form, a process we term 'Ag preservation' in contrast to the 'Ag processing' of Ag for T cells. DCs can internalize and sequester soluble ovalbumin (OA) intracellularly, until they receive a second signal via e.g., CD40, which induces cross presentation (Delamarre et al., 2003). The fact that DCs express low levels of proteases (Delamarre et al., 2005) can explain why they degrade antigens relatively slowly. Bergtold et al. (2005) found that whether or not DC-associated Fc receptors process Ag for presentation to T cells or preserve Ag for presentation to B cells depends on whether the Fc receptor cytoplasmic tail has an ITAM or ITBvI for signaling. These results suggest that targeting Ags to DC receptors with different cytoplasmic signaling elements can differentially affect the program for Ag presentation to B cells vs. Ag processing and presentation to T cells. Our results indicate that CD22 and CD72 can be key ITIM receptors on B cells for Ag targeting and regulation of the type of immune response elicited.

[0073] The Ag- scFv technology will make it possible to compare the efficacy of targeting antigens to DCs vs. B cells with DNA vaccination. Because as with DCs, activation signals to B cells enhance their capacity as APCs (Heit et al,
2004), we will compare the following prime-boost protocols with anti-CD40 scFv: 1) prime HA-anti-33D1 (DCs), boost with either a) HA-anti-33D1 (DCs) or b) HA-anti-CD22 (B cells); and 2) prime with HA-anti-CD22 (B cells), boost with either a) HA-anti-33D1 (DCs) orb) HA-anti-CD22 (B cells).

[0074] Based on a number of previous studies showing that DCs are especially efficient at stimulating naive T cells, we predict that priming with DCs will consistently be more efficient than priming with B cells. However, it is less clear if targeting DCs in a booster will be more or less effective than targeting B cells or will lead to qualitatively different antibody responses. It will be important to compare by ELISA the levels of HA-specific antibodies of different isotypes and IgG subclasses. While the initial screen here will be a simple ELISA, more complete humoral antibody responses will be performed for top vaccine candidates.

[0075] Recent studies have shown that dendritic cells (DCs) can regulate B cell functions. Here we report that bone marrow (BM)-derived immature DCs (iDCs), but not mature DCs (mDCs), can inhibit BCR-induced proliferation of B cells in a contact-dependent manner. This inhibition is also dependent on the B cell antigen co-receptor, CD22. However, expression of α2-6 sialic acids on iDCs is not required for this inhibition as iDCs from ST6Gal-I KO mice also inhibited BCR-induced proliferation of WT B cells. We also report that a ligand for CD22 is expressed on the surface of iDCs. This ligand does not contain α2-6 sialic acids and is distinct from the ligand expressed on the surface of B cells. Upon examination of the splenic B cell subsets in the various KO mice, we found that CD22 and ST6Gal-I KO mice had deficiencies in marginal zone and transitional 2 precursor B cells, suggesting that the development of these splenic B cells is dependent on both CD22 and α2-6 sialic acid-containing glycoproteins. However, the numbers of long-lived mature B cells in the BM were lower in the CD22 KO, but normal in the ST6Gal-I KO mice, indicating that the maintenance of these cells is dependent only on CD22. Thus, we propose there are two distinct ligands for CD22 that have different functions. The α2-6 sialic acid-containing glycoprotein can be important for the development of splenic B cell subsets while the ligand that does not contain α2-6 sialic acids, perhaps on iDCs, is required for the maintenance of long-lived mature B cells in the BM.

[0076] The role of DCs in antigen (Ag) capture, processing and presentation to T cells has been extensively studied (Clark and Ledbetter, 1994, Kapsenberg,
Upon entry into LNs, DCs capture and process Ag and then migrate to T cell zones where they present the Ag to T helper (T_h) cells. The primed T_h cells, in turn, induce B cells growth, differentiation and antibody (Ab) production through the release of cytokines and also through direct cell-cell contact requiring CD40-CD40 ligand (CD40L/CD154) interactions. Thus, DCs can regulate B cells responses indirectly through T cell priming. Several studies have shown, however, that DCs can also directly influence B cell responses. One way by which DCs regulate B cells is through the production of soluble factors including cytokines. DCs can stimulate CD40-activated B cells to proliferate and produce Ab by secreting soluble factors (Dubois et al, 1998). The differentiation of naive B cells into IgM-secreting plasma cells is dependent on IL-12 production by DCs (Dubois et al, 1998).

Other soluble factors produced by DCs that can regulate B cells are the TNF family members B cell activating factor, BAFF (BlyS/TALL-1/THANK/zTNF4) and APRIL (Balazs et al, 2002, Litinskiy et al, 2002, Avery et al, 2003, Craxton et al, 2003, Hanada et al, 2003). BAFF and APRIL produced by DCs greatly enhance B cell Ag receptor (BCR)-induced proliferation of B cells (Craxton et al, 2003, Hanada et al, 2003). Balazs et al (2002) also found that BAFF and APRIL play a role in the differentiation of naive marginal zone (MZ) B cells into plasmablasts. These TNF family members also induce IgG secretion in B cells (Hanada et al, 2003). Furthermore, CD40-independent class switching is also affected by DC-generated BAFF and APRIL (Litinskiy et al, 2002). Type I IFNs and IL-6 produced by a subset of DCs also influence B cells to differentiate into Ig-secreting plasmablasts (Jego et al, 2003).

DCs can also exert their effects on B cells by direct cell-to-cell interactions. CD40L expressed on DCs can induce CD40 positive B cells to secrete IgG and IgA (Pinchuk et al, 1996). DC-mediated CD40 triggering can also provide B cells with a survival signal (Wykes and MacPherson, 2000). Ag-specific B cells can physically interact with DCs in vitro that have been pulsed with the cognate Ag (Huang et al, 2005). This direct interaction induces the release of intracellular Ca^{2+} by the B cells. DCs and B cells can form clusters in vitro, resulting in the induction of B cell proliferation, differentiation and isotype class switching (Dubois et al, 1999). In vivo, B cells and DCs interact in a few distinct locations. DCs can cluster with B cells in the lymph in an LFA-I-dependent manner (Kushnir et al, 1998). Both VLA-4 and LFA-1 play important roles in enabling B cells to recognize membrane-bound
Ags expressed on target cells (Carrasco et al., 2004; Carrasco et al., 2006). When LFA-I and VLA-4 on B cells bind to their ligands (CD54/ICAM-1 and VCAM-I, respectively) on the surface of target cells also expressing membrane-bound Ag, the threshold for A-specific BCR signaling required for activation is lowered. The binding of both the integrin and BCR facilitates synapse formation between the B cell and target cell and allows the B cell to effectively spread over the membrane expressing the Ag. This subsequently results in B cell activation and allows the B cell to acquire Ag from the target cell (Batista et al., 2001; Fleire et al., 2006).

[0079] DCs and B cell also interact in the red pulp of the spleen (Garcia de Vinuesa et al., 1999). This interaction is thought to support plasmablast survival and their differentiation into Ab-secreting plasma cells. Balazs et al. (2002) demonstrated that association between DC and B cells can occur at T cell-B cell borders in the spleen. Blood DCs capture bacteria and deliver them to MZ B cells, after which, the DC-B cell-Ag complexes moves to T cell-B cell borders and the bridging channel connecting the white and red pulp, where plasmablast production subsequently takes place (Balazs et al., 2002). In LNs, B cells and DCs associate in follicles (Berney et al., 1999, Lindquist et al., 2004). B cells can also interact with DCs once they have exited the high endothelial venule, but before they enter LN follicles (Qi et al., 2006). This interaction results in the activation of the Ag-specific B cells by the DCs expressing cognate Ag, and in the transfer of Ag on the surface of DCs to the Ag-specific B cells (Wykes et al., 1998; Qi et al., 2006).

[0080] Here we report that bone marrow-derived immature DCs (iDCs), but not mature DCs (mDCs), can inhibit BCR-induced proliferation of B cells in a contact-dependent manner. This inhibition is also dependent on the BCR co-receptor, CD22, which contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) (for a review, see M. in Daeron, Annu. Rev. Immunol. 15:203-234 , 1997). We also report the novel finding that a ligand for CD22, not containing α2-6-sialic acids, is expressed on the surface of iDCs. This CD22 ligand on iDCs is distinct from the ligands expressed on the surface of B cells, implicating differential roles for cis versus trans interactions between CD22 and its ligand.
BISPECIFIC COMPOUNDS AS MODULATOR OF B-LYMPHOCYTE ACTIVITY

In one aspect, a method for identifying candidate or test bispecific compounds is provided which reduce the concentration of an agent in the serum and/or circulation of a non-human animal. Compounds selected or optimized using the instant methods can be used to treat subjects that would benefit from administration of such a compound, e.g., human subjects.

Candidate compounds that can be tested in an aspect of the methods of the present invention are bispecific compounds. As used herein, the term "bispecific compound" includes compounds having two different binding specificities. Exemplary bispecific compounds include, e.g., bispecific antibodies, heteropolymers, and antigen-based heteropolymers.

Bispecific molecules that can be tested in an aspect of the invention preferably include a binding moiety that is specific for B-lymphocyte receptors, e.g., surface receptors CD22 and CD72.

In another aspect, novel B-lymphocyte receptor binding molecules can be identified based on their ability to bind to surface receptors CD22 and CD72. For example, any number of test compounds, e.g., scFv-antigen fusion proteins or peptidomimetics or other drugs can be used for testing and can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small chemical molecule libraries of compounds (Lam, Anticancer Drug Des. 12: 145, 1997).

In many drug screening programs which test libraries of modulating agents and natural extracts, high throughput assays are desirable in order to maximize the number of modulating agents surveyed in a given period of time. Assays which are performed in cell-free systems, such as can be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test modulating agent. Moreover, the
effects of cellular toxicity and/or bioavailability of the test modulating agent can be
generally ignored in the in vitro system, the assay instead being focused primarily on
the effect of the drug on the molecular target as can be manifest in an alteration of
binding affinity with upstream or downstream elements.

[0086] In another aspect, phage display techniques known in the art can be
used to identify novel binding molecules of B-lymphocyte receptor, e.g., surface
receptors CD22 and CD72.

[0087] In one aspect, the invention provides assays for screening candidate
or test compounds which bind to surface receptors CD22 and CD72 or biologically
active portion thereof.

[0088] Cell-based assays for identifying molecules that bind to B-
lymphocyte receptor, e.g., surface receptors CD22 and CD72, can be used to identify
additional agents for use in bispecific compounds of the invention. For example, cells
expressing surface receptors CD22 and CD72 can be used in a screening assay. For
example, compounds which produce a statistically significant change in binding to
surface receptors CD22 and CD72 can be identified.

[0089] In one aspect, the assay is a cell-free assay in which a B-lymphocyte
receptor binding molecule is identified based on its ability to bind to surface receptors
CD22 and CD72 in vitro. The surface receptors CD22 and CD72 protein binding
molecule can be provided and the ability of the protein to bind B-lymphocyte receptor
can be tested using art recognized methods for determining direct binding.

Determining the ability of the protein to bind to a target molecule can be
accomplished, e.g., using a technology such as real-time Biomolecular Interaction
Curr. Opin. Struct. Biol. 5: 699-705, 1995. As used herein, "BIA" is a technology for
studying bispecific interactions in real time, without labeling any of the interactants
(e.g., BIAcore). Changes in the optical phenomenon of surface plasmon resonance
(SPR) can be used as an indication of real-time reactions between biological
molecules.

[0090] The cell-free assays of the present invention are amenable to use of
both soluble and/or membrane-bound forms of proteins. In the case of cell-free assays
in which a membrane-bound form a protein is used it can be desirable to utilize a
solubilizing agent such that the membrane-bound form of the protein is maintained in
solution. Examples of such solubilizing agents include non-ionic detergents such as n-
octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-1 14, Thesit®, Isotridecypoly(ethylene glycol ether),n, 3-[(3-cholamidopropyl)dimethylammonio]-l-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-l-propane sulfonate (CHAPSO), or N-dodecyl=NN,N-dimethyl-3-ammonio-l-propane sulfonate.

[0091] Suitable assays are known in the art that allow for the detection of protein-protein interactions (e.g., immunoprecipitations, two-hybrid assays and the like). By performing such assays in the presence and absence of test compounds, these assays can be used to identify compounds that modulate (e.g., inhibit or enhance) the interaction of a protein of the invention with a target molecule(s).

[0092] Determining the ability of the protein to bind to or interact with a target molecule can be accomplished, e.g., by direct binding. In a direct binding assay, the protein could be coupled with a radioisotope or enzymatic label such that binding of the protein to a target molecule can be determined by detecting the labeled protein in a complex. For example, proteins can be labeled with $^{125}$I, $^{35}$S, $^{14}$C, or $^{3}$H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, molecules can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

[0093] Typically, it will be desirable to immobilize either a protein of the invention or its binding protein to facilitate separation of complexes from uncomplexed forms of one or both of the proteins, as well as to accommodate automation of the assay. Binding to an upstream or downstream binding element, in the presence and absence of a candidate agent, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In one aspect, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/ B-lymphocyte receptor (GST/ B cell receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, Mo.) or glutathione derivatized microtitre plates, which are then combined with the cell lysates, e.g. $^{35}$S-labeled, and the test modulating agent, and the mixture incubated under conditions conducive to complex formation, e.g., at physiological
conditions for salt and pH, though slightly more stringent conditions can be used. Following incubation, the beads are washed to remove any unbound label, and the matrix immobilized and radiolabel determined directly (e.g., beads placed in scintillants), or in the supernatant after the complexes are subsequently dissociated. Alternatively, the complexes can be dissociated from the matrix, separated by SDS-PAGE, and the level of B-lymphocyte receptor-binding protein found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

[0094] Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, biotinylated molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, 111.), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical).

[0095] It is also within the scope of this invention to determine the ability of a compound to modulate the interaction between antigen proteins and B-lymphocyte receptor, e.g., surface receptors CD22 and CD72, without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of a protein of the invention with its target molecule without the labeling of either the protein or the target molecule. McConnell et al, Science 257: 1906-1912, 1992. As used herein, a "microphysiometer" (e.g., Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indicator of the interaction between compound and receptor.

that can be used to cross-link the components of a bispecific molecule include: polyethylene glycol, SATA, SMCC, as well others known in the art, and available, e.g., from Pierce Biotechnology. Exemplary forms of bispecific molecules that can be tested are described in U.S. Ser. No. 60/411,731, filed on Sep. 16, 2002, the contents of which are incorporated herein by reference.

[0097] In another aspect, different multimeric forms of bispecific molecules can be made (e.g., dimer, trimer, tetramer, pentamer, or higher multimer forms). In another aspect, purified forms of bispecific molecules can be tested, e.g., as described in U.S. Ser. No. 60/380,211, filed on Can 13, 2002, the contents of which are incorporated herein by reference.

[0098] In another aspect, when one of the binding moieties of the heteropolymer is an antibody, antibodies of different isotypes (e.g., IgA, IgD, IgE, IgGl, IgG2 (e.g., IgG2a), IgG3, IgG4, or IgM) can be used. In another aspect, portions of an antibody molecule (e.g., Fab fragments) can be used for one of the binding moieties. In a preferred aspect at least one of the binding moieties is an antibody comprising an Fc domain. In one aspect, the antibody is a mouse antibody.

[0099] In another aspect, the effect of modifications to antibodies can be tested, e.g., the effect of deimmunization of the antibody, e.g., as described in U.S. Ser. No. 60/458,869, filed on Mar. 28, 2003 can be tested.

[0100] In methods provided in the present invention, the concentration of an agent, e.g. pathogenic agent, in the serum, circulation and/or tissue of the non-human animal can be reduced by at least e.g. about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90% or about 100%.

[0101] In another aspect, the concentration of an agent in the serum, circulation and/or tissue of a subject can be measured indirectly. For example, pathology resulting from the presence of the agent in the serum and/or circulation can be measured, e.g., by examining tissue samples from the animal. Another indirect measurement of the concentration of an agent in the serum, circulation and/or tissue of the non-human animal is measurement of the ability of the agent to cause infection in the non-human animal. For example, the effect of the bispecific compound on clinical signs and symptoms of infection can be measured. The ability of the bispecific compound to inhibit the spread of infection, e.g., from one organ system to another or from one individual to another can also be tested.
In another aspect the ability of the bispecific compound to bind to
cells bearing scFv-antigen fusion proteins in the non-human animal is measured. For
example, in one aspect, determining the ability of the bispecific compound to bind to
a B-lymphocyte receptor target molecule can also be accomplished using a
technology such as real-time Biomolecular Interaction Analysis (BIA) (Sjolander et
699-705, 1995). As used herein, “BIA” is a technology for studying biospecific
interactions in real time, without labeling any of the interactants (e.g., BIAcore).
Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used
as an indication of real-time reactions between biological molecules.

In another aspect, the destruction of the agent by cells in the non-
human animal (e.g., killing by macrophage) is measured.

Compounds that reduce the concentration of the agent in the serum
and/or circulation of the non-human animal (as compared with concentrations
observed in non-human animals that do not receive the bispecific compound) can be
selected.

Compounds for testing in the subject assays can be selected from
among a plurality of compounds tested. In another aspect, bispecific compounds for
testing in the instant assays can have already been identified as being capable of
binding B-lymphocyte receptor, e.g., surface receptors CD22 and CD72, e.g., in an in
vitro assay and can be further evaluated or optimized using the instant assays. In such
cases, the ability of a bispecific compound to reduce the concentration of an agent in
the serum and/or circulation can be compared to another bispecific compound or a
non-optimized version of the same compound to determine its ability reduce the
concentration of the agent in the serum and/or circulation.

In preferred aspects, the bispecific compounds of the instant
invention are administered at concentrations in the range of approximately 1 µg
compound/kg of body weight to approximately 100 µg compound/kg of body weight.
As defined herein, a therapeutically effective amount of a bispecific compound (i.e.,
an effective dosage) ranges from about 0.01 to 5000 µg/kg body weight, preferably
about 0.1 to 500 µg/kg body weight, more preferably about 2 to 80 µg/kg body
weight, and even more preferably about 5 to 70 µg/kg, 10 to 60 µg/kg, 20 to 50 µg/kg,
24 to 41 µg/kg, 25 to 40 µg/kg, 26 to 39 µg/kg, 27 to 38 µg/kg, 28 to 37 µg/kg, 29 to
36 µg/kg, 30 to 35 µg/kg, 31 to 34 µg/kg or 32 to 33 µg/kg body weight. The skilled
artisan will appreciate that certain factors can influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

[0107] In a preferred example, the animal is treated with bispecific compound in the range of between about 1 to 500 µg/kg body weight following intravenous (iv) injection of an agent. It will also be appreciated that the effective dosage of a bispecific compound used for treatment can increase or decrease over the course of a particular treatment. Changes in dosage can result and become apparent from the results of diagnostic assays as described herein.

[0108] The route of administration of test compounds and/or agents can be intravenous (iv) injection into the circulation of the animal. Other administration routes include, but are not limited to, topical, parenteral, subcutaneous, or by inhalation. The term "parenteral" includes injection, e.g. by subcutaneous, intravenous, or intramuscular routes, also including localized administration, e.g., at a site of disease or injury. Sustained release of compounds from implants is also known in the art. One skilled in the pertinent art will recognize that suitable dosages will vary, depending upon such factors as the nature of the disorder to be treated, the patient's body weight, age, and general condition, and the route of administration. Preliminary doses can be determined according to animal tests, and the scaling of dosages for human administration are performed according to art-accepted practices.

[0109] The candidate compounds and agents can be administered over a range of doses to the animal. When the agent is also administered to the animal, the candidate compound can be administered either before, at the same time, or after, administration of the agent.

[0110] Surface receptors CD22 and CD72 in B-lymphocytes expressed in transgenic animals, e.g. mice, of the present invention can be used to screen or evaluate candidate compounds useful for treating disorders or diseases in humans that are associated with the presence of unwanted agents in the serum and/or circulation of a subject, such as autoantibodies, infectious agents, or toxins.

[0111] Exemplary targeted agents that can be bound by the bispecific compounds of the present invention include blood-borne agents, including, but not
limited to, any of the following: viruses, viral particles, toxins, bacteria, polynucleotides, antibodies, *e.g.*, autoantibodies associated with an autoimmune disorder. In one aspect, exemplary targeted viral agents include, but are not limited to, any one of the following: cytomegalovirus, influenza, Newcastle disease virus, vesicular stomatitis virus, rabies virus, herpes simplex virus, hepatitis, adenovirus-2, bovine viral diarrhea virus, human immunodeficiency virus (HIV), dengue virus, Marburg virus, Epstein-Barr virus.

[0112] Exemplary Gram-positive bacterial targets *Streptococcus pyogenes*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, or *Bacillus subtilis*. Any of the methods and compositions described above are useful for the treatment of skin infections, community-acquired pneumonia, upper and lower respiratory tract infections, skin and soft tissue infections, hospital-acquired lung infections, bone and joint infections, respiratory tract infections, acute bacterial otitis media, bacterial pneumonia, urinary tract infections, complicated infections, noncomplicated infections, pyelonephritis, intra-abdominal infections, deep-seated abscesses, bacterial sepsis, central nervous system infections, bacteremia, wound infections, peritonitis, meningitis, infections after burn, urogenital tract infections, gastro-intestinal tract infections, pelvic inflammatory disease, endocarditis, and other intravascular infections. The infections to be treated can be caused by Gram-positive bacteria. These include, without limitation, infections by, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Enterococcus faecium*, *Clostridium perfringens*, *Clostridium difficile*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, other *Streptococcus* spp., and other *Clostridium* spp. More specifically, the infections can be caused by a Gram-positive coccus, or by a drug-resistant Gram-positive coccus. Exemplary Gram-positive cocci are, without limitation, *S. aureus*, *S. epidermidis*, *S. pneumoniae*, *S. pyogenes*, *M. catarrhalis*, *C. difficile*, *H. pylori*, *Chlamydia* spp., and *Enterococcus* spp.

[0113] Bacteremia can be caused by gram-negative or gram-positive bacteria. Gram-negative bacteria have thin walled cell membranes consisting of a single layer of peptidoglycan and an outer layer of lipopolysacchacide, lipoprotein, and phospholipid. Exemplary gram-negative organisms include, but are not limited to, Enterobacteriacea consisting of Escherichia, Shigella, Edwardsiella, Salmonella, Citrobacter, Klebsiella, Enterobacter, Hafnia, Serratia, Proteus, Morganella, Providencia, Yersinia, Erwinia, Butlaxella, Cedecea, Ewingella, Kluyvera,
Tatumella and Rahnella. Other exemplary gram-negative organisms not in the family Enterobacteriaceae include, but are not limited to, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Burkholderia, Cepacia, Gardenerella, Vaginalis, and Acinetobacter species. Gram-positive bacteria have a thick cell membrane consisting of multiple layers of peptidoglycan and an outside layer of teichoic acid. Exemplary gram-positive organisms include, but are not limited to, Staphylococcus aureus, coagulase-negative staphylococci, streptococci, enterococci, corynebacteria, and Bacillus species.

[0114] In one aspect, the targeted agent is resistant to traditional therapies, e.g., is resistant to antibiotics.

[0115] In one aspect, in performing an assay of the invention, the agent is administered to the transgenic animal, e.g., prior to, simultaneously with, or after administration of a bispecific compound.

[0116] The bispecific compounds of the present invention, or any portion thereof, can be modified to enhance their half life. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compounds are termed "peptide mimetics" or "peptidomimetics" (Fauchere, Adv. Drug Res. 15: 29, 1986; Veber et al, TINS p.392, 1985; and Evans et al, J. Med. Chem 30: 1229, 1987, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides can be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as an antigen polypeptide, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH₂NH⁻, -CH₂S⁻, -CH₂=CH⁻, -CH=CH⁻, -COCH₂⁻, -CH(OH)CH₂⁻, and -CH₂SO⁻, by methods known in the art and further described in the following references:

Almquist et al., J. Med. Chem. 23: 1392-1398, 1980 (--COCH$_2$); Jennings-White et al., Tetrahedron Lett. 23: 2533, 1982 (--COCH$_2$); Szelke et al., European Patent Application No. EP 45665 CA: 97: 39405, 1982 (--CH(OH)CH$_2$); Holladay et al., Tetrahedron. Lett. 24: 4401-4404, 1983 (--COCH$_2$); and Hruby, Life Sci. 31: 189-199, 1982 (--CH$_2$S--); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH$_2$NH-. Such peptide mimetics can have significant advantages over polypeptide aspects, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) to which the peptidomimetic binds to produce the therapeutic effect. Derivatization (e.g., labeling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic.

[0117] Systematic substitution of one or more amino acids of an amino acid sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) can be used to generate more stable peptides. In addition, constrained peptides can be generated by methods known in the art (Rizo et al., Annu. Rev. Biochem. 61: 387, 1992, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.


[0119] Polypeptides can be produced, typically by direct chemical synthesis, and used as a binding moiety of a heteropolymer. Peptides can be produced as modified peptides, with nonpeptide moieties attached by covalent linkage to the N-terminus and/or C-terminus. In certain preferred aspects, either the carboxy-terminus or the amino-terminus, or both, are chemically modified. The most common modifications of the terminal amino and carboxyl groups are acetylation and amidation, respectively. Amino-terminal modifications such as acylation (e.g., acetylation) or alkylation (e.g., methylation) and carboxy-terminal modifications such as amidation, as well as other terminal modifications, including cyclization, can be incorporated into various aspects of the test compounds. Certain amino-terminal and/or carboxy-terminal modifications and/or peptide extensions to the core sequence can provide advantageous physical, chemical, biochemical, and pharmacological properties, such as: enhanced stability, increased potency and/or efficacy, resistance to serum proteases, desirable pharmacokinetic properties, and others.

**METHODS OF TREATMENT**

[0120] Also described herein are both prophylactic and therapeutic methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72.

[0121] The invention relates to methods for modulating an immune response in a subject, the method comprising targeting antigens to B cell surface receptors using an scFvIg derived from an antibody which binds to the receptor, and which genetically fuses the antigen to the carboxyl end of the scFvIg moiety. Therapeutic antibodies and scFv-antigen fusion proteins can be identified by, for example, any of a combination of diagnostic or prognostic assays as described herein or are known in the art. In general, such disorders involve treatment for infectious disease, *e.g.*, bacterial, viral, or parasite disease, or cancer.

[0122] Another aspect of the invention pertains to methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72. The agent can be a compound that specifically binds to B-lymphocyte receptor, *e.g.*, surface receptors CD22 and CD72.
The agent can be an antibody or a protein, e.g., scFv-antigen fusion proteins. These modulatory methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject).

[0123] The present invention provides methods for methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72 and for treatment for infectious disease, e.g., bacterial, viral, or parasite disease, or cancer.

[0124] Successful treatment of disorders related to infectious disease or cancer can be brought about by techniques that serve to activate binding of antigen to B-lymphocyte receptor, e.g., surface receptors CD22 and CD72. For example, compounds, e.g., an agent identified using an assay described herein, such as an antibody, that prove to exhibit negative modulatory activity, can be used to prevent and/or ameliorate symptoms of disorders. Such molecules can include, but are not limited to peptides, phosphopeptides, small organic or inorganic molecules, or antibodies (including, for example, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and F(ab')2 and F(ab')expression library fragments, scFV molecules, and epitope-binding fragments thereof). In particular, antibodies and derivatives thereof (e.g., antigen-binding fragments thereof) that specifically bind to B-lymphocyte receptor, e.g., surface receptors CD22 and CD72 can modulate or activate immune response to infectious disease, e.g., bacterial, viral, or parasite disease, or cancer.

**KITS**

[0125] The invention provides kits comprising the compositions, e.g., nucleic acids, expression cassettes, vectors, cells, polypeptides (e.g., B-lymphocyte receptor, e.g., surface receptors CD22 and CD72,) and/or scFv-antigen fusion antibodies of the invention. The kits also can contain instructional material teaching the methodologies and uses of the invention, as described herein.

**THERAPEUTIC APPLICATIONS**

[0126] The compounds and modulators identified by the methods of the present invention can be used in a variety of methods of treatment. Thus, the present invention provides compositions and methods for treating an infectious disease, e.g., bacterial, viral, or parasite disease, or cancer using methods for modulating an
immune response in a subject by targeting antigens into the immune system through
the surface receptors CD22 and CD72

[0127] Exemplary infectious disease, include but are not limited to, viral or
bacterial diseases. The polypeptide or polynucleotide of the present invention can be
used to treat or detect infectious agents. For example, by increasing the immune
response, particularly increasing the proliferation and differentiation of B and/or T
cells, infectious diseases can be treated. The immune response can be increased by
either enhancing an existing immune response, or by initiating a new immune
response. Alternatively, the polypeptide or polynucleotide of the present invention can
also directly inhibit the infectious agent, without necessarily eliciting an immune
response.

[0128] Similarly, bacterial or fungal agents that can cause disease or
symptoms and that can be treated or detected by a polynucleotide or polypeptide of
the present invention include, but not limited to, the following Gram-Negative and
Gram-positive bacterial families and fungi: Actinomycetales (e.g., Corynebacterium,
Mycobacterium, Norcardia), Aspergillus, Bacillaceae (e.g., Anthrax, Clostridium),
Bacteroidaceae, Blastomyces, Bordetella, Borrelia, Brucellosis, Candidiasis,
Campylobacter, Coccidiodomycosis, Cryptococcus, Dermatocycoses,
Enterobacteriaceae (Klebsiella, Salmonella, Serratia, Yersinia), Erysipelothrix,
Helicobacter, Legionella, Leptospirosis, Listeria, Mycoplasmatales, Neisseriaceae
(e.g., Acinetobacter, Gonorrhea, Meningococcal), Pasteurellaceae Infections (e.g.,
Actinobacillus, Haemophilus, Pasteurella), Pseudomonas, Rickettsiaceae,
Chlamydiaceae, Syphilis, and Staphylococcal. These bacterial or fungal families can
cause the following diseases or symptoms, including, but not limited to: bacteremia,
endocarditis, eye infections (conjunctivitis, tuberculosis, uveitis), gingivitis,
opportunitic infections (e.g., AIDS related infections), paronychia, prosthesis-related
infections, Reiter's Disease, respiratory tract infections, such as Whooping Cough or
Empyema, sepsis, Lyme Disease, Cat-Scratch Disease, Dysentry, Paratyphoid Fever,
food poisoning, Typhoid, pneumonia, Gonorrhea, meningitis, Chlamydia, Syphilis,
Diphtheria, Leprosy, Paratuberculosis, Tuberculosis, Lupus, Botulism, gangrene,
tetanus, impetigo, Rheumatic Fever, Scarlet Fever, sexually transmitted diseases, skin
diseases (e.g., cellulitis, dermatocycoses), toxemia, urinary tract infections, wound
infections. A polypeptide or polynucleotide of the present invention can be used to
treat or detect any of these symptoms or diseases.
Moreover, parasitic agents causing disease or symptoms that can be treated or detected by a polynucleotide or polypeptide of the present invention include, but not limited to, the following families: Amebiasis, Babesiosis, Coccidiosis, Cryptosporidiosis, Dientamoebiasis, Dourine, Ectoparasitic, Giardiasis, Helminthiasis, Leishmaniasis, Theileriasis, Toxoplasmosis, Trypanosomiasis, and Trichomonas. These parasites can cause a variety of diseases or symptoms, including, but not limited to: Scabies, Trombiculiasis, eye infections, intestinal disease (e.g., dysentery, giardiasis), liver disease, lung disease, opportunistic infections (e.g., AIDS related), Malaria, pregnancy complications, and toxoplasmosis. A polypeptide or polynucleotide of the present invention can be used to treat or detect any of these symptoms or diseases.

Preferably, treatment using a polypeptide or polynucleotide of the present invention could either be by administering an effective amount of a polypeptide to the patient, or by removing cells from the patient, supplying the cells with a polynucleotide of the present invention, and returning the engineered cells to the patient (ex vivo therapy). Moreover, the polypeptide or polynucleotide of the present invention can be used as an antigen in a vaccine to raise an immune response against infectious disease.

FORMULATION AND ADMINISTRATION OF PHARMACEUTICAL COMPOSITIONS

The invention provides pharmaceutical compositions comprising polypeptides or scFv-antigen fusion proteins of the invention. As discussed above, the polypeptides or scFv-antigen fusion proteins of the invention can be used methods for modulating an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72 to treat infectious disease, e.g., bacterial, viral, or parasite disease, or cancer. Such modulation in a cell or a non-human animal can generate a screening modality for identifying compounds to treat or ameliorate infectious disease, e.g., bacterial, viral, or parasite disease, or cancer. Administration of a pharmaceutical composition of the invention to a subject is used to generate an immune response in the subject.

The polypeptides or scFv-antigen fusion proteins of the invention can be combined with a pharmaceutically acceptable carrier (excipient) to form a pharmacological composition. Pharmaceutically acceptable carriers can contain a
physiologically acceptable compound that acts to, e.g., stabilize, or increase or decrease the absorption or clearance rates of the pharmaceutical compositions of the invention. Physiologically acceptable compounds can include, e.g., carbohydrates, such as glucose, sucrose, or dextrose, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins, compositions that reduce the clearance or hydrolysis of the peptides or polypeptides, or excipients or other stabilizers and/or buffers. Detergents can also used to stabilize or to increase or decrease the absorption of the pharmaceutical composition, including liposomal carriers. Pharmacologically acceptable carriers and formulations for peptides and polypeptide are known to the skilled artisan and are described in detail in the scientific and patent literature, see e.g., the latest edition of Remington's Pharmaceutical Science, Mack Publishing Company, Easton, Pa. ("Remington's").

[0133] Other physiologically acceptable compounds include wetting agents, emulsifying agents, dispersing agents or preservatives which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, e.g., phenol and ascorbic acid. One skilled in the art would appreciate that the choice of a pharmaceutically acceptable carrier including a physiologically acceptable compound depends, for example, on the route of administration of the peptide or polypeptide of the invention and on its particular physio-chemical characteristics.

[0134] In one aspect, a solution of polypeptides or scFv-antigen fusion proteins of the invention are dissolved in a pharmaceutically acceptable carrier, e.g., an aqueous carrier if the composition is water-soluble. Examples of aqueous solutions that can be used in formulations for enteral, parenteral or transmucosal drug delivery include, e.g., water, saline, phosphate buffered saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions and the like. The formulations can contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents, detergents and the like. Additives can also include additional active ingredients such as bactericidal agents, or stabilizers. For example, the solution can contain sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate or triethanolamine oleate. These compositions can be sterilized by conventional, well-known sterilization techniques, or can be sterile filtered. The resulting aqueous solutions can be packaged for use as is, or lyophilized,
the lyophilized preparation being combined with a sterile aqueous solution prior to
administration. The concentration of peptide in these formulations can vary widely,
and will be selected primarily based on fluid volumes, viscosities, body weight and
the like in accordance with the particular mode of administration selected and the
patient's needs.

[0135] Solid formulations can be used for enteral (oral) administration. They
can be formulated as, e.g., pills, tablets, powders or capsules. For solid compositions,
conventional nontoxic solid carriers can be used which include, e.g., pharmaceutical
grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum,
cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral
administration, a pharmaceutically acceptable nontoxic composition is formed by
incorporating any of the normally employed excipients, such as those carriers
previously listed, and generally 10% to 95% of active ingredient (e.g., peptide). A
non-solid formulation can also be used for enteral administration. The carrier can be
selected from various oils including those of petroleum, animal, vegetable or synthetic
origin, e.g., peanut oil, soybean oil, mineral oil, sesame oil, and the like. Suitable
pharmaceutical excipients include e.g., starch, cellulose, talc, glucose, lactose,
sucrose, gelatin, malt, rice, flour, chalk, silica gel, magnesium stearate, sodium
stearate, glycerol monostearate, sodium chloride, dried skim milk, glycerol, propylene
glycol, water, ethanol.

[0136] Polypeptides or scFv-antigen fusion proteins of the invention, when
administered orally, can be protected from digestion. This can be accomplished either
by complexing the polypeptides or scFv-antigen fusion proteins with a composition to
render it resistant to acidic and enzymatic hydrolysis or by packaging the polypeptides
or scFv-antigen fusion proteins in an appropriately resistant carrier such as a
liposome. Means of protecting compounds from digestion are well known in the art,
see, e.g., Fix, PharmRes. 13: 1760-1764, 1996; Samanen, J. Pharm. Pharmacol. 48:
119-135, 1996; U.S. Pat. No. 5,391,377, describing lipid compositions for oral
delivery of therapeutic agents (liposomal delivery is discussed in further detail, infra).

[0137] Systemic administration can also be by transmucosal or transdermal
means. For transmucosal or transdermal administration, penetrants appropriate to the
barrier to be permeated can be used in the formulation. Such penetrants are generally
known in the art, and include, e.g., for transmucosal administration, bile salts and
fusidic acid derivatives. In addition, detergents can be used to facilitate permeation.
Transmucosal administration can be through nasal sprays or using suppositories. See, e.g., Sayani, *Crit. Rev. Ther. Drug Carrier Syst.* 13: 85-184, 1996. For topical, transdermal administration, the agents are formulated into ointments, creams, salves, powders and gels. Transdermal delivery systems can also include, e.g., patches.

[0138] The polypeptides or scFv-antigen fusion proteins of the invention can also be administered in sustained delivery or sustained release mechanisms, which can deliver the formulation internally. For example, biodegradable microspheres or capsules or other biodegradable polymer configurations capable of sustained delivery of a peptide can be included in the formulations of the invention (see, e.g., Putney, *Nat. Biotechnol.* 16: 153-157, 1998).

[0139] For inhalation, the polypeptides or scFv-antigen fusion proteins of the invention can be delivered using any system known in the art, including dry powder aerosols, liquids delivery systems, air jet nebulizers, propellant systems, and the like. See, e.g., Patton, *Biotechniques* 16: 141-143, 1998; product and inhalation delivery systems for polypeptide macromolecules by, e.g., Dura Pharmaceuticals (San Diego, Calif.), Aradigm (Hayward, Calif.), Aerogen (Santa Clara, Calif.), Inhale Therapeutic Systems (San Carlos, Calif.), and the like. For example, the pharmaceutical formulation can be administered in the form of an aerosol or mist. For aerosol administration, the formulation can be supplied in finely divided form along with a surfactant and propellant. In another aspect, the device for delivering the formulation to respiratory tissue is an inhaler in which the formulation vaporizes. Other liquid delivery systems include, e.g., air jet nebulizers.

[0140] In preparing pharmaceuticals of the present invention, a variety of formulation modifications can be used and manipulated to alter pharmacokinetics and biodistribution. A number of methods for altering pharmacokinetics and biodistribution are known to one of ordinary skill in the art. Examples of such methods include protection of the compositions of the invention in vesicles composed of substances such as proteins, lipids (for example, liposomes, see below), carbohydrates, or synthetic polymers (discussed above). For a general discussion of pharmacokinetics, see, e.g., Remington's, Chapters 37-39.

[0141] The polypeptides or scFv-antigen fusion proteins of the invention can be delivered alone or as pharmaceutical compositions by any means known in the art, e.g., systemically, regionally, or locally (e.g., directly into, or directed to, a tumor); by intraarterial, intrathecal (IT), intravenous (IV), parenteral, intra-pleural cavity, topical,
oral, or local administration, as subcutaneous, intra-tracheal (e.g., by aerosol) or transmucosal (e.g., buccal, bladder, vaginal, uterine, rectal, nasal mucosa). Actual methods for preparing administrable compositions will be known or apparent to those skilled in the art and are described in detail in the scientific and patent literature, see e.g., Remington's. For a "regional effect," e.g., to focus on a specific organ, one mode of administration includes intra-arterial or intrathecal (IT) injections, e.g., to focus on a specific organ, e.g., brain and CNS (see e.g., Gurun, *Anesth Analg.* 85: 317-323, 1997). For example, intra-carotid artery injection if preferred where it is desired to deliver a polypeptide or scFv-antigen fusion protein of the invention directly to the brain. Parenteral administration is a preferred route of delivery if a high systemic dosage is needed. Actual methods for preparing parenterally administrable compositions will be known or apparent to those skilled in the art and are described in detail, in e.g., Remington's, See also, Bai, *J. Neuroimmunol.* 80: 65-75, 1997; Warren, *J. Neurol. Sci.* 152: 31-38, 1997; Tonegawa, *J. Exp. Med.* 186: 507-515, 1997.

[0142] In one aspect, the pharmaceutical formulations comprising polypeptides or scFv-antigen fusion proteins of the invention are incorporated in lipid monolayers or bilayers, e.g., liposomes, see, e.g., U.S. Pat. Nos. 6,110,490; 6,096,716; 5,283,185; 5,279,833. The invention also provides formulations in which water soluble polypeptides or scFv-antigen fusion proteins of the invention have been attached to the surface of the monolayer or bilayer. For example, peptides can be attached to hydrazide-PEG-(distearoylphosphatidyl) ethanolamine-containing liposomes (see, e.g., Zalipsky, *Bioconjug. Chem.* 6: 705-708, 1995). Liposomes or any form of lipid membrane, such as planar lipid membranes or the cell membrane of an intact cell, e.g., a red blood cell, can be used. Liposomal formulations can be by any means, including administration intravenously, transdermally (see, e.g., Vutla, *J. Pharm. Sci.* 85: 5-8, 1996), transmucosally, or orally. The invention also provides pharmaceutical preparations in which the polypeptides or scFv-antigen fusion proteins of the invention are incorporated within micelles and/or liposomes (see, e.g., Suntres, *J. Pharm. Pharmacol.* 46: 23-28, 1994; Woodle, *Pharm. Res.* 9: 260-265, 1992). Liposomes and liposomal formulations can be prepared according to standard methods and are also well known in the art, see, e.g., Remington's; Akimaru, *Cytokines Mol. Ther.* 1: 197-210, 1995; Alving, *Immunol. Rev.* 145: 5-31, 1995;

[0143] In one aspect, the active peptide compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyoorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[0144] It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

[0145] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD$_{50}$ (the dose lethal to 50% of the population) and the ED$_{50}$ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD$_{50}$/ED$_{50}$. Compounds that exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0146] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage can vary within this range depending
upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models, e.g., of inflammation or disorders involving undesirable inflammation, to achieve a circulating plasma concentration range that includes the \( \text{IC}_{50} \) (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography, generally of a labeled agent. Animal models useful in studies, e.g., preclinical protocols, are known in the art, for example, animal models for inflammatory disorders such as those described in Sonderstrup (Springer, *Sem. Immunopathol.* 25: 35-45, 2003) and Nikula *et al.*, *Inhal. Toxicol.* 4(12): 123-53, 2000), and those known in the art, e.g., for fungal infection, sepsis, cytomegalovirus infection, tuberculosis, leprosy, viral hepatitis, and infection (e.g., by mycobacteria).

**[0147]** As defined herein, a therapeutically effective amount of protein or polypeptide such as an antibody (i.e., an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, for example, about 0.01 to 25 mg/kg body weight, about 0.1 to 20 mg/kg body weight, or about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The protein or polypeptide can be administered one or several times per day or per week for between about 1 to 10 weeks, for example, between 2 to 8 weeks, between about 3 to 7 weeks, or about 4, 5, or 6 weeks. In some instances the dosage can be required over several months or more. The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively treat a subject, including, but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an agent such as a protein or polypeptide (including an antibody) can include a single treatment or, preferably, can include a series of treatments.

**[0148]** For antibodies, the dosage is generally 0.1 mg/kg of body weight (for example, 10 mg/kg to 20 mg/kg). Partially human antibodies and fully human antibodies generally have a longer half-life within the human body than other antibodies. Accordingly, lower dosages and less frequent administration is often
possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (e.g., into the brain). A method for lipidation of antibodies is described by Cruikshank et al. J. Acquired Immune Deficiency Syndromes and Human Retrovirology, 14: 193, 1997).

[0149] The present invention encompasses agents or compounds that modulate an immune response in a subject by targeting antigens into the immune system through the surface receptors CD22 and CD72. An agent can, for example, be a polypeptide or scFv-antigen fusion proteins. Such compounds include, but are not limited to, peptides, peptidomimetics (e.g., peptoids), amino acids, amino acid analogs, small non-nucleic acid organic compounds or inorganic compounds (i.e., including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

[0150] Exemplary doses include milligram or microgram amounts of the small chemical molecule per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is furthermore understood that appropriate doses of a small chemical molecule depend upon the potency of the small chemical molecule with respect to the expression or activity to be modulated. When one or more of these small chemical molecules is to be administered to an animal (e.g., a human) in order to modulate expression or activity of a polypeptide or scFv-antigen fusion proteins of the invention, a physician, veterinarian, or researcher can, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.
[0151] An antibody or fragment thereof can be linked, e.g., covalently and/or with a linker to another therapeutic moiety such as a therapeutic agent or a radioactive metal ion, to form a conjugate. Therapeutic agents include, but are not limited to, antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)).

[0152] The conjugates described herein can be used for modifying a given biological response. For example, the moiety bound to the antibody can be a protein or polypeptide possessing a desired biological activity. Such proteins can include, for example, a toxin such as abrin, ricin A, Pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, .alpha.-interferon, .beta.-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers.

[0153] Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Pat. No. 4,676,980.

[0154] The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

[0155] Compounds as described herein can be used for the preparation of a medicament for use in any of the methods of treatment described herein.

[0156] The pharmaceutical compositions are generally formulated as sterile, substantially isotonic and in full compliance with all Good Manufacturing Practice (GMP) regulations of the U.S. Food and Drug Administration.

TREATMENT REGIMENS: PHARMACOKINETICS

[0157] The pharmaceutical compositions of the invention can be administered in a variety of unit dosage forms depending upon the method of administration. Dosages for typical polypeptides or scFv-antigen fusion protein as pharmaceutical compositions are well known to those of skill in the art. Such dosages are typically advisory in nature and are adjusted depending on the particular therapeutic context or patient tolerance. The amount of polypeptide or scFv-antigen fusion protein adequate to accomplish this is defined as a "therapeutically effective dose." edThe dosage schedule and amounts effective for this use, i.e., the "dosing regimen," will depend upon a variety of factors, including the stage of the disease or condition, the severity of the disease or condition, the general state of the patient's health, the patient's physical status, age, pharmaceutical formulation and
concentration of active agent, and the like. In calculating the dosage regimen for a patient, the mode of administration also is taken into consideration. The dosage regimen must also take into consideration the pharmacokinetics, *i.e.*, the pharmaceutical composition’s rate of absorption, bioavailability, metabolism, clearance, and the like. See, *e.g.*, the latest Remington’s; Egleton, *Peptides* 18: 1431-1439, 1997; Langer, *Science* 249: 1527-1533, 1990.

[0158] In therapeutic applications, polypeptide or scFv-antigen fusion protein compositions are administered to a patient suffering from infectious disease, *e.g.*, bacterial, viral, or parasite disease, or cancer, in an amount sufficient to at least partially arrest the condition or a disease and/or its complications. For example, in one aspect, a soluble peptide pharmaceutical composition dosage for intravenous (IV) administration would be about 0.01 mg/hr to about 1.0 mg/hr administered over several hours (typically 1, 3, or 6 hours), which can be repeated for weeks with intermittent cycles. Considerably higher dosages (*e.g.*, ranging up to about 10 mg/ml) can be used, particularly when the drug is administered to a secluded site and not into the blood stream, such as into a body cavity or into a lumen of an organ, *e.g.*, the cerebrospinal fluid (CSF).

[0159] The following examples of exemplary aspects for carrying out the present invention are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

**EXEMPLARY ASPECTS**

### Materials and Methods

**[0160] Reagents.** Recombinant mouse GM-CSF was purchased from Fitzgerald. LPS was obtained from Sigma-Aldrich. Goat anti-mouse IgM F(ab’)2 fragments and PE-conjugated goat anti-human IgG were purchased from Jackson ImmunoResearch Laboratories. COS cells used for generation of CD22 fusion proteins were obtained from the American Type Culture Collection. All flow cytometry reagents were purchased from BD Biosciences. *Arthobacter ureafaciens (Au)* neuraminidase was purchased from Roche Diagnostics and the PE-conjugated *Sambucus nigra* lectin was obtained from Vector Labs.

**[0161] Mice.** WT (C57BL/6) and CD22 KO (C57BL/6) (Oipoby *et al.*, 1996) mice were housed under specific pathogen-free conditions, and all experiments were performed in compliance with the University of Washington Institutional
Animal Care and Use Committee. ST.setSizeGal-I KO mice (Hennet et al., 1998) were housed under specific pathogen-free conditions at the University of California, San Diego facility. The BM and splenocytes were collected at UCSD and shipped overnight to UW as single cell suspensions in complete RPMI medium.

[0162] **DC cell culture.** BM cells from the femurs and tibias of mice were collected under sterile conditions and cultured in complete RPMI media supplemented with 20 ng/ml recombinant mouse GM-CSF. The cells were fed every other day for 8 days to generate iDCs. To obtain mDCs, day 7 cultured iDCs were stimulated with 1 µg/ml LPS for 24 hs. Prior to co-culturing with the CFSE-labeled B cells, the DCs were extensively washed with complete RPMI.

[0163] **B cell purification.** B cells from mouse spleens were purified using the EasySep Negative Selection kit for mouse B cells (StemCell Corporation) according to the manufacturer's protocol. Following the purification, the B cells were labeled by incubation with 10 µM CFSE for 10 minutes at 37°C, followed by quenching. For the B cell-DC co-cultures, washed DCs and CFSE-labeled B cells were cultured in 24 well tissue culture dishes or 0.4 µm transwells (Corning) at 37°C. Following 72 hs of incubation, the cells were collected and CFSE dilution was measured by flow cytometry. Data were analyzed using FlowJo software.

[0164] **Surface staining of cells.** Generation of the CD22 Rg constructs, transfections and protein purification were previously described (Law et al., 1995). To stain with the CD22 Rg fusion proteins, cells were washed twice with PBS containing 0.1% FCS and sodium azide, incubated with the fusion proteins on ice and then subsequently washed again. Next, the cells were stained with PE-conjugated goat anti-human IgG, incubated on ice, and then washed a final time before analyzing the CD22 Rg binding by flow cytometry. Data were analyzed using FlowJo software.

[0165] **Neuraminidase and metaperiodate treatments.** For neuraminidase treatment, cells were washed twice with PBS containing 2 mg/ml BSA and resuspended at a concentration of 5x10^6 cells/ml. Au neuraminidase was added to the cells at a final concentration of 400 mU/ml. The cells were then incubated at 37°C for 2 hs, after which they were washed and stained. For the sodium metaperiodate oxidation treatment, cells were washed twice with cold PBS, diluted to a concentration of 1x10^6 cells/ml in 2 mM sodium metaperiodate, and then incubated on ice for 15 or 30 minutes, after which they were washed and stained.
Immature DCs, but not mature DCs, inhibit BCR-induced proliferation of B cells

Recent studies reported that DCs can present preserved antigen to B cells (Bergtold et al., 2005) and can activate B cells and transfer Ag to them in vivo (Qi et al., 2006). Similarly, we found that DCs can modulate BCR-dependent proliferative responses in splenic B cells. Mouse bone marrow-derived DCs (iDCs) were co-cultured with purified CFSE-labeled murine splenic B cells along with graded doses of anti-mouse IgM, and B cell division was determined 72 hs later. As few as one iDC per 16 B cells inhibited BCR-induced proliferation (Fig. 1A), and when even more iDCs were in the culture with B cells (4 B cells: 1 iDC ratio), the inhibition was more pronounced. This inhibition was evident as early as 48 hs after BCR crosslinking (data not shown). We also examined the effects on cell proliferation of mature bone marrow-derived DCs (mDCs) obtained from stimulating iDCs with LPS for 24 hs. The mDCs were washed extensively before co-culturing them with the B cells. Interestingly, BCR-induced proliferation was slightly enhanced by the presence of mDCs (Fig. 1B), and enhancement was most evident when lower doses of anti-IgM were used. Similar results were obtained using mDCs matured with peptidoglycan, another Toll-like receptor agonist (data not shown).

iDC-mediated inhibition of BCR-induced proliferation is contact dependent

Because iDCs has been previously implicated in the induction of T cell tolerance (Steinman et al., 2003), we decided to focus on characterizing the inhibitory effects of iDCs on B cells. This inhibition effect could be the result of either direct contact between the two cell types and/or the production of soluble factors by the iDCs. To distinguish between these two possibilities, we performed the CFSE-based assay using transwells with a pore size of 0.4 µm so that the cells could not traverse the barrier, but soluble factors could. When CFSE-labeled splenic B cells and iDCs were physically separated in the transwells, iDCs did not inhibit the proliferation of WT B cells (Fig. 2); the amount of B cell division was similar to that in cultures containing only B cells. This was observed at both B cell:DC ratios. Thus, iDC-mediated inhibition of B cells requires direct contact between B cells and iDCs. These data suggest that perhaps surface molecule(s) on B cells interact with structures
on iDCs, which then activate negative inhibitory pathways within B cells. The activation of this pathway would result in the inhibition of BCR-induced proliferation.

**iDC-mediated inhibition of BCR-induced proliferation** is dependent on the BCR co-receptor, CD22

[0168] The results indicate that the iDC-mediated inhibitory effects on B cell proliferation require cell-cell contact, possibly via inhibitory cell surface receptors on B cells. Two well-known inhibitory receptors expressed on B cells are CD22 and CD72 (Nitschke and Tsubata, 2004). Both receptors are known to activate inhibitory pathways within B cells since they both contain ITIMs within their cytoplasmic domains. CD22 was an attractive candidate because it as an adhesion molecule with ligands expressed on hematopoietic cells and also because it modulates BCR signaling and associates with the BCR complex (Cornall et al., 1999). BCR crosslinking results in CD22 tyrosine phosphorylation and recruitment of the SHP-I protein tyrosine phosphatase (PTPase) to the plasma membrane (Law et al., 1996; Cornall et al., 1999). To determine if CD22 might be involved in iDC-mediated regulation of B cells, we compared the ability of iDCs to inhibit WT and CD22 KO B cells. Unlike WT B cells, CD22 KO B cells proliferated equally well in the presence and absence of iDCs and at both the high and low B cell:DC ratio cultures (Fig. 3A). Similar results were obtained when the CD22 KO B cells were co-cultured with CD22 KO bone marrow-derived iDCs (Fig. 8). Furthermore, physically separating the CD22 KO B cells and WT iDCs had no effect on BCR-induced proliferation of the CD22 KO B cells (Fig. 3A). Thus, CD22 appears to be required for DCs to inhibit BCR-induced cell division.

In the absence of α2-6 sialic acid linkages, iDCs still inhibit BCR-induced proliferation

[0169] CD22 can mediate interactions with erythrocytes, neutrophils, T cells, B cells and monocytes (Stamenkovic and Seed, 1990; Engel et al., 1993; Law et al., 1995); thus it is possible that CD22 on B cells binds to ligands on iDCs. This binding might be required for iDCs to inhibit B cell proliferation in response to BCR crosslinking. To examine this possibility, we compared WT iDCs to iDCs derived from mice deficient for the sialyltransferase STöGal-I, which generates α2-6 sialic acid ligands for CD22 (Hennet et al., 1998). Surprisingly, STöGal-I KO iDCs, like
WT iDCs, inhibited BCR-induced proliferation of WT B cells (Fig. 3B). To determine if the inhibition mediated by the ST6Gal-I KO iDCs is contact-dependent, we used transwells to provide a barrier between the iDCs and B cells. When the ST6Gal-I KO iDCs and WT B cells were physically separated, BCR-induced cell division was not inhibited (Fig. 3B), as was the case with WT iDCs (Fig. 2). Thus, physical interaction between WT B cells and ST6Gal-I-deficient iDCs is required for iDCs to inhibit BCR-induced B cell proliferation.

**CD22 interacts with molecule(s) on iDCs**

[0170] To determine if CD22 binds to molecules on the surface of iDCs, we used chimeric CD22 fusion proteins to stain bone marrow-derived DCs (Law et al., 1995). One of the chimeric fusion proteins contains all seven extracellular immunoglobulin (Ig) domains fused to the Fc region of human IgG (CD22 Rg1-7). The other fusion protein contained deletions of Ig domains 1 and 2 (CD22 Rg3-7), the regions previously shown to be required for CD22 ligand binding (Law et al., 1995). These proteins, along with a human IgG (Fc fragment) control, were used to stain bone marrow-derived iDCs from WT and ST6Gal-I KO mice. As expected, the CD22 Rg1-7 fusion protein bound to WT iDCs, but the CD22 Rg3-7 fusion protein did not (Fig. 4A). Surprisingly, CD22 Rg1-7 also bound to ST6Gal-I KO iDCs (Fig. 4A). To ensure that the iDCs from ST6Gal-I-deficient mice did not express α2-6 sialic acids, we compared the binding of *Sambucus nigra* lectin (SNA)—a lectin that specifically binds to α2-6 sialic acids—to WT and ST6Gal-I KO iDCs. As expected, SNA bound to WT, but not to ST6Gal-I KO iDCs. We also compared the binding of the CD22 fusion proteins to WT and ST6Gal-I KO B cells. As expected, CD22 Rg1-7 binding was higher on the WT B cells compared to the ST6Gal-I KO B cells (Fig. 4B). However, some binding of CD22 Rg1-7 to ST6Gal-I KO B cells was also consistently detected. Thus, CD22 binds to a ligand(s) on iDCs that does not contain α2-6 sialic acids. This ligand on iDCs is distinct from the major CD22 ligand on B cells, which is comprised mostly of α2-6 sialic acid-containing glycoproteins.

**CD22 binding to iDCs is not disrupted by cleavage of α2-6 sialic acids**

[0171] To test further if CD22 interacts with ligands distinct from those expressing α2-6 sialic acids on iDCs, we treated the cells with a neuraminidase from *Arthrobacter ureafaciens* (Au) to specifically cleave α2-6 sialic acids (Sgroi et al.,
1993). Following treatment of iDCs with the Au neuraminidase, the cells were stained with the CD22 Rg fusion proteins, SNA or human IgG control and analyzed by flow cytometry. Binding of CD22 Rg1-7 and CD22 Rg3-7 were largely unaffected by neuraminidase treatment of the iDCs (Fig. 5A, top panels). On B cells, CD22 Rg1-7 binding was significantly reduced following neuraminidase treatment (Fig. 5A bottom panels) as previously reported (Hennet et al., 1998). Thus, the requirements for CD22 to bind to B cells are different than the requirements for binding to iDCs.

[0172] To determine if CD22 binds to sialic acids other than α2-6 linkages, we stained cells with the CD22 fusion proteins following mild sodium metaperiodate oxidation, which cleaves all sialic acids on the surface of cells (Sgroi et al., 1993). Binding of control human IgG and CD22 Rg3-7 to the surface of iDCs was increased slightly following sodium metaperiodate oxidation (Fig. 5B top left and middle panels). The increase in binding of these control proteins can be due to changes in the negative charges produced by cleaved of the sialic acids on the surface of the cells. Following sodium metaperiodate treatment, slightly less CD22 Rg1-7 bound to iDCs (Fig. 5B top right panel). This decrease in binding was particularly evident compared to the controls and can reflect some sialic acid-dependent binding of CD22 on iDCs. When B cells were subjected to sodium metaperiodate oxidation, binding of the human IgG and CD22 Rg3-7 control proteins was not change (Fig. 5B right and middle panels), but CD22 Rg1-7 binding decreased significantly compared to untreated cells (Fig. 5B bottom right panel). Thus, the CD22 ligand on iDCs is not an αc2-6 sialic acid-containing glycoprotein, but can contain some other sialic acids. In contrast, the CD22 ligand on B cells is comprised mostly αc2-6 sialic acid-containing glycoproteins. Furthermore, the binding of CD22 to B cells requires Ig domains 1 and 2 of CD22 since binding of the deletion mutant (CD22 Rg3-7) was unaltered by both neuraminidase treatment and mild sodium metaperiodate oxidation. In summary, B cells and iDCs clearly express distinct sets of CD22 ligands.

**CD22 ligand interactions can play a role in the development of transitional T2 B cell precursors**

[0173] To assess where in B cell development ST6Gal-I-generated CD22 ligands versus alternative ligands not containing αc2-6-sialic acids might be required, we compared BM and splenic B cell subsets from WT, CD22 KO and ST6Gal-I KO mice. As we previously showed, CD22 KO mice had fewer long-lived mature B cells
in the BM compared to WT (Otipoby et al., 1996 and data not shown). However, the numbers of mature B cells in the BM of ST6Gal-I KO mice were comparable WT mice, as previously reported (Hennet et al., 1998; Collins et al., 2006; data not shown). Interestingly, CD22 and ST6Gal-I KO mice similar defects in the splenic B cell development. We determined the cell numbers of the various B cell subsets in the spleens according to the methods of Cariappa et al. (2001) and Saito et al. (2003), which separates transitional 2 (T2) B cell populations into two subsets (Fig. 6A). Cells were stained for surface IgM, IgD and CD21 and analyzed by flow cytometry. CD22 KO mice had normal of numbers follicular mature B cells (FO) and slightly more transitional 1 (TI) B cells (Fig. 7). The MZ B cell population was reduced by one-half in the CD22 KO mice, as previously reported (Samardzic et al., 2002). Interestingly, CD22 KO mice had dramatic deficiencies in the two T2 subsets. There were 65% fewer T2 follicular precursor cells (T2-FOP) and 87% less T2 marginal zone precursor cells (T2-MZP) compared to WT mice. The ST6Gal-I KO showed similar deficiencies in MZ and T2 precursor B cell populations (Fig. 6B).

Discussion

Our data showed that iDCs, but not mDCs, inhibit BCR-induced proliferation of WT B cells in a cell contact dependent manner (Fig. 1 and T). These results suggested that a negative inhibitory receptor on the surface of B cells can be binding to the iDCs, thereby activating an inhibitory pathway to raise the threshold of BCR stimulation required to induce B cell proliferation. The inhibition of B cell division by iDCs but not mDCs is reminiscent of DC regulation of T cells (Steinman et al., 2003). iDCs can inhibit and even tolerize T cells, while mDCs stimulate T cell proliferation. The ability of DCs to control the initiation of an immune response requires that iDCs undergo maturation by toll-like receptor (TLR) or CD40 stimulation (Hawiger et al., 2001; Bonifaz et al., 2002; Lui et al., 2002). An
inhibitory role for DCs is more likely to occur in a steady state during induction of
tolerance or anergy. Indeed, DCs can induce peripheral T cell anergy in vivo they are
stimulated by Ag in the absence of CD40 or TLR co-stimulation (Hawiger et al,
2001; Lui et al., 2002).

[0176] Since physical contact between iDCs and B cells was necessary for
iDCs to inhibit B cells, we speculated that an inhibitory receptor on B cells was
involved. Two inhibitory receptors expressed on B cells that we thought might be
involved in this iDC-mediated inhibition were CD22 and CD72. Both these receptors
contain ITIMs within their cytoplasmic domains and have been shown to be involved
in negative signaling (Nitschke and Tsubabta, 2004). CD22 was an especially
attractive possibility since it physically associates with BCRs to inhibit BCR signaling
(Leprince et al., 1993; Hanasaki et al., 1995; Collins et al., 2006) and is an adhesion
molecule with ligands expressed on hematopoietic cells. Strikingly, iDC-mediated
inhibition of B cell proliferation required CD22 (Fig. 3A). Surprisingly, ST6Gal-I KO
iDCs, which lack cc2-6 sialic acid ligands for CD22, inhibited WT B cell proliferation
(Fig. 3B). Furthermore, CD22 bound equally well to WT and ST6Gal-I KO iDCs
(Fig. 4A) suggesting that a ligand for CD22 is expressed on both of these iDCs.
However, CD22 binding to ST6Gal-I KO B cells was lower compared to WT B cells
(Fig. 4B). Thus, our findings suggest that iDCs do not express α2-6 sialic acid-
containing glycoprotein ligands for CD22 and that there is differential expression of
CD22 ligands on B cells and iDCs. B cells express both α2-6 sialic acid and non-αc2-6
sialic acid glycoprotein ligands since some CD22 binding was evident in both
ST6Gal-I KO B cells (Fig. 4B) and Au treated WT B cells (Fig. 5A).

[0177] To determine where CD22 ligand binding on iDCs might be
important for regulating B cell development, we examined splenic B cell subsets in
CD22 and ST6Gal-I KO mice (Fig. 6A, 6B and Fig. 7). CD22 KO mice had fewer
MZ and T2 B cell precursors compared to WT animals. Interestingly, the T2 subset
that was most deficient in the CD22 KO mice was also the very same subset of B cells
from WT mice that has the highest expression of CD22 (Fig. 6C and Fig. 7).
Therefore, it is possible that CD22 ligand interactions play a role in the development
of T2 B cells as they differentiate into either MZ or FO B cells. One attractive idea
requiring further study is that CD22 engagement on T2 B cells via accessory cells in
the spleen reduces the strength of BCR signaling and thereby contributes to MZ B cell
maturation. Indeed, Casola et al. (2004) showed that reducing the strength of BCR signaling favors MZ B cell maturation, a result consistent with our finding that CD22 KO mice have defective MZ B cell development.

[0178] CD22 can bind to its ligands in cis (on the same cellular surface) or in (on a different cell) (Cyster and Goodnow, 1997). The distinct CD22 ligands on different cell types can have functional significance. CD22 binding to ligands expressed on the B cell surface in cis can induce different signaling pathways and cellular responses compared to binding ligands expressed on other cells types (i.e. iDCs) in trans. CD22 ligand binding in cis is thought to be important for the regulation of BCR signaling (Cyster and Goodnow, 1997; Jin et al., 2002; KeIm et al., 2002). Trans binding of CD22 to its ligand has not been as extensively studied as cis interactions. One previous study reported that the activation of B cells was attenuated if the Ag-bearing cell were also induced to express oc2-6 sialic acid on their cell surface (Lanoue et al., 2002). Our data are in agreement with these findings. The binding of CD22 to its ligand on iDCs in trans can result in the engagement of CD22, subsequently leading to the attenuation of BCR signaling. Thus resulting in decreased B cell activation and proliferative responses. Lanoue et al. (2002) suggest that trans CD22 ligand interactions can be important for attenuating B cell responses to self Ag. Thus, binding of CD22 to its ligand on iDCs in trans can be a novel mechanism used by the immune system to maintain tolerance to self Ag, thereby preventing the occurrence of autoimmune diseases.

[0179] The fact that CD22 might function both in cis and in trans can help to explain some of the discordant phenotypes observed between CD22- and ST6Gal-I KO mice. For example, CD22 KO mice have reduced numbers of recirculating B cells in the bone marrow while ST6Gal-I KO mice have normal numbers compared to WT animals (Hennet et al., 1998; Collins et al., 2006). Binding of CD22 in trans to ligands not containing α-2-6 sialic acid linkages, perhaps on iDCs, can regulate the development, survival or maintenance of recirculating B cells in the bone marrow. In contrast, CD22 ligand interaction with αc2-6 sialic acid-containing glycoproteins in cis or trans, can be responsible for regulating the development of different B cell subsets (i.e. MZ B cells which are deficient in both CD22 and ST6Gal-I KO mice). CD22 ligand trans interactions could also potentially regulate MHC class II levels on B cells. MHC class II expression is elevated on CD22 KO B cells, but normal on
ST6Gal-I KO B cells (Otipoby et al., 1996; Hennet et al., 1998; Ghosh et al., 2006). This CD22 trans ligand interaction can act to down-regulate MHC class II expression, thereby reducing B cell activation.

[0180] The cellular requirements for the generation and maintenance of long-lived BM B cells are poorly understood and can involve DCs present in the BM. Cell-to-cell interactions requiring novel CD22 ligands on iDCs and CD22 on B cells can be required. The inhibitory signal induced by this interaction can be important for the homeostatic maintenance of long-lived mature B cells in the BM. It is possible that CD22 cis interactions can regulate BCR signaling thresholds and thus the development of splenic B cell subsets, but the maintenance of mature BM B cells can be regulated by trans interactions. Thus, binding of CD22 to ligands on iDCs can play an important role in memory B cell development.

[0181] From the data presented here and those of other groups, we propose a model whereby the CD22 siglec domain is normally "masked" by its binding to sialylated glycans ligands in cis, such as CD45, IgM or CD22 itself. In the absence of ST6Gal-I, CD22 interactions would be eliminated but CD22 could still function through its interactions with ligands on iDCs. The trans interactions would inhibit BCR signaling that could oppose the activation signals produced by the lack of CD22 cis interactions and thus result in normal BCR signaling levels. Thus, CD22 ligand interactions in cis and in trans can work in concert to regulate BCR signaling threshold levels and development of B cell subsets in the spleen and bone marrow.

[0182] B cells and production of neutralizing antibodies. Pre-existing antibodies in the circulation or at the mucosa provide a first line of defense against viral re-infection. For instance, injection of FLU-infected, susceptible mice with an HA-specific neutralizing mAb prevented death (Gerhard, 2001). In other studies, long long-lasting smallpox vaccine-induced antibodies are sufficient to protect macaques against lethal monkeypox virus (Edghill-Smith et al., 2005). Memory B cells which can replenish high affinity antibody producing plasma cells at sites of viral entry are important for providing sterilizing protective immunity. Neutralizing antibodies are necessary and sufficient to protect mice against infection with vesicular stomatitis virus (VSV). However, neutralizing activity properties of anti-VSV antibodies in vitro did not correlate necessarily with neutralizing activity in vivo (Bachmann et al., 1997). Instead, protection depended principally on the presence of a minimum serum
level of neutralizing antibodies. Thus, it is important to understand the factors leading to the generation and maintenance of memory B cells.

[0183] DCs not only process and present Ags to T cells but also present Ags to B cells. While much is known about how DCs process and present Ags to T cells (Trombetta and Mellman, 2005), less is known about how Ag-specific responses of B cells occur and are regulated. Others and we reported that human DCs activated through CD40 could drive B cells to proliferate and mature (Pinchuk et al., 1996, Clark, 1997, Dubois et al., 1997, 1998, Fayette et al., 1998, Wykes et al., 1998).

Since then a number of studies have suggested that DCs can play an important role in 'antigen preservation and presentation' to B cells (Carrasco and Batista, 2006). Balazs et al. (2002) found that a rapid antibody response to intravenous injection of intact, particulate Streptococcus pneumoniae involves both dendritic cells (DCs) and B cells. Blood DCs efficiently capture bacteria and deliver them to the spleen, where DCs encounter and trigger marginal zone (MZ) B cells to mature and produce protective IgM antibodies. As with Ag-specific activation of T cells by DCs (Clark and Ledbetter, 1994), this B cell maturation requires 'co-stimulation': the bacterial Ag and a second signal provided by BAFF/BLys. The DCs are a likely source of BAFF since bacterial products stimulate DCs to produce BAFF (e.g., Craxton et al., 2003, Boule et al., 2004). More recently Qi et al. (2006) showed that B cells entering lymph nodes (LNs) interact with DCs bearing antigen and are activated through a mechanism apparently requiring cell-cell contact.

[0184] B cells clearly can acquire Ags from DCs and other target cells and recognize membrane-associated Ags (Batista et al., 2001, Bergtold et al., 2005, Carrasco and Batista, 2006). The fact that DCs can activate Ag-specific B cells suggests that DCs have a means of retaining Ags in an intact form, a process we term 'Ag preservation' in contrast to the 'Ag processing' required for recognition of Ag by T cells (Clark and Santos, in preparation). DCs can internalize and sequester soluble ovalbumin (OA) intracellularly, until they receive a second signal via e.g., CD40, which induces cross presentation (Delamarre et al., 2003). The fact that DCs express low levels of proteases (Delamarre et al., 2005) can explain why they degrade antigens relatively slowly. Bergtold et al (2005) found that whether or not DC-associated Fc receptors process Ag for presentation to T cells or preserve Ag for presentation to B cells depends on whether the Fc receptor cytoplasmic tail has an ITAM (T cell) or ITIM (B cell) for signaling. These results suggest that targeting Ags
to DC receptors with different cytoplasmic signaling elements can differentially affect the program for Ag presentation to B cells vs. Ag processing and presentation to T cells. By constructing and expressing a panel of scFvIg-Ag fusion plasmids each targeted to a particular DC or B cell surface receptor, we can be able to optimize protective neutralizing antibody responses or modulate autoimmune responses by depletion or downregulation of B cell proliferation and memory responses.

[0185] **MAb-based Ag targeting to DCs.** Historically, the development of effective vaccines has required the use of potent adjuvants, but some of the best adjuvants have deleterious side effects (Warren et al., 1986, Petrovsky and Aguilar, 2004). Since DCs are orders of magnitude better than other cells in inducing adaptive immunity and have been used effectively as part of vaccines (see Pulendran and Ahmed, 2006), many studies have focused on how best to deliver Ags to DCs. Ags can be very efficiently targeted to DCs by attaching them to mAbs specific for DC-associated surface molecules. Initial results demonstrated that targeting Ags to certain DC surface molecules supplanted the need for external adjuvants to elicit protective immunity (Carayanniotis and Barber, 1987, Wang et al., 2000).

[0186] MAbs to a range of different surface molecules have been used to deliver Ags to DCs in vivo and/or in vitro including MHC class II, CD1Ic, DEC205/CD205, DC-SIGN/CD209, F4/80-like receptor (FIRE), CIRE, Dectin-1, Dectin-2, 33D1/DCIR2, and the mannose receptor (Finkelman et al., 1996, Bonifaz et al., 2002, 2004, Engering et al., 2002, Chieppa et al., 2003, Ramakrishna et al., 2004, Demangel et al., 2005, Tacken et al., 2005, Corbett et al., 2005, Boscardin et al., 2006, Trumpfheller et al., 2006, Carter et al., 2006a,b, Dudziak et al., 2006). The mAb-based Ag delivery system can be two orders of magnitude more effective than non-targeted Ags in activating protective immunity. Simultaneous injection of Ag-anti-DC conjugates with an anti-CD40 mAb significantly enhanced host immune responses more than the classic Alum+Ag vaccine (Bonifaz et al., 2004, Boscardin et al., 2006). This important finding is highly pertinent to the field of DNA vaccination, where many studies have found that DNA alone can induce Ab responses comparable with unadjuvanted protein immunogens, but ‘for sheer magnitude of Ab titers DNA alone could not equal a potent protein plus adjuvant’ (Donnelly et al., 2005). We propose to adapt this Ag-mAb delivery method for use in DNA vaccines and thereby provide a means to overcome the limitations of Ag only DNA vaccines. Optimizing a DNA vaccine will require testing several DC targets, since the type of immune
response induced appears to vary depending on which DC receptor is used to deliver Ag. For instance, Ag delivered via anti-FIRE, anti-CIRE or anti-Dectin-1 mAbs triggers specific antibody responses without an adjuvant, while Ag with anti-DEC205 does not (Corbett et al., 2005, Carter et al., 2006b).

[0187] **33D1/DCIR2, an attractive target for antigen delivery to DCs.**

CLRs are especially attractive targets for Ag delivery to DCs since: 1) they have very restricted tissue distribution, e.g., BDCA-2 is only expressed on pDCs (Dzionek et al., 2001), and langerin, only on Langerhans cells (LCs, Valladeau et al., 2000); 2) many CLRs internalize after crosslinking and can deliver Ags to proteosomes (Engering et al., 2002, Pulendran, 2005, Brown, 2006); 3) CLRs differ in how they signal DCs (Kanazawa et al., 2004), and thus, can be useful not only for delivering Ags to a restricted set of DCs, but also for inducing tailor made protective immune responses; and 4) human CLRs when expressed in mice retain their restricted expression on mouse DC subset counterparts (e.g., Kaplan et al., 2005), and many anti-human CLR mAbs bind to macaque DC homologues. Thus, pre-clinical animal models are available for testing anti-human mAb-based delivery of antigens to DC subsets.

[0188] Several studies suggest that receptors with associated ITAM domains like FcDRIIA and the B cell receptor (BCR) complex, target Ags to lysosomes for efficient degradation and presentation to T cells via a process requiring protein tyrosine kinases like Syk (Bergtold et al., 2005, Clark et al., 2004). In contrast, receptors with ITIM domains like FcDRIIB can be able to deliver Ags to a nondegradative intracellular compartment, where they can be retained in a native state for subsequent presentation to B cells. This model suggests that delivery of Ags to DC receptors with ITIMs might promote more robust antibody responses, while delivery of Ags to DC receptors with ITAMs would favor inducing more CD4 T cell responses. Several DC-associated CLRs have intracellular ITIMs including human DCIR (Bates et al., 1999) and DCAL-2 (Chen et al., 2006) and their closely related homologues in mice, DCIRI 33D1/DCIR 2, DCIR3 and MICL. These CLRs are attractive potential targets for Ag delivery. Similarly, delivery of Ags to B cell surface receptors with ITIMs versus ITAMs might show differential effects on antigen specific immune responses.

[0189] **scFv technology.** The Clark lab discovered CD40, cloned human (hCD40) and mouse CD40 (mCD40) and made some of the first hybridomas producing mAb to hCD40 and mCD40 (Clark and Ledbetter, 1986, 1994, Heath et al,
1993, Hasbold et al., 1994). The lab has extensive experience with antibody engineering technologies (Hayden et al., 1997). Figure 10 diagrams the structure of a prototype scFv-Ag fusion gene. cDNA cassettes encoding rat anti mCD40 V_L and V_H segments were cloned from the IC10 hybridoma, fused by a (glyiser)₆ linker, and attached to a mutated murine IgG2a Fc domain, including an SCC hinge and (P238S/K322S/P331S) Fc. The single chain fusion gene also encodes a linker-Ag domain attached to the carboxyl end of the CH3. We used the rat IgG2a anti-mCD40 hybridoma IC10 to isolate V_H and V_L segments and create a CD40 scFv (Fig. 10) from a rat V_L-rat V_H-hinge and mouse IgG2a CH2-CH3 region (CD40 scFv-mG2a, see Fig. 15 below).

[0190] We have also isolated cDNA from the 33D1 and the P4G2 hybridomas for use in preparing 33D1 and DCAL-2 scFvs, respectively. The mIgG2a CH2 and CH3 domains were mutated at residues important for mediating antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-directed cytoxicity (CDC) (Figure 11). The homologous sites for human and macaque IgGl domains indicated in Figure 11). We are preparing anti-CD40 scFv with either mutated macaque (CD40 scFv-macGl) or human IgGl tails (CD40 scFv-humGl) as part of a Primate Center funded project (RR00166) using our G28-5 mAb, which binds to and stimulates both human and macaque CD40 (macCD40, Clark and Draves, 1987).

[0191] Figure 11 shows the alignment of murine IgG2a, human IgGl, and macaque IgGl- hinge-CH2-CH3- amino acid sequences. Sequence matches between all 3 sequences are highlighted in red; matches between two sequences are highlighted in light blue; conservative sequence changes are in green. The consensus sequence is listed at the bottom. Residues enclosed in boxes are a subset of conserved residues identified as important in ADCC, CDC, or FcR binding. These residues are P238 (ADCC), N297 (FcR binding, ADCC, CDC, and glycosylation), K322 (Clq binding, CDC), and P331 (Clq binding, CDC). The preferred aspect can contain one or more of these mutations or be the wild type residue depending on whether depletion of the target cell subset is desired as part of the outcome.

[0192] We previously prepared a cDNA construct encoding CD40 scFv humGl (V_L-V_H-CH2-CH3) together with human CTLA4. The expressed protein bound to both CD40 and CD80 on human BJAB B cells and to CD40-Ig or CD80-Ig fusion proteins (data not shown). The chimeric protein (CTLA-4/GCD40-Ig) was much more efficient at inhibiting allogeneic T cell proliferation compared to a CTLA-
4-Ig fusion protein (data not shown). Thus, it is possible for us to construct and express a functional receptor-scFv hybrid protein construct. Therefore, it is very likely that we will be able to design constructs capable of expressing functional Ag-scFv hybrid proteins.

[0193] **In vivo treatment of macaques with scFvs.** We produced single chain scFvs to human CD20 that binds to macaque CD20. When we inoculated a CD20 scFv with a mutant human IgGl hinge and wildtype CH2 and CH3 domains i.v. into two macaques (10 mg/kg), it rapidly depleted macaque B cells in vivo. B cell levels remained low for 4-6 wks and then returned to normal. By contrast a CD20 scFv with an effector CH domain mutated so that ADCC was reduced to background levels, when inoculated into macaques, depleted B cells less efficiently and cell numbers recovered within 2-3 weeks (data not shown). Thus, a DC-specific scFv engineered not to mediate either ADCC or CDC is very unlikely to deplete DCs in vivo. In addition, we have also made functional, bispecific molecules, which fuse an scFvIg domain to an antigen such as viral antigens including HIV gp120 (Hayden-Ledbetter, unpublished data). Development of viral antigen-scFvIg fusion constructs which target to selected DC markers should be straightforward.

[0194] Our approach combines state-of-the-art antibody engineering technology along with new insights into dendritic cell (DC) and B cell biology. We propose to develop single chain antibody fragments (scFv)- specific for DC and B cell receptor targets containing an ITEv1 motif; fuse these to intact antigens; and test whether these novel fusion proteins are able to elicit or modulate antigen specific immune responses to the antigen of interest. This approach requires optimization of the scFv delivery system including adapting it for use as a DNA vaccine or a soluble fusion protein; optimization of antigen expression so that neutralizing epitopes are expressed on the back end of the scFv and preserved during delivery to B cells; and testing fusion protein candidates for their ability to induce neutralizing antibodies and protective immunity using pre-clinical mouse and macaque models. We propose that by improving antigen (Ag) delivery to B cell target receptors that can activate or inhibit B cell specific responses, we can more effectively regulate the spectrum of immune responses elicited by different pathogenic organisms or by autoantigens.

[0195] **Preparation of scFv constructs.** B cells at different stages of maturation and DC subsets express particular patterns of surface receptors that mediate specific patterns of activation of other lymphocyte subsets. Targeting Ags to
these specialized B cells and DC subsets with similar scFv-Ig-Ag molecules will allow us to determine which receptors mediate optimal neutralizing Ab responses in vivo or might be used to dampen autoimmune responses and promote a more tolerogenic program. In order to be able to compare targeting to different DC subsets and B cell surface molecules we will prepare the set of scFv we will prepare are listed in Fig. 15. We have mRNA from the hybridomas for all the targets listed and have verified consensus sequences for a number of VH and VL segments necessary for this study. We already have developed an scFv to mouse CD40; we are preparing anti-human CD40 (hCD40) scFv with either macaque IgGl CH2 and CH3 domains (hCD40 scFv-macGl) or human IgGl CH2 and CH3 domains (hCD40 scFv-humGl) as part of a Primate Research Center grant. We will use mouse IgG2a since it is homologous to human and macaque IgGl and binds well to protein A. We will construct \( V_L-V_H \)-hinge CH2-CH3 scFv constructs, and mutate the CH2 and CH3 domain at homologous residues identified as being important for mediation of ADCC and CDC (Fig. 11, Idusogie et al., 2001, Presta, 2002).

[0196] The results in Figure 16 show that WT mice and CD22 KO mice differ in their IgGl antibody response when the antigen is targeted to dendritic cells, in this example epitopes on the 33D1 rat IgG2b mAb. This was evident at a low dose of antigen (100 ng, i.v.) and also at higher doses of Ag (10 and 100 µg, i.v.). This result was unexpected since several studies have reported that antigen-specific IgGl antibody responses of CD22 KO mice are not different from those of WT mice (e.g., Otipoby et al., 1996, Sato et al., 1996).

[0197] This unexpected finding led us to the invention that blockade of CD22 without destroying CD22 target cells will modulate the immune response, would need a CD22 inhibitor which would not mediate ADCC or fx complete in order to actively suppress CD22 without destroying target cells. Such a CD22 binding molecule would bind to an epitope on CD22 which modulates B cell interactions with other cells such as dendritic cells and would not fix complement or mediate antibody-dependent cell-mediated cytotoxicity (ADCC). A preferred aspect of a CD22 inhibitor would be comprised of molecule that has humanized VH and VL domains that bind to CD22 and a hinge and human CH2 and CH3 domains that have been mutated so that they do not mediate ADCC or fix complement.
At one dose of Ag targeted to DCs (Figure 16, 1 µg, i.v.), WT mice were as unresponsive to antigen immunization as CD22 KO mice. This unexpected result led us to the invention that a CD22 inhibitor when inoculated with an antigen targeted to dendritic cells will modulate immune response, and for instance inhibit or alter antibody responses to the targeted antigen.

Since the mice can recognize epitopes on the rat IgG2b mAb antibody, this form of administration targets the rat epitopes to mouse DCs. After 10 days, sera from the immunized mice were isolated and levels of mouse IgGl anti-rat IgG2b were measured by ELISA. As indicated in Fig. 16, WT mice make a strong Ag-specific IgGl antibody response to 100 ng of inoculated antigen, but CD22 KO mice did not. Thus, the absence of CD22 prevented an antigen-specific IgGl antibody response to an antigen targeted to dendritic cells. These results teach that inhibiting or modulating CD22 can affect Ag specific antibody responses.

To prepare an scFv, total RNA will be isolated from hybridoma cells growing in log phase using QIAGEN RNeasy kits, including QIA shredder column purification to homogenize cell lysates, and purification over RNeasy mini-columns. Then cDNA will be synthesized and anchor tailed, and PCR will be performed using an anchor-tail (CCCCCC)-complementary primer and a primer, which anneals specifically to the antisense strand of the constant region of either mouse (or rat) CK or mouse CH1 (or rat) for the appropriate isotype. The amplified variable region fragments are then TOPO cloned (Invitrogen), and clones with inserts of the correct size sequenced. Consensus sequence for each variable domain will be determined from sequences of at least 4 independent clones. The scFv is constructed by insertion of a variable length (gly4ser) linker (10-20 amino acids) or by sewing PCR using overlapping primers containing a synthetic (gly4ser)3, a (gly4ser)4 or a hydrophilic linker containing a (glyser) motif at each end, linker domain inserted between the V_L and V_H regions. Constructs are fused to a synthetic V_L light chain signal peptide derived from the sequences of several different leaders, with a Kozak signal sequence included to improve expression level of the expressed fusion proteins. Human and macaque IgGl and mouse IgG2a Fc domains (hinge, CH2, and CH3 domains) have been isolated from blood (human and macaque) or splenic (mouse) RNA. Sequences have been altered using SOEing PCR to introduce mutations at residues implicated in mediating ADCC and CDC effector functions.
[0201] Once assembled, the scFv-Ig-Ag fusion constructs will be inserted into one of two expression vectors for creation of the DNA vaccines. The first will permit strong constitutive expression of the fusion proteins under the control of the CMVintA immediate-early promoter, which is a superior promoter and still favored for inducing immune responses (Lee et al., 1997, Donnelly et al., 2005) and which has been used successfully for FLU DNA vaccination in mice (Kodihalli et al., 1999). Alternatively, it can be necessary to use a second type of construct where scFv expression is under the control of a tissue specific, promoter such as the species-specific muscle creatine kinase (MCK) promoter (Bojak et al., 2002). And of course the coding region will have a secretion signal sequence as described above so that the Ag-scFvs are secreted from transduced cells.

[0202] After a construct is prepared, it will be transfected into CHO-DG44 cells (Urlaub et al., 1983, 1986), since these cells make possible establishing high producing stable transfectants via selection of resistant clones in increasing levels of methotrexate. The CHO-DG44 cells we will use are suitable for scale up under clean GMP conditions. Thus, if warranted our technology will be readily translated for use in the development and testing of candidate protein vaccines. We will grow high producing CHO cells in spinner and/or shaker flasks and purify scFv from supernatants by protein A-sepharose chromatography. We anticipate being able to obtain about 10 to 250 mg/l of scFv based on prior experience, i.e., sufficient amounts for in vivo experiments in mice or even macaques. It will be important to verify that each of the scFvs we produce does not fix complement or mediate ADCC against appropriate mouse, macaque or human cells or cell lines; decreased or absent effector functions is essential for an scFv to be used in vivo for Ag delivery without depletion of DCs. We will verify that the scFvs have the expected activity, e.g., that the scFvs to CD40, CD22, CD72, 33D1, DCAL-2 and CD40 internalize and that CD40 scFv-mG2a, as expected, stimulates mouse iDCs to mature and express high levels of MHC class IFCD86. Most of the constructs will be designed to eliminate ADCC and C binding effector function but not Fc receptor binding, which if it occurs can deliver Ag (You et al., 2001). Retention of this function can help to maintain the half-life of the fusion protein. In some preferred aspects, such as to achieve elimination of target cells producing autoantibodies with high affinity for autoantigen, preservation of effector functions would be desirable in order to achieve depletion of the dysfunctional reactivity.
The linker between the scFv-Ig and Ag portions of the molecule might need to be optimized to prevent problems with protease cleavage or improper folding. In general, successful linkers are usually hydrophilic, more disordered short peptide segments. Because we wish to generate an immune response against the Ag linked to the scFv, the linker segment might also be presented and be immunogenic. Thus, the optimal linker might best be chosen from a linker like a domain from prokaryotes (bacterial or viral sequences) rather than a mammalian linker segment such as an Ig hinge domain.

The following examples illustrate the production of a set of scFvIg molecules targeted to a surface receptor, murine DCAL2, expressed on several lymphocyte subsets, including B cells, and CD8+ and CD8+ dendritic cells. In addition, we describe the construction and expression of several [scFvIg-antigen] fusion genes and proteins which can mediate delivery of the antigens to cells expressing the target receptor by high affinity binding of the scFv portion of the molecules. We demonstrate that we can genetically couple the scFv to the antigen and achieve expression and specific binding of the resulting fusion proteins. A similar approach could be used for human, mouse, and rat V regions targeted to a variety of different lymphocyte surface receptors. A variety of different antigens or antigen fragments could be fused to an scFvIg in order to target delivery of the antigen to a particular subset of cells.

**Construction of DCAL2 specific scFv**

Total RNA was isolated from approximately 10^7 cultured cells in logarithmic growth, with a viability greater than or equal to 90%. The hybridomas include P4G2, a rat-anti-mouse DCAL2 antibody producing hybridoma, the rat anti-mouse CD22 hybridoma. Cells were cultured in RPMI containing 10% fetal bovine serum, 5x10^-4 M 2-mercaptoethanol, 2 mM glutamine, penicillin/streptomycin, sodium pyruvate, and DMEM non-essential amino acids for several passages prior to isolation of RNA. Cells were harvested by centrifugation at 100 RPM, washed in PBS, and resuspended in 600 microliters of RLT buffer containing 2-mercaptoethanol to lyse the cells.

RNA was isolated using RNAeasy kits and QIAshredder homogenization kits (QIAGEN, Valencia, CA) according to manufacturer’s instructions. CDNA was prepared using 250 ng oligo(dT)(12-18), 1 microgram total RNA, and Superscript III Reverse Transcriptase (Invitrogen). The variable domains of
the P4G2 scFvs were cloned using a family of degenerate 5' oligonucleotides able to cross-hybridize for each V region gene family and a single 3' primer specific for the constant region of either the light or heavy chain. PCR amplified products were reamplified by nested PCR, using a second set of 3' primers internal to the first set. Fragments were cloned into the PCR 2.1-TOPO vector (Invitrogen), clones screened for inserts of the proper size by EcoRI digestion, and positive clones sequenced as previously described.

[0207] Specific primers were then designed from the consensus sequence obtained for each V region, and included desired linkers and/or restriction sites at the primer ends. The following oligonucleotides were used for the secondary PCR reactions of the VL domain for the P4G2 scFv:

P4G2 VL 5' (no leader peptide, AgeI restriction site at beginning of VL)
5'-ttcgaaacggtaggtcagttcagtcacctct-3'

P4G2 VL3'(3' primer, antisense strand)
5'-ctccggagagatctttcagttccagtttagtcccatc-3'

The primers used for the VH domain are shown below:

P4G2 VH5'
5'-ttcgaaacggtagttcagcgtgtgagctgg-3'

33DEC VH3': (3' primer, antisense strand, attaches BglII site out of frame to end of VH)
5'-gtttctcagatctgagagactgtgacaccatc-3'

[0208] Small cassettes were designed which incorporated a 5' restriction site, Kozak consensus sequence, and the leader peptide from the human VK3 germline sequence which includes an in frame Age I site at the end of the leader peptide, just upstream of the framework region for the light chain variable domain. Overlapping, partially complementary oligonucleotides were used in PCR extension reactions to create these short cassettes. PCR products of the correct size were isolated by gel electrophoresis and cloned into the pCR-2.1 TOPO (Invitrogen) vector. The nucleotide and predicted amino acid sequence of this cassette is listed below:
Similarly, a second series of cassettes were designed which incorporated one of three linker domain inserted between the VL and VH or the VH and VL domains, depending on the scFv, and incorporating restriction sites BgIII at the 5’ end and Agel at the 3’ end. The nucleotide and predicted amino acid sequences of each of these cassettes is listed below:

\[(\text{GS})-(\text{HP})-(\text{GS})\] 5’-
\[\text{agatctctcgagaggttgtggatcagaggtgagaggtgagaggtgagaggtgagaggtgttccacggt}\]

NH2-DLSGGSGSGEGPKGSVEGGSTG-COOH

\[(\text{G4S})3\] 5’-
\[\text{agatctctcgagaggttgtggatcagaggtgagaggtgagaggtgagaggtgagaggtgagaggtgttccacggt}\]

NH2-DLSGGGGSGGGSGGGGGSTG-COOH

\[(\text{G4S})4\] 5’-
\[\text{agatctctcgagaggttgtggatcagaggtgagaggtgagaggtgagaggtgagaggtgagaggtgttccacggt}\]

NH2-DLSGGGGSGGGSGGGGGGSGGGSTG-COOH

Each of these cassettes were synthesized de novo from overlapping, partially complementary oligonucleotides, which were annealed and extended by PCR extension reactions, fragments of the correct size isolated by gel electrophoresis and QIAquick (QIAGEN, Valencia, CA) isolation, followed by cloning into pCR 2.1-TOPO vectors for DNA sequence verification.

PCR reactions were performed on the TOPO cloned DNA using a 30 cycle program with the following profile: 94C, 30 sec; 55C, 30-60 sec; 72C, 30-60 sec, followed by a final extension at 72C for 8 minutes. PCR products were gel purified and fragments recovered using a QIAQUICK gel extraction kit (QIAGEN, Valencia, CA). Fragments were subcloned into pUC derived vectors for creating
fusion genes. Usually, a VH was subcloned into a particular vector with the leader peptide cassette as a three way ligation, positive clones identified by restriction digestion, and these clones recut with the appropriate enzymes for inserting linker and VL domains together as a second three-way ligation. Fragments were mixed together in the appropriate ratios to optimize three-way ligation reactions.

[0212] Fragments were diluted 1:50 and 1 microliter used for SEWING PCR reactions when linkers were attached in this manner instead of by multi-fragment ligation. Diluted overlapping PCR products were added to standard PCR reactions without additional primers and run for 2 cycles. Cycling was then paused, and the flanking VL 5'and VH 3' primers were added. Cycling was resumed and allowed to complete the remainder of the 30 cycle program. The temperature profile was 94C, 60 sec; 55C, 60 sec; and 72C, 60 sec for 30 cycles.

[0213] Initial expression studies were performed by transient transfection of COS cells, using Polyfect (QIAGEN, Valencia, CA) transfection reagent according to manufacturer's instructions. Culture supernatants were harvested at 72 hours post-transfection and screened for binding to the A20 mouse B cell lymphoma line which expresses high levels of mouse DCAL2 (see Figure x). Constructs were then transfected into CHO DG44 cells to create stable cell lines as described previously (cite our Patents). COS transfections were performed using the P4G2 scFvIg and P4G2scFvIg-OVA or P4G2scFvIg-HEL DNAs. Culture supernatants were harvested 72 hours after transfection, and screened for production levels of fusion protein by SDS-PAGE electrophoresis and for binding activity to target cells expressing the antigen.

Construction of a murine and rat IgG2a with mutations that eliminate ADCC and CDC activity:

[0214] The sequence encoding the Fc domain of mouse IgG2a was cloned, using C57BL/6 mouse spleen RNA as template, from the hinge to the end of the CH3 domain, but incorporating a cys to ser mutation in the hinge region so that the first hinge cysteine residue that normally pairs with the constant region of the light chain is converted to a serine residue, and a second mutation at the beginning of the CH2 domain at position P238, converting it to a serine residue. This mutation eliminates ADCC activity mediated through the Fc domain of the -Ig tail in the molecules which contain it. PCR fragment s were cloned into the pCR2.1-TOPO vector and the
sequence confirmed by DNA sequencing. The following oligonucleotides were used for the initial PCR reactions:

\[
\text{MUIGG2ACH2: } 5'\text{-cctccatgcaaatgcccagcacctaacctcttgggtggatcatccgtcttcatcttcc}-3' \\
\text{MUIgG2A5: } 5'\text{-agatctcgagcccagaggtcccacaatcaagccctctcctccatgcaaatgcc}-3' \\
\text{MUIgG2A3S: } 5'\text{-gtttctagattatcatttacccggaagtccgagagaagctcttagtcgt}-3' \\
\text{MUIgG2ANS: } 5'\text{-gatatctctagatttacccggagtccgagagaagctcttagtcgt}-3' \\
\]

[0215] Mutations were then inserted by oligonucleotide-directed mutagenesis into the CH2-CH3 junction region that have been implicated in effective CDC activity. The following oligonucleotides were used to introduce mutations into this region of the Fc domain. Two smaller fragments were created with overlapping ends, each containing the mutations in residues K322 and P331. Overlap extension PCR of these mutated fragments, with addition of the MUIgG2a5 sense primer and the MUIgG2a3S or MUIGg2aNS antisense primers permitted the reconstruction of molecules with targeted point mutations which interfere with both ADCC and CDC effector functions. For the targeting purposes described where an immune response is modulated to augment reactivity, it is important to prevent depletion of the target cell populations, so effector function of the targeting molecules is deleterious. However, in cases where a particular target receptor is expressed on cells that process and present autoantigens, or on cells that produce autoantibody, it might be advantageous to deplete the target population by preserving effector functions of the targeting constructs.

[0216] The following two oligonucleotides were used to create small, overlapping Fc subfragments that incorporate both the K322S and P331S mutations.

\[
\text{MIGG2A KP5: } 5'\text{-agtgccagaggtagtcaatgtgctggtcaacaataaagactctccagctc}-3' \\
\]

This primer was used in combination with the MUIGG2A3S or NS primers above to create a short subfragment encoding the CH3 domain of the mouse IgG2a.

\[
\text{MIGG2A KP-3: } 5'\text{-gtttctctgagtaggctgtcggagctgctttgccgagcatgtgaccgac}-3' \\
\]

[0217] This primer was used as an antisense primer in combination with the MUIgG2A5 sense primer to create a larger Fc subfragment encoding the hinge and CH2 domains. The two subfragments overlap with one another. Overlap extension
PCR was performed to recreate a full length mutated Fc. After two rounds of PCR, the flanking primers were added to the PCR reactions to amplify the full length mutated Fc molecules. Full length PCR fragments were isolated from the subfragments and oligonucleotides by gel electrophoresis and QIAquick column purification. Fragments were TOPO cloned and clones with correct fragment sizes were sequenced to verify the incorporation of the desired mutations.

[0218] A secondary series of constructs were created with a restriction site engineered at the end of the coding sequence, but without a STOP codon, so that antigens or antigen fragments could be fused at the carboxyl end of the -Ig tail.

[0219] MUIgG2ANS: 5'-gatatctctagatttacccggagtccgagagaagctcttagtcgt-3'

[0220] These constructs were used to fuse in frame, a variety of antigens to the scFvIg sequences.

Subcloning of antigen subdomains in frame with scFvIg-NS (no stop) fusion genes

[0221] The antigens for hen egg lysozyme (HEL) and three mutant derivatives were cloned from FLAG-tagged sequences inserted in pCDNA3hygro vectors, obtained as a kind gift from Robert Brink. The sequence encoding only the HEL specificity without any signal peptide or the FLAG tag was PCR amplified using the following primers, and a hydrophilic linker attached by serial PCR reactions, using overlapping 5' primer sets.

HEL5'+LNK: tctagagctagacatggccaagaaggacagtcttgaggt-3'
HEL5': 5'-aaggagacagtctgagctgagaagctttttgagatgtggag
HEL3': 5'-tcatagactttactgagccfcagyctgatccac-3'

The hydrophilic linker sequence used is as follows:
5'-tcatagagctagacatggccaagaaggacagtctgagctgaggatctggtagggt-3' (OVA)
OR
5'-tcatagagctagacatggccaagaaggacagtctgagctgagaaa-3' (HEL)

[0222] The predicted amino acid sequence encoded is:
NH2-SRASDMAKKETVWRLEEFGRF-COOH (OVA)
NH2-SRASDMAKKETVWRLEK-COOH (HEL)

Similarly, the antigen for chicken ovalbumin was amplified from a construct obtained from Michel Nussenzweig, Rockefeller University. The ovalbumin sequence was also amplified with an amino terminal linker sequence attached to separate the end of the murine CH3 domain from the beginning of the ovalbumin coding sequence. The oligonucleotides used were as follows:

OVA5’-gtttctagctagcgacatggccaagaaggagacag-3’

OVA3’-gttgctagctctagattatcactacagatcctcttctgagatg-3’

Construction and Screening of [scFvIg-antigen] fusion genes/proteins for targeting

The generic structure for these constructs is illustrated in the schematic diagram shown in Fig. 10. Several alternative constructs are summarized in Table 1 diagrammed below. The sequence modifications of interest are indicated for each molecule, classified by their subdomain location in the final fusion gene/protein. Several different molecular forms of the anti-mouse DCAL2 scFvIg targeting constructs were constructed. New modified cassettes were cloned into TOPO vectors (Invitrogen, Carlsbad, CA) and sequenced using an ABI xxx sequencer. Once sequence modifications were confirmed, the cassettes were subcloned into existing constructs, replacing one or more unmodified regions in the previous molecules. Constructs were transformed into competent TOPIO bacteria (Invitrogen, Carlsbad, CA) and plasmids isolated using QIAGEN (Valencia, CA) miniprep kits. DNA was then transiently transfected into COS cells Multiple independent transfections of each new molecule were performed in order to determine the average expression level for each new form. Culture supernatants from COS transfections were screened by SDS-PAGE, Western blotting, and FACS binding assays using A20 cells as targets.

The scFvIg, scFvIg-HEL, and scFvIg-OVA constructs were produced by construction of recombinant expression plasmids and stable transfection of Chinese hamster ovary cells (CHO DG44). Master wells producing the highest level of fusion proteins were identified by IgG sandwich ELISA and cell binding assays using serial dilutions of harvested culture supernatants. Fusion proteins were purified from spent CHO culture supernatants by Protein A affinity chromatography after
adjustment of the pH to 9.0 using 0.2M sodium carbonate buffer. Protein was eluted from the columns in 0.1M citrate buffer, pH 2.5, and dialyzed into PBS, pH 7.0.

[0226] A20 murine B cell lymphoma cells were suspended in staining media, PBS with 2%FBS (#16140-071, Gibco/Invitrogen, Grand Island, NY). Culture supernatants were concentrated 10-15 fold, dialyzed in PBS, and serial dilutions added to the cells. Binding reactions were allowed to proceed on ice for 45 minutes prior to centrifugation and washing in staining media. Cells were resuspended in staining medium containing biotin conjugated monoclonal antibody anti mouse IgG2a (5.7, Becton Dickinson) at 1:100. Cells were washed by centrifugations and resuspended in PBS with 2% FBS containing SA-PE conjugate at 1:400 (Becton Dickinson). Samples were incubated 45 minutes on ice, washed in staining media and then cells were transferred to FACS cluster tubes (#4410, CoStar, Corning, NY). Cells were fixed in 0.1% paraformaldehyde. Samples were analyzed by flow cytometry using a FACsCalibur and CellQuest software (Becton Dickinson).

[0227] Additionally, A20 cells were incubated with culture supernatants, washed as described above, and incubated in a second step containing directly conjugated antibodies specific for HEL or for OVA for 45 minutes on ice. Samples were washed in staining media, fixed, and analyzed as described above. The binding analysis using different second steps to detect distinct portions of the fusion protein demonstrated that a molecule with a mouse IgG2a and either OVA or HEL attached bound specifically to the A20 cells. Further blocking studies using labeled P4G2 whole antibody demonstrated that the binding to A20 cells is specific for the mDCAL2 receptor and not some other portion of the molecule.
Table 1. of anti-mDCAL2-Ag constructs

P4G2 scFvlg- molecules

<table>
<thead>
<tr>
<th>Name</th>
<th>Leader</th>
<th>VL</th>
<th>Linker</th>
<th>VH</th>
<th>Hinge cysteines</th>
<th>CH2</th>
<th>CH3</th>
<th>STOP/NS/Ag</th>
</tr>
</thead>
<tbody>
<tr>
<td>P4G2-VL-(G4S)-3-VH-mlgG2a-TM+S</td>
<td>huVK3</td>
<td>WT</td>
<td>(GLY4SER)</td>
<td>WT</td>
<td>mlgG2a-SCC</td>
<td>P238S</td>
<td>P331 S</td>
<td>STOP</td>
</tr>
<tr>
<td>P4G2-VL-(G4S)-4-VH-mlgG2a-TM,S</td>
<td>huVK3</td>
<td>WT</td>
<td>(GLY4SER)</td>
<td>WT</td>
<td>mlgG2a-SCC</td>
<td>P238S</td>
<td>P331 S</td>
<td>STOP</td>
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<tr>
<td>P4G2-VL-(G4S)-3-VH-mlgG2a-TM,NS-HELWT</td>
<td>huVK3</td>
<td>WT</td>
<td>(GLY4SER)</td>
<td>WT</td>
<td>mlgG2a-SCC</td>
<td>P238S</td>
<td>K322S</td>
<td>STOP</td>
</tr>
<tr>
<td>P4G2-VL-(G4S)-3-VH-mlgG2a-TM,NS-HEL1X</td>
<td>huVK3</td>
<td>WT</td>
<td>(GLY4SER)</td>
<td>WT</td>
<td>mlgG2a-SCC</td>
<td>P238S</td>
<td>K322S</td>
<td>NS-HEL1 X</td>
</tr>
<tr>
<td>P4G2-VL-(G4S)-3-VH-mlgG2a-TM,NS-HEL2X</td>
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<td>WT</td>
<td>(GLY4SER)</td>
<td>WT</td>
<td>mlgG2a-SCC</td>
<td>P238S</td>
<td>K322S</td>
<td>NS-HEL2X</td>
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<td>(GLY4SER)</td>
<td>WT</td>
<td>mlgG2a-SCC</td>
<td>P238S</td>
<td>K322S</td>
<td>NS-HEL3X</td>
</tr>
<tr>
<td>P4G2-VL-(G4S)-3-VH-mlgG2a-TM,NS-OVA</td>
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<td>WT</td>
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<td>WT</td>
<td>mlgG2a-kv, SCC</td>
<td>P238S</td>
<td>P331 S</td>
<td>NS-OVA</td>
</tr>
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</table>
References


[0228] All publications and patent applications cited in this specification are herein incorporated by reference in their entirety for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference for all purposes.

[0229] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.
What is Claimed:

1. A method for modulating an immune response in a subject which comprises targeting antigens into the immune system through the surface receptors CD22 and CD72.

2. A method for modulating an immune response in a subject which comprises targeting antigens into the immune system through surface receptors preferentially expressed on B lymphocytes, wherein the surface receptors comprise an immunoreceptor tyrosine inhibitory motif (ITIM).

3. The method of claim 1 or 2, wherein the targeting antigen is from an infectious agent.

4. The method of claim 1 or 2, wherein the targeting antigen is a tumor-associated antigen.

5. The method of claim 1 or 2, wherein the targeting antigen is an autoantigen.

6. The method of claim 1 or 2, wherein modulating the immune response generates protective immunity to a pathogen.

7. The method of claim 1 or 2, wherein modulating an immune response suppresses autoantibody production.

8. A method for modulating an immune response in a subject, the method comprising targeting antigens to B cell surface receptors using an scFvIg derived from an antibody which binds to the receptor, wherein the antigen is genetically fused to the carboxyl end of the scFvIg moiety.

9. A method for producing an scFvIg-Ag fusion protein which comprises genetically fusing the scFvIg directly to the antigen specificity of interest, without any intervening sequence.

10. A method for producing an scFvIg-Ag fusion protein which comprises genetically fusing the scFvIg to the antigen through use of a spacer linker to physically separate functional domains.
11. A method for producing an scFvIg-Ag fusion protein which comprises genetically fusing the scFvIg to the antigen by a hydrophilic linker to improve solubility and decrease steric hindrance between protein domains.

12. A composition comprising a CD22 binding molecule that does not fix complement, or mediate ADCC, or lead to destruction of the cells to which it binds.

13. A method for modulating an immune response in a subject which comprises targeting antigens into the immune system through surface receptors on dendritic cells together with an inhibitor of CD22 function or binding.

14. The method of claim 13, wherein antigen is from an infectious agent.

15. The method of claim 13, wherein antigen is a tumor-associated antigen.

16. The method of claim 13, wherein antigen is an autoantigen.

17. The method of claim 13, wherein modulating the immune response generates protective immunity to a pathogen.

18. The method of claim 13, wherein altering an immune response suppresses autoantibody production.

19. A method for modulating an immune response in a subject which comprises targeting antigens into the immune system through surface receptors on dendritic cells together with an inhibitor of CD22 function or binding.

20. The method of claim 19, wherein antigen is from an infectious agent.

21. The method of claim 19, wherein antigen is a tumor-associated antigen.

22. The method of claim 19, wherein antigen is an autoantigen.

23. The method of claim 19, wherein modulating the immune response generates protective immunity to a pathogen.
24. The method of claim 19, wherein altering an immune response suppresses autoantibody production.

25. A method for modulating an immune response in a subject which comprises targeting antigens into the immune system through CD22 receptors on B lymphocytes whereby CD22 binding to dendritic cells is inhibited.

26. The method of claim 14, wherein the immune system is altered or suppressed.
FIGURE 2
FIGURE 3
FIGURE 4
FIGURE 5
<table>
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<tr>
<th>B cell subset</th>
<th>WT</th>
<th>CD22-/-</th>
</tr>
</thead>
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<tr>
<td>T1</td>
<td>3.1 ± 0.6 X 10^6</td>
<td>4.8 ± 1.2 X 10^6</td>
</tr>
<tr>
<td>T2-FOP</td>
<td>5.1 ± 1.0 X 10^6</td>
<td>1.8 ± 0.6 X 10^6**</td>
</tr>
<tr>
<td>T2-MZP</td>
<td>4.6 ± 1.7 X 10^6</td>
<td>0.6 ± 0.3 X 10^6**</td>
</tr>
<tr>
<td>FO</td>
<td>22.6 ± 8.1 X 10^6</td>
<td>23.0 ± 5.3 X 10^6</td>
</tr>
<tr>
<td>MZ</td>
<td>3.9 ± 1.5 X 10^6</td>
<td>1.9 ± 0.7 X 10^6**</td>
</tr>
</tbody>
</table>

**FIGURE 7**
FIGURE 8
scFvLg fusion gene for expression of Ag targeting constructs:

V regions
- Anti-33D1
- Anti-DEC205
- 1C10 (anti-mouse CD40)
- P4G2 (anti-mouse DCAL2)
- Anti-CD22
- Anti-CD72
- Anti-CD40

Lnk
- (glyser) hydrophilic linker
- (gly4ser)3 linker
- (gly4ser)4 linker

Ig tail
- +/- STOP codon
- mlgG2a SCC H TM
- mlgG2a SSS H TM
- mlgG2a SSS H Y405/407
- hlgG1 mutants
- maclG1 mutants

Ag
- CS
- OVA
- HA
- HEL
- other

FIGURE 10
FIGURE 11
Schematic Structure of prototype scFvLg-Ag fusion protein

anti-CD22 VH

anti-CD22 VL

hinge

mlgG2a CH2-CH3

Ag

FIGURE 12
FIGURE 13

DCs

NK cells

CD8-
CD8+
PDCA-1+

IgD^{High} IgM^{High}

IgD^{Low} IgM^{High}

CD4
CD8

B-cells

Splenic T cells
mDCAL-2 is internalized upon crosslinking by P4G2 + anti-rlgG2a
## FIGURE 15

Figure 15. scFvs developed for antigen targeting studies.

<table>
<thead>
<tr>
<th>Hybridoma</th>
<th>Target</th>
<th>Source for CH2/CH3</th>
<th>Designation</th>
<th>V regions/Current Status</th>
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<td><strong>Costimulation</strong></td>
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<td></td>
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<tr>
<td>rat 1C10</td>
<td>mCD40</td>
<td>mouse (m)IgG2a</td>
<td>mCD40 scFv-mG2a</td>
<td>scFv constructed</td>
</tr>
<tr>
<td>mouse G28-5</td>
<td>hCD40</td>
<td>macaque IgG1</td>
<td>hCD40 scFv-macG1</td>
<td>scFv constructed</td>
</tr>
<tr>
<td>(RR00166)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mouse G28-5</td>
<td>hCD40</td>
<td>human IgG1</td>
<td>hCD40 scFv-humG1</td>
<td>scFv constructed</td>
</tr>
<tr>
<td>(RR00166)</td>
<td></td>
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<td></td>
</tr>
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<td><strong>DC subset</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat 33D1</td>
<td>m33D1</td>
<td>mIgG2a</td>
<td>m33D1 scFv-mG2a</td>
<td>scFv constructed</td>
</tr>
<tr>
<td>mouse 6B4</td>
<td>hDCIR</td>
<td>macaque IgG1</td>
<td>hDCIR scFv-macG1</td>
<td>anti-macaque clone</td>
</tr>
<tr>
<td>identified</td>
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<td></td>
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<tr>
<td>mouse 6B4</td>
<td>hDCIR</td>
<td>human IgG1</td>
<td>hDCIR scFv-humG1</td>
<td>scFv obtained</td>
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<tr>
<td>mouse 6B4</td>
<td>hDCIR</td>
<td>mIgG2a</td>
<td>hDCIR scFv-mG2a</td>
<td>hybridoma obtained</td>
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<tr>
<td>rat NLDC145</td>
<td>DEC205</td>
<td>mIgG2a</td>
<td>mDEC205 scFv-mG2a</td>
<td>hybridoma obtained</td>
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<tr>
<td>rat P4G2</td>
<td>mDCAL-2 2</td>
<td>mIgG2a</td>
<td>mDCAL-2 scFv-mG2a</td>
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<td>rat 2A11</td>
<td>Dectin-1</td>
<td>mIgG2a</td>
<td>Dectin-1 scFv-mG2a</td>
<td>scFv obtained</td>
</tr>
<tr>
<td>Dr. S. Wong</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>B cell subsets</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rat P4G2</td>
<td>mDCAL.2</td>
<td>mIgG2a</td>
<td>mCD22 scFv-mG2a</td>
<td>hybridoma obtained</td>
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<tr>
<td>rat anti-mouse CD72</td>
<td>mCD22</td>
<td>mIgG2a</td>
<td>mCD72 scFv-mG2a</td>
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<tr>
<td>mouse anti-human CD22</td>
<td>hlgG1</td>
<td>humanCD22 scFv-hlgG1</td>
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<td></td>
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<tr>
<td>mouse anti-human CD72</td>
<td>hlgG1</td>
<td>humanCD72 scFv-hlgG1</td>
<td>hybridoma obtained</td>
<td></td>
</tr>
<tr>
<td>rat 1C10</td>
<td>mCD40</td>
<td>mIgG2a</td>
<td>mCD40-mG2a</td>
<td>scFv constructed</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>mouse G28-5</td>
<td>hCD40</td>
<td>macaque IgG1</td>
<td>hCD40 scFv-macG1</td>
<td>scFv constructed</td>
</tr>
<tr>
<td>(RR00166)</td>
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<td></td>
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<tr>
<td><strong>Negative controls</strong></td>
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<tr>
<td>rat Y13-238</td>
<td>v-Ras</td>
<td>mIgG2a</td>
<td>control scFv-mG2a</td>
<td>hybridoma obtained</td>
</tr>
<tr>
<td>mouse 10.2.16</td>
<td>ml-Ak</td>
<td>macaque IgG1</td>
<td>control scFv-macG1</td>
<td>hybridoma obtained</td>
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<tr>
<td>mouse 10.2.16</td>
<td>ml-Ak</td>
<td>human IgG1</td>
<td>control scFv-humG1</td>
<td>hybridoma obtained</td>
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<tr>
<td>VLP-Ig-Ag</td>
<td>mIgG2a</td>
<td></td>
<td>control Ig-Ag</td>
<td>construct made</td>
</tr>
</tbody>
</table>
WT and CD22 KO 1° Immunized Mice

WT
CD22 KO

mIgG1 anti-rat IgG2b (µg/ml)

33D1 Doses per Mouse

FIGURE 16
FIGURE 17

P4G2 scFvlg, P4G2 scFvlg-OVA and P4G2scFvlg–HEL fusion proteins bind to A20 cells.

PE-SA + biotin-anti-mouse IgG2a

Counts

Counts

huBDCA-2-ScFv-PE  huBDCA-2-ScFv-PE-anti-HEL

purple = huBDCA-2
green = DCAL-2

purple = huBDCA-2
green = DCAL-2 HEL (WT)
red = DCAL-2 HEL (2x)
blue = DCAL-2 HEL (3x)

Counts

Counts

huBDCA-2-ScFv-PE

huBDCA-2-ScFv-PE-anti-HEL

purple = huBDCA-2
green = DCAL-2 OVA (2)
red = DCAL-2 OVA (4)

Counts

Counts

huBDCA-2-ScFv-PE

huBDCA-2-ScFv-PE-anti-HEL

purple = huBDCA-2
green = DCAL-2 OVA (2)
red = DCAL-2 OVA (4)
FIGURE 18

Serial Dilutions of P4G2scFvIg and P4G2scFvIg-Ag CHO Culture Supernatants Bind in a dose-dependent manner to A20 B cells

Red: Neg Cont
Blue: Neat
Green: 1:5
Orange: 1:10
Nucleotide and Predicted amino acid sequence of P4G2 scFvIg-OVA fusion gene.
A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/00(2006.01)i, C07K 14/47(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A61K 39/00, C07K 14/47

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS(KIPO internal), Delphion, Pubmed (CD22, scFvIg Ag fusion protein, spacer linker, autoimmunity, B cell)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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</thead>
<tbody>
<tr>
<td>X/A</td>
<td>Goyal A et al., Inclusion of a fusin-sensitive spacer enhances the cytotoxicity of τbotoxin rest:Fcetoxin containing recombinant single-chain Immunotoxins Biochem J 2000 Jan 15, 345 R 2 247-254 See abstract &amp; figure 1</td>
<td>9-1 1/12</td>
</tr>
<tr>
<td>X/A</td>
<td>Mοττz D et al., Cytotoxic T lymphocytes with a grafted recognition specificity for ERBB2-expressing tumor cells Proc Natl Acad Sci U SA 1994 May 10, 91(10) 4318-4322 See abstract, result &amp; figure 1</td>
<td>9-1 1/12</td>
</tr>
<tr>
<td>Y/A</td>
<td>Arndt MA et al., Generation of a highly stable, internalizing anti-CD22 single-chain Fv fragment for targeting non-Hodgkin's lymphoma Int J Cancer 2003 Dec 10,107(5) 822-829 See abstract</td>
<td>12/9-1 1</td>
</tr>
</tbody>
</table>

* Special categories of cited documents

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 28 APRIL 2008 (28 04 2008)

Date of mailing of the international search report 29 APRIL 2008 (29.04.2008)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seomsa-ro, Seogu, Daejeon 302-701, Republic of Korea

Facsimile No  82-42-472-7140

Authorized officer

CHO, Kyung Joo

Telephone No  82-42-481-8287

Form PCT/ISA/210 (second sheet) (April 2007)
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<tr>
<td>Y/A</td>
<td>Carnahan J et al., Epratuzumab, a CD22-targeting recombinant humanized antibody with a different mode of action from  ( \alpha )-tuximab Mol Immunol 2007 Feb, 44(6) 1331-1341 Epub 2006 Jun See abstract, introduction, &amp; discussion</td>
<td>12/9-11</td>
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<td>Y/A</td>
<td>WO2006073941A2 (GENENTECH, INC.) 13 July 2006 See claims 1, 62</td>
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<td>A</td>
<td>Tedder TF et al., CD22 A Multifunctional Receptor That Regulates B Lymphocyte Survival and Signal Transduction Adv Immunol 2005, 88 1-50 See abstract</td>
<td>9-12</td>
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<td>A</td>
<td>US7074403BA (Immunomedics, Inc.) 11 July 2006 See abstract, &amp; claim 1</td>
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</table>
This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos. 1-8, 13-26 because they relate to subject matter not required to be searched by this Authority, namely:
   - Claims 1 to 8, 13 to 26 pertain to methods for treatment of the human or animal body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39(1)(iv) of the Regulations under the PCT, to search.

2. [ ] Claims Nos. because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos. because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6-4(a).

This International Searching Authority found multiple inventions in this international application, as follows:

- Claims 1-8, 12-26 (invention 1) are directed to a method for modulating an immune response by through the surface receptor CD22.
- Claims 9-11 (invention 2) is directed to a method for producing an scFvIg-Ag fusion protein.

- [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
- [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
- [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.
- [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims, it is covered by claims Nos.

**Remark on Protest**

- [ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- [ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- [ ] No protest accompanied the payment of additional search fees.