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(54) Title: METHODS AND COMPOSITIONS TO GENERATE AND CONTROL THE EFFECTOR PROFILE OF T CELLS BY SIMULTANEOUS LOADING AND ACTIVATION OF SELECTED SUBSETS OF ANTIGEN PRESENTING CELLS

(57) Abstract: The present invention is directed to novel compositions and methods and their use in treating an autoimmune disorder or achieving tolerance to an antigen. More specifically, the present invention is directed to human immunoglobulin constructs for loading a delivered T cell epitope on to the surface of an antigen presenting cell for achieving tolerance to that delivered T cell epitope. The present invention may be applied to any T cell epitope mediated autoimmune disorder.



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Methods and compositions to generate and control the effector profile of T Cells by simultaneous loading and activation of selected subsets of antigen presenting cells

5 Related Cases

The present application claims priority to U.S. patent application serial number 60/708,980 filed August 17, 2005, all of which are hereby incorporated by reference.

Field of Invention

10 The present invention is generally directed to methods and compositions to generate an immune response. More specifically, the present invention is directed to methods and compositions of loading an antigen presenting cell to display a delivered epitope on a MHC class I molecule in a context appropriate for the generation of desired T cell responses.

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Background of the Invention

No direct evidence has been shown that delivery of antigen via Fc gamma receptors ("FcγR") triggers an effective antitumoral or antiinfectious response. For example, it was previously shown that delivery of a viral NP (nucleoprotein) derived epitope within immunoglobulin or IgG backbone did not result in detectable induction of cytotoxic immunity (Zaghoulani ET AL., Eur J. Immunol. 1993; 23(11):2746-50). In contrast, delivery of the same epitope in context of NP expressing cells (transfectomas) resulted in significant cytolytic activity. It was therefore concluded at that time that "APC (antigen presenting cells) are unable to present an influenza nucleoprotein (NP) peptide from the same context (1 microM Ig-NP) to an MHC class I-restricted T cell " and thus, "the endocytic compartment, when offered MHC class I-and II-restricted peptides within the same carrier protein context, favors presenting by class II by at least 1000-fold".

25 Access of the NP epitope to MHC class I presentation pathway is dependent on delivery strategy and was thus believed to be severely limited subsequent to FcγR internalization. More recently, it has been proposed that cross-linking or simultaneous

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engagement of FcγR on antigen presenting cells ("APC") may greatly optimize signal transduction and result in stimulation of cross-priming and APC stimulation, resulting in effective loading of MHC class I molecules (Regnault et al., J Exp Med. 1999, Jan 18;189(2):371-80). This could be achieved using immune complexes (multivalent antigen-antibody non-covalent complexes); however, due to the potential of C ("complement") mediated disease, the complexes could only be administered to the APC *ex vivo* (Naama et al., J Clin Lab Immunol. 1985 Jun;17(2):59-67; Rafiq et al., J Clin Invest. 2002 Jul;110(1):71-9). Alternatively, (Fab)2-antigen recombinant fusion constructs directed to receptors onto APC, can result in receptor cross-linking internalization, and presentation in context of MHC class II molecules (Lunde et al., Biochem Soc Trans 2002;30(4):500-6). The insertion of antigen, however, modifies the Fc portion of the constant domains (CH2 and CH3) of the immunoglobulin ("Ig") that can result in serious and unpredictable effects on the half life and pharmacokinetics, two parameters that are tightly associated with the integrity of this segment (Spiegelberg HL, J Clin Invest 1975 Sep;56(3):588-94). Finally, there is no conclusive evidence to date that either one of the strategies described above, when applied *in vivo*, induce protective or therapeutic anti-tumoral or anti-microbial immunity that would be associated with the generation of optimal MHC class I and II-restricted T cells that produce specific cytokines such as IFN-γ. Even when applied *ex vivo*, the immune complex strategy has displayed limited efficacy due to the balance in the activity of ITAM+ and ITIM+ FcγR (Kalergis and Ravetch, J Exp Med 2002 Jun 17;195(12):1653-9). Thus, it has yet to be determined whether *in vivo* delivery of antigen to APC via the monovalent ligation of Fcγ receptors can be used to induce effective anti-tumoral or antiviral immunity.

PCT Application Serial Number PCT/US03/07995 filed March 14, 2002 and U.S. patent application serial number 60/364,490 filed April 30, 2002 are hereby incorporated by reference. Swiss-Protein/Trembl Protein Knowledgebase™ on CD-ROM, available from Geneva BioInformatics, is hereby incorporated by reference in its entirety.

Summary of the Invention

The present invention demonstrates, contrary to expectations, that *in vivo* and *ex vivo* loading of APC via monovalent engagement of FcγR, using peptide epitopes

covalently attached to the IgG backbone without modification of the Fc portion, results in access of the epitope to the MHC I processing and presentation pathway, with effective loading of MHC class I molecules. Unexpectedly, this results in generation of robust Tc2 responses characterized by IL-4, but not IL-2 or IFN- γ -producing, MHC class I restricted T cells that recognize the epitope within IgG backbone.

In addition, the generation of this “deviated” response was not effective in controlling a pathologic process associated with tumor growth, nor was it associated with significant priming of cytolytic T cells. This explains largely the previous failure to detect induction of immunity in similar context previously and demonstrates, unexpectedly, that cross-linking or multivalent engagement of Fc γ R on APC (such as in context of immune complexes or Fab2-antigen compounds) is not a prerequisite for effective loading of the peptide onto MHC class I molecules. This is important since the concept could be applied *in vivo* (in contrast to immune complexes) and the integrity of Fc portion and thus PK profile could be retained (in contrast to Fab2-antigen recombinant molecules). Despite effective loading of MHC class I molecules, the APC were not able to trigger protective anti-tumoral and anti-microbial immunity when loaded *in vivo* by peptide epitope within IgG backbone.

Further, the present application discloses novel compositions that result in effective redirection of class I-immunity to Tc1 effectors that take advantage of the unexpected loading of MHC I by peptide within IgG backbone. Such compositions are able to transform seemingly ineffective MHC class II and class I-restricted peptides into highly effective ones. Fc γ R-mediated loading of APC associated with stimulation of APC by novel synthetic polynucleotides, result in generation of class I-restricted cytolytic cells and IFN- γ , IL-2 producing T cells, further associated with protection against a highly virulent microbe or recovery from malignant tumoral process. It is also shown that variants of the technology, applied incorrectly or as previously proposed, are not optimal in generation of immunity protective against viruses or tumors, in particular of MHC class I-restricted nature. The present application demonstrates the reason for past failures and teaches how to obtain and apply the different components of the technology in order to obtain optimal effect.

Various embodiments of the invention include:

1. A method of loading an antigen presenting cell and generating a T cell response against an antigen or peptide epitope by use of at least one peptide epitope attached to an Ig, Ig backbone backbone or portion thereof thereby forming an Ig-peptide molecule/complex or portion thereof wherein when administered to a patient *in vivo* or *ex vivo*, the epitope is effectively processed and presented by the MHC I pathway of the antigen presenting cell resulting in effective loading of MHC class I molecules on the antigen presenting cell thereby resulting in an MHC class I – peptide complex.
2. The method of paragraph 1 wherein the Ig-peptide molecule/complex or portion thereof is administered with RNA strands.
3. The method of paragraph 2 wherein the RNA is dsRNA strand and is pA:pU.
4. The method of paragraph 3 wherein the dsRNA is pA:pU and the dsRNA is between approximately 20 - 100 base pairs in size.
5. The method of paragraphs 1, 2, 3 or 4 wherein the Ig backbone is derived from human Ig.
6. The method of paragraphs 1, 2, 3 or 4 wherein the Ig backbone is derived from human IgG.
7. The method of paragraph 1, 2, 3, or 4 wherein the Ig backbone is humanized Ig.
8. The method of paragraph 1 wherein the antigen presenting cell is loaded via monovalent engagement of FcγR.
9. The method of paragraph 1 wherein the antigen presenting cell may be loaded *in vivo* or *ex vivo*.

10. The method of paragraph 1 wherein the peptide epitopes are covalently attached to the Ig backbone.

5 11. The method of paragraph 1 wherein the peptide epitope is attached to the Ig backbone without modification of the Fc portion of the Ig.

12. The method of paragraph 1 wherein the peptide epitope is inserted within a CDR region of the immunoglobulin molecule.

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13. The method of paragraphs 1, 2, 3 or 4 wherein the peptide epitope is inserted within a CDR region of the immunoglobulin molecule by insertion or deletion.

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14. The method of paragraphs 1, 2, 3 or 4 wherein the MHC class I –peptide complex results in generation of robust Tc2 responses characterized by IL-4 but not IL-2 or IFN- γ -production.

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15. The method of paragraph 1 wherein the peptide epitope is selected from the group consisting of: influenza virus M1 or M2; hepatitis C virus NS3; hepatitis B virus core antigen; human papilloma virus HPV 18-E7, HPV 16 – E7, HPV 18 E6, HPV 16 E6; melanoma –gp100; MART-1; TRP-2; carcinoembryonic antigen precursor; Her –2; tetanus toxin universal T helper epitope; HIV-1: reverse transcriptase; HIV1: gag; insulin precursor – human; human Gad 65; prostate tumor antigens; mucin 1; herpes simplex antigens; and, respiratory syncytial virus antigens.

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16. The method of paragraph 1 wherein the negative effects of sera are avoided.

17. The method of paragraphs 1, 2, 3 or 4 wherein the Ig peptide molecule and dsRNA are administered by subcutaneous or intraperitoneal injection.

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18. The method of paragraph 1 wherein the antigen presenting cell is selected from the group consisting of dendritic cells, monocytes, macrophages and B cells.

19. The method of paragraph 1 wherein the antigen presenting cell is selected from the group consisting of CD11c+ and CD11b+ APC.

20. The method of paragraph 1 wherein the resulting MHC-peptide complexes formed by *in vivo* delivery are expressed for up to 1 to 2 weeks.

21. The method of paragraphs 1, 2, 3 or 4 wherein the MHC-peptide complex results in activation of T cells.

22. The method of paragraph 21 wherein the T cell response is determined by ITAM+ and ITIM+ Fcγ receptors on APC.

23. The method of paragraph 21 wherein expression of the gamma chain of ITAM+ FcγR isoforms induces the T cell response wherein ITIM+ FcγRII limits the T cell response.

24. The method of paragraphs 18 or 19 wherein monocytes induce Th2 and Tr1 cells, both dendritic cells and monocytes induce Th3 cells, and wherein CD11b+ monocytes are more potent than dendritic cells in triggering a regulatory response following IgG-mediated delivery of T cell epitope.

25. The method of paragraphs 1, 2, 3 or 4 wherein the loading of APC with a peptide delivered within an Ig backbone *in vivo* results in induction of Th2 immunity.

26. The method of paragraphs 1, 2, 3 or 4 wherein the loading of APC with a peptide delivered within an Ig backbone *in vivo* results in induction of Th3 and Tr1 immunity.

27. The method of paragraph 1 wherein the T cell response is enhanced by co-stimulation with one of the following selected from the group consisting of anti-CD40mAb, recombinant IL-12 or synthetic dsRNA.

28. The method of paragraphs 1, 2, 3 or 4 wherein IL-2, IFN- γ and IL-4 are down-regulated in a dose dependent manner and IL-10 and TGF-beta are upregulated in a dose-dependent manner.

29. The method of paragraphs 1, 2, 3, or 4 wherein the peptide epitope is recNP and induces NP-specific MHC class I-restricted T cell immunity consisting of IL-4 producing Tc2 cells.

30. The method of paragraph 1 further comprising the use of RNA motifs thereby resulting in a modified immune response.

31. The method of paragraph 30 wherein the RNA motifs are dsRNA.

32. The method of paragraph 27 wherein the IgG1 and IgG2a antibody responses were increased and associated with an enhanced Th1 and Th2 response.

33. The method of paragraph 2, 27 or 30 wherein the dsRNA was selected from the group consisting of pA:pU, pI:pC and pC:pG.

34. The method of paragraphs 27 or 30 wherein the dsRNA is pA:pU and induced MHC class I-restricted Tc1 cells thereby producing IFN- γ .

35. The method of paragraphs 33 or 34 wherein the dsRNA are from 10 - 50Kd.

36. The method of paragraphs 2 or 30 wherein the RNA motifs are ssRNA selected from the group consisting of p(A), p(C), p(G), p(I) and p(U).

37. The method of paragraph 1 wherein the peptide-epitope is NP and further comprising the coadministration of dsRNA motifs thereby resulting in effective induction of IL-2 and IFN-gamma.

5 38. The method of paragraph 1 wherein the APC are loaded *ex vivo* resulting in the formation of MHC class I-peptide complexes and generation of a Tc response.

39. The method of paragraph 38 wherein the APC are administered to the patient by adoptive transfer.

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40. The method of paragraph 38 wherein the formation of MHC class I-peptide complexes results in differentiation of Tc2 cells producing IL-4 but not IFN-gamma.

41. The method of paragraph 38 wherein further comprising the step of administering
15 RNA motifs thereby resulting in a broadening of the T cell profile to include IFN-gamma producing Tc1 cells.

42. A method of immunization of a patient comprised of loading an antigen presenting cell by use of at least one peptide epitope of an antigen attached to an Ig backbone or
20 portion thereof thereby forming an Ig-peptide molecule and administering to the patient *in vivo* the Ig-peptide molecule in conjunction with a dsRNA motif wherein the epitope is effectively processed and presented by the MHC I pathway resulting in effective loading of MHC class I molecules and thereby resulting in an effective secondary expansion of MHC class I-restricted T cells subsequent to *in vivo* exposure to the antigen.

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43. The method of paragraph 42 wherein the antigen is a virus.

44. The method of paragraph 43 wherein the virus is the influenza virus.

30 45. The method of paragraph 42 wherein the peptide-epitope is recIgG-NP(Kd).

46. The method of paragraph 42 wherein the dsRNA is pA:pU.

47. The method of paragraph 42 wherein the T cells are cytotoxic T lymphocytes.

48. The method of paragraph 42 wherein the secondary expansion of MHC class I-
5 restricted T cells subsequent to *in vivo* exposure to the antigen is greater than
administration of the recombinant antigen in sterile saline only.

49. A method of controlling and treatment of a tumor after clinical diagnosis, by loading
an antigen presenting cell by use of at least one tumor associated T cell epitope attached
10 to an IgG backbone or portion thereof thereby forming an IgG-peptide molecule and
administering the Ig-peptide molecule *in vivo* in conjunction with dsRNA.

50. The method of paragraph 49 wherein the tumor associated T cell epitope is
effectively processed and presented by the MHC I pathway resulting in effective loading
15 of MHC class I molecules on the antigen presenting cell thereby resulting in an MHC
class I-peptide complex.

51. The method of paragraph 49 wherein the method results in an immune response to the
tumor associated T cell epitope and tumor rejection.

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52. The method of paragraphs 49, 50 or 51 wherein the dsRNA is pA:pU.

53. The method of paragraph 49 wherein the Ig-G peptide complex and dsRNA are
administered repeatedly as an anti-tumor therapy.

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54. The method of paragraph 49 wherein upon tumor rejection, Tc1 immunity is
developed against the tumor associated epitope.

55. The method of paragraph 49 where upon administration of IgG-peptide and dsRNA,
30 Tc2 immunity is developed against the tumor associated epitope.

56. The method of paragraph 49 wherein the method further induces an effective memory response to the same tumor associated epitope.

57. The method of paragraph 49 wherein the method results in continued immunity to
5 tumor cell variants.

58. The method of paragraphs 49, 50, 51, 52, 53, 54, 55, 56, or 57 wherein the tumor associated T cell epitope is selected from the group consisting of melanoma -gp100, MART-1, TRP-2, carcinoembryonic antigen precursor XP 064845/NCB1, Her -2,
10 prostate tumor antigens, and MUC 1.

59. A recombinant human Ig molecule or portion thereof capable of binding to an FcγR of an APC, comprising of a CH₃ region adjacent to a CH₂ region whereby a hinge region attaches an antigen to the CH₂ region wherein the antigen has an oligo-glycine linker
15 attached to the hinge region.

60. The recombinant human Ig molecule of paragraph 59 whereby the antigen has a flanking sequence extending therefrom followed by a leader.

20 61. The recombinant human Ig molecule of paragraph 59 wherein the human Ig molecule is an IgG molecule.

62. The recombinant human Ig molecule of paragraph 59 wherein the antigen is a viral or tumor antigen.
25

63. The recombinant human Ig molecule of paragraph 59 wherein the amino acid sequence of the CH₃ region is:

GQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNY
KTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSP

30 GK and conservatively modified variants thereof.[Seq. I.D. No. 1].

64. The recombinant human Ig molecule of paragraph 59 wherein the amino acid sequence of the CH₂ region is: APELLGGPSVFLFPPKPKDTLMISRTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWL NGKEYKCKVFNKALPAPIEKTISKAK and conservatively modified variants thereof.

5 [Seq. I.D. No. 2].

65. The recombinant human Ig molecule of paragraph 59 wherein the amino acid sequence of the hinge region is: EPKSCDKTHTCPPCP and conservatively modified variants thereof. [Seq. I.D. No. 3].

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66. The recombinant human Ig molecule of paragraph 53 wherein the amino acid sequence of the flanking sequence is: QVQLQ and conservatively modified variants thereof. [Seq. I.D. No. 4].

15 67. A composition for enhancing an immune response to an antigen wherein the composition is a polynucleotide wherein the polynucleotide is made up of compounds selected from the group consisting of adenine, uracil, guanine, cytosine and inosine.

68. The composition of paragraph 67 wherein the polynucleotide is dsRNA.

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69. The composition of paragraph 68 wherein the dsRNA is selected from the group consisting of pA:pU and pI:pC.

25 70. The composition of paragraph 69 wherein the dsRNA is pA:pU and wherein some of the adenine and uracil is occasionally replaced by guanine, cytosine or inosine along the polynucleotide chain.

71. The composition of paragraph 69 wherein the antigen is a virus.

30 72. The composition of paragraph 69 wherein the antigen is attached to an immunoglobulin or portion thereof and administered *in vivo*.

73. The composition of paragraph 72 wherein the antigen is protein or a peptide.

74. The composition of paragraphs 67, 68, 69 or 70 wherein the antigen is a tumor
5 associated epitope.

75. The composition of paragraph 74 wherein the antigen is a T cell epitope.

76. The composition of paragraphs 67, 68, 69 or 70 wherein the dsRNA is administered
10 together with said antigen.

77. The composition of paragraph 67 wherein the polynucleotide is dsRNA and is
coadministered with the antigen.

15 78. The composition of paragraph 67 wherein the antigen is already present in the body.

79. The composition of paragraph 67 wherein the antigen is administered in a
pharmaceutically acceptable carrier.

20 80. Use of dsRNA in the manufacture of a medicament for enhancing an immune
response to an antigen in a patient, comprising administering said dsRNA to a patient in
conjunction with said antigen.

81. The use of paragraph 80 wherein an epitope of said antigen is delivered to the patient
25 in an immunoglobulin or portion thereof.

82. The use of paragraphs 80 or 81 wherein the dsRNA is comprised of pA:pU.

83. The use of paragraphs 80 or 81 wherein the dsRNA is comprised of pI:pC.
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84. The use of paragraph 81 wherein the dsRNA consists of bases selected from the group consisting of adenine, cytosine, uracil, guanine and inosine.

85. The use of paragraphs 81, 82 or 83 wherein the use enhances the Th1 and/or Tc1
5 response to the antigen.

86. The use of paragraphs 81, 82 or 83 wherein the use induces a Tc1 cell response to the antigen.

10 87. The use of paragraphs 81, 82 or 83 wherein the immune response includes an enhanced B cell response.

88. The use of paragraphs 81, 82 or 83 wherein the antigen is administered with additional antigen.

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89. The use of paragraphs 81, 82 or 83 wherein the use induces expression of CXC and CC chemokines.

90. The use of paragraphs 81, 82 or 83 wherein the administering of dsRNA enhances T
20 or B cell responses or both T and B cell responses by recruitment and activation of CD11b⁺ monocytes.

91. The use of paragraphs 81, 82 or 83 wherein the administering of dsRNA enhances T
or B cell responses or both T and B cell responses by recruitment and activation of
25 dendritic cells.

92. The use of paragraphs 81, 82 or 83 wherein the dsRNA compositions enhance an immune response by recruiting antigen presenting cells.

30 93. The use of paragraph 92 wherein the antigen presenting cell is a professional antigen presenting cell.

94. The use of paragraph 92 wherein the antigen presenting cell is a naive antigen presenting cell.

5 95. The use of paragraphs 81, 82 or 83 wherein the antigen is a non-infectious antigen and wherein the MHC Class 1 restricted T cells are cross-primed by the dsRNA.

96. The use of paragraphs 81, 82 or 83 wherein the composition and antigens are administered by one of the following selected from the group consisting of mucosal
10 administration, respiratory administration, intravenous administration, subcutaneous administration, and intramuscular administration.

97. The use of paragraph 81 wherein the antigen is administered in an immunoglobulin or portion thereof or in an immunoglobulin backbone.

15

98. The use of paragraph 97 wherein the wherein the antigen is a peptide epitope.

99. A method of preventing high zone tolerance in a patient to an antigen comprising administering said antigen together with a dsRNA composition wherein the dsRNA
20 composition comprises at least one compound selected from the group consisting of poly-adenine, poly-uracil, poly-guanine, poly-cytosine, poly-inosine.

100. The method of paragraph 99 wherein the antigen is non-infectious.

25 101. The method of paragraph 99 wherein the antigen is administered in high doses or already present in the body.

102. The method of paragraphs 99, 100 or 101 wherein the dsRNA is selected from the group consisting of pA:pU and pI:pC.

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103. The method of paragraphs 99, 100, 101 or 102 wherein the method prevents B cell unresponsiveness.

104. A method of enhancing the immune system in a patient exposed to a pathogen
5 comprising the administration of dsRNA to the patient.

105. The method of paragraph 104 wherein the dsRNA is selected from the group consisting of pA:pU and pI:pC.

106. The method of paragraphs 104 or 105 wherein the dsRNA is administered to a
10 patient in concentrations ranging from 100 ug/ml to 1 mg/ml.

107. The method of paragraphs 104, 105 or 106 wherein the pathogen is unknown.

108. The method of paragraphs 104, 105, 106 or 107 wherein the dsRNA is administered
15 in a pharmaceutically acceptable carrier.

109. The method of paragraph 104 wherein a T cell response to the pathogen is enhanced.

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110. A method of enhancing an immune response in a patient in need thereof comprising loading an antigen presenting cell by use of at least one peptide epitope of an antigen attached to an Ig backbone thereby forming an Ig-peptide complex or molecule and administering the Ig-peptide complex or molecule *in vivo* in conjunction with a
25 dsRNA motif wherein the epitope is effectively processed and presented by the MHC pathway of the antigen presenting cell resulting in effective loading of MHC molecules and thereby resulting in an effective secondary expansion of MHC molecules subsequent to *in vivo* exposure to the antigen.

111. The method of paragraph 110 wherein the MHC pathway is the MHC I pathway.
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112. The method of paragraph 110 wherein the MHC pathway is the MHC II pathway.

113. The method of paragraph 111 wherein the method results in effective loading of MHC Class I molecules on the antigen presenting cell.

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114. The method of paragraph 112 wherein the method results in effective loading of MHC Class II molecules on the antigen presenting cell.

115. The method paragraphs 110, 111 or 112 wherein the dsRNA is pA:pU.

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116. The method of paragraphs 110, 111 or 113 wherein the method results in secondary expansion of MHC Class I restricted T cells.

117. The method of paragraph 115 wherein the antigen is a virus.

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118. The method of paragraph 117 wherein the virus is an influenza virus.

119. The method of paragraph 115 wherein the antigen is a tumor associated epitope.

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120. The method of paragraph 115 wherein the T cell is a cytotoxic T lymphocyte.

121. A method of generating an immune response to an antigen in a patient comprising:

administering to the patient an immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of said antigen attached to said immunoglobulin or portion thereof and administering said immunoglobulin or portion thereof in conjunction with a dsRNA segment.

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122. The method of paragraph 121 wherein the immunoglobulin or portion thereof and said dsRNA segment are administered together.

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123. The method of paragraph 121 wherein the immunoglobulin or portion thereof and said dsRNA segment are administered separately.

124. The method of paragraph 121 wherein said patient is human.

125. The method of paragraph 121 wherein upon administration of said immunoglobulin or portion thereof to said patient the immunoglobulin or portion thereof loads the antigen presenting cell by engagement with the antigen presenting cell's FcγR said peptide epitope is effectively processed and presented by the MHC I pathway of the antigen presenting cell resulting in effective loading of the MHC class I molecules.

126. The method of paragraph 121 wherein the peptide epitope is attached within the CDR region of the immunoglobulin or portion thereof.

127. The method of paragraph 121 wherein the immune response generates an effective T cell response to the antigen.

128. The method of paragraph 121 wherein the T cells are cytotoxic T lymphocytes.

129. The method of paragraph 121 wherein the dsRNA segment is selected from the group consisting of pA:pU and pI:pC.

130. The method of paragraph 121 wherein the peptide epitope is a T cell epitope.

131. The method of paragraph 121 wherein the peptide epitope is selected from the group consisting of influenza virus M1 or M2; hepatitis C virus NS3; hepatitis B virus core antigen; human papilloma virus HPV 18-E7, HPV 16 – E7, HPV 18 E6, HPV 16 E6; melanoma –gp100; MART-1; TRP-2; carcinoembryonic antigen precursor; Her – 2; tetanus toxin universal T helper epitope; HIV-1: reverse transcriptase; HIV1: gag; insulin precursor – human; human Gad 65; prostate tumor antigens; mucin 1; herpes simplex antigens; and, respiratory syncytial virus antigens.

132. The method of paragraph 121 wherein the immunoglobulin or portion and dsRNA segment thereof is administered by one of the methods selected from the group consisting of intravenous administration and bolus injection.

5 133. The method of paragraph 121 wherein the immunoglobulin or portion thereof and the dsRNA are administered in a pharmaceutically acceptable carrier.

134. The method of paragraph 121 wherein the method induces an effective memory response to the peptide epitope.

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Brief Description of the Drawings:

Fig. 1A shows (a) representation of natural IgG (light chain – heavy chain heterodimer); (B) antigen (Ag) derived peptide inserted within CDR (complementarity determining region) 3, 2, 1 or framework region; (C) VH (heavy chain, variable region) segment replaced with an antigen or fragment; (D) VH and CH1 segments replaced with antigen or antigen fragment;

Fig. 1B illustrates diagrammatically the IgG-peptide and Fc peptide;

Fig. 1C shows properties of selected human IgG backbone;

Fig. 1D shows the sequence of the constant region of the heavy chain as well as schematic depiction of a prospective construct;

Figs. 1E - 1M show the sequences of various antigens and epitopes discussed in the present application and which can be inserted into an immunoglobulin [sequences can be accessed on the internet at ncbi.nlm.nih.gov (add the proper address prefix: [http://www.](http://www.ncbi.nlm.nih.gov)) by searching the "proteins" section by use of the provided accession number. The content of this database is hereby incorporated by reference in its entirety.] ;

Fig. 1N – 1O shows the heavy and light chain of human IgG1;

Fig. 1P shows the strategy of engineering of an immunoglobulin cassette for insertion of two peptides;

Fig. 1Q shows a strategy for engineering of multiple peptides into a human immunoglobulin construct;

Fig. 1R shows strategies of expression of human immunoglobulins with various cell lines;

Fig. 1S shows a diagram of double or multiple peptides in a human immunoglobulin;

5 Fig. 1T shows human constructs with various combinations of B and or T cell epitopes;

Fig. 1U shows some additional examples of B or T cell epitopes;

Fig. 1V shows various other peptides that can be delivered;

10 Fig. 1W - 1Z₃ is a table of various T cell epitopes/autoimmune peptides which may be inserted into the human IgG to form an IgG-peptide;

Figs. 2A - 2B show that while the injection of the peptide epitope in saline was not immunogenic, a similar dose of peptide used for *ex vivo* loading of APC effectively triggered a substantial immune response upon adoptive transfer;

15 Fig. 3 shows that delivery of epitope within Ig backbone considerably favored its stability in the systemic circulation;

Figs. 4A - 4B show that pre-incubation of peptide with serum resulted in decreased TcH activation;

Figs. 5A - 5B show that the relative efficiency of MHC-peptide complex formation greatly varied depending on the nature of antigen and APC;

20 Figs. 6A - 6B show that the peptide epitope within IgG backbone was more effective on a molar basis (1 order of magnitude) than the peptide alone in inducing TcH activation when handled by blood-derived APC;

25 Figs. 7A - 7B show that the use of oil-in-water adjuvant (incomplete Freund's adjuvant, IFA) only modestly enhanced the *in vivo* formation of MHC-peptide complexes on APC of lymph nodes but not the spleen or thymus;

Figs. 8A - 8D show that use of FcγR mediated delivery of peptides results in preferential formation of immunogenic MHC II - peptide complexes on CD11c+ and CD11b+ APC;

30 Figs. 9A - 9C show long lasting expression of peptide onto endogenous MHC II, on both DC (dendritic cells) and monocytes;

Fig. 10 shows that formation of MHC II - peptide complexes on dendritic cells and monocytes, subsequent to IgG mediated delivery of peptide epitope, is critically dependent on ITAM+ FcγR that encompass the gamma chain;

Fig. 11 shows that results show that the expression of the gamma chain of ITAM+ FcγR isoforms is necessary for the induction of T cell response to APC loaded with peptide within the IgG backbone;

Figs. 12A - 12D show that unexpectedly and in contrast with the potency / cell basis (Example 8), at the organism level, the CD11b⁺ monocytes have the highest impact on the immune response to a peptide epitope delivered within the IgG backbone;

Figs. 13A - 13B shows that FcγR-mediated delivery of a T cell epitope within the recombinant Ig backbone results in Th2 rather than Th1 response;

Fig. 14 shows that FcγR-mediated delivery of T cell epitope within recombinant Ig backbone results in Th2 rather than Th1 response;

Fig. 15 shows that a peptide epitope within the IgG backbone triggers a cellular response of Th2 profile that is enhanced but not switched by a conventional adjuvant (CFA);

Fig. 16 shows that peptide presentation by APC, subsequent to loading with antigen by using recombinant IgG as delivery platform, occurs in context of limited co-stimulation;

Figs. 17A-17B show that the activity of HA (110-120 hemagglutinin peptide) specific IL-4 producing T cells triggered by administration of recHA(I-Ed)-IgG is dependent on CD4 rather than CD8;

Fig. 18 shows that the IgG mediated delivery of T cell epitope has a profound and differential effect on the expansion and cytokine production by activated T cells: IL-2, IFN-γ and surprisingly IL-4, were down-regulated in a dose-related manner;

Figs. 19A – 19B show that in contrast to viral immunization with an influenza virus strain bearing the cognate peptide, Ig-mediated peptide delivery was ineffective in triggering cytotoxic response;

Figs. 20A – 20D show that co-administration of MBP and PLP epitopes by using recombinant IgG curbed the chronic progression of disease;

Fig. 21 summarizes the impact of IgG / FcγR-mediated delivery of epitopes on the T cell response, based on data provided in Examples 2-20;

Fig. 22 shows that shows that natural, non-infectious double stranded RNA produced during infection with influenza virus, has substantial effects on the specific
5 immune response to a protein antigen;

Fig. 23A shows an extensive library of synthetic RNA motifs;

Figs. 23B – 23D show that different synthetic RNAs have an enhancing effect on the B and T cell response to a prototype protein antigen;

10 Figs. 24A - 24B show effects of selected RNA motifs on the innate immune response;

Fig. 25 shows that distinct RNA motifs bind to different receptors on antigen presenting cells;

Fig. 26 shows that distinct RNA motifs induce differential upregulation of chemokines;

15 Fig. 27 shows that the control of replication of influenza virus can be achieved by using selected synthetic RNA motifs;

Fig. 28 shows that selected synthetic RNA motifs pI:pC and pA:pU largely prevent high zone tolerance that is usually associated with administration of large amounts of purified protein;

20 Fig. 29 shows that selected synthetic RNA motifs effect on human monocytic cells;

Figs. 30A – 30B show that non-tagged pA:pU, but not non-tagged pI:pC, was able to compete out the binding of tagged pA:pU to human THP-1 monocytic cells;

Fig. 31 shows the purification and fractionation steps of dsRNA;

25 Fig. 32 shows that lower molecular weight fractions of a selected synthetic RNA compounds are endowed with different biological activity;

Fig. 33 shows that pI:pC but not pA:pU induced antibody response against itself, with a cross-reactive component against another RNA motif;

30 Figs. 34A – 34B show that co-use of selected synthetic RNAs promote effective induction of IL-2 and IFN-gamma subsequent to IgG mediated delivery of an MHC class I-restricted epitope;

Fig 35 shows that *ex vivo* APC loading by recombinant IgG is more effective in formation of MHC class I-peptide complexes and generation of Tc response, compared to use of free peptide itself;

Fig. 36 show that IgG mediated delivery of a class I restricted epitope is most effective in priming class I restricted Tc1 responses when co-administration of selected synthetic RNA was carried out;

Fig. 37 shows that effective priming of anti-viral cytotoxic T cells requires both effective *in vivo* loading of APC with class I restricted epitope delivered via IgG, together with appropriate instruction by selected synthetic RNA motif;

Fig. 38 shows that immunization with a recombinant IgG bearing a viral class I restricted epitope together with selected synthetic dsRNA, resulted in priming of an immune response capable of limiting the replication of a virus subsequent to infectious challenge;

Fig. 39 describes the tumor models used for testing the efficiency of Ig-peptide-based molecules;

Fig. 40 shows that both effective *in vivo* loading of APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA motifs, are necessary and sufficient for effective control of tumor growth and induction of tumor rejection;

Fig. 41 shows that both effective *in vivo* loading of APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA, can trigger an effective immune response to tumor-associated antigens;

Fig. 42 shows that tumor infiltrating lymphocytes displaying the T cell receptor marker TCR β acquired expression of the activation marker CD25 upon treatment with recombinant immunoglobulin bearing tumor associated epitope, together with selected synthetic dsRNA motif;

Fig. 43 shows that the treated mice that successfully rejected the tumor developed Tc1 responses against the tumor-associated epitope on the therapeutic Ig, along with Tc2 immunity;

Fig. 44 shows that successful rejection of tumor induced by indicated treatment is followed by effective protection against subsequent challenge with the same tumor, indicating development of effective immune memory;

5 Figs. 45A - 45B show that the emerging immunity, subsequent to the indicated treatment that results in tumor rejection, protects against challenge with loss of antigen variants and is associated with overall expansion of cytokine producing cells;

Figs. 46A – 46B shows GAD stimulated cytokine production;

Fig. 47 shows the effect of mitomycin C on T cell activation demonstrating loading of APCs by the Ig-P constructs;

10 Fig. 48 shows that blockade of the APC Fcγ receptor eliminated the ability of the APCs to activate T cells;

Figs. 49A – 49B shows that in a fully human system (i.e. both stimulator and responder cells are of human origin), IgGIN is able to deliver the GAD epitope to the DR4 MHC and produce an expansion of the responder T cell population;

15 Figs. 50A-50B show that in a fully human system (i.e. both stimulator and responder cells are of human origin), IgGIN is able to deliver the Ins9-23 epitope to the DR4 MHC and produce an expansion of the responder T cell population; and,

Fig. 51 shows that IgGIN treatment was effective in lowering the development of diabetes in these mice by 70%.

20

Detailed Description of the Invention

Definitions:

5

The following definitions are intended to act as a guide and are not to be considered limiting of terms found throughout the specification:

10

adjuvant - a substance that enhances the adaptive arm of the immune response to an antigen;

adoptive transfer - transfer of a cell population from one animal to another of the same haplotype;

15

antigen - a molecule that can be specifically recognized by the adaptive elements of the immune system (B cells, T cells or both);

antigen presenting cell - heterogeneous population of leukocytes with very efficient immunostimulatory capacity;

20

BALB/C mouse - Widely distributed and among the most widely used inbred mouse strains;

25

B cell - a type of lymphocyte developed in the bone marrow. Each B cell encodes a surface receptor specific for a particular antigen. Upon recognition of a specific antigen, B cells multiply and produce large amounts of antibodies which in turn bind to the antigen which activated the B cell;

30

B cell unresponsiveness - antigen-specific lack of response by B cell;

CDR - Complementarity Determining Region; hypervariable regions in an immunoglobulin which create the antigen binding site. There are three CDR regions: CDR1, CDR2 and CDR3;

chemokines - a group of at least 25 small cytokines, all of which bind to heparin;

5 complete Freund's adjuvant - an oil-in-water emulsion containing mycobacterial cell wall components;

cross primed - antigen presenting cells that have acquired antigens from infected tissues and then present them to cognate T cells;

10

dendritic cells - A subtype of antigen presenting cells (i.e. CD11c+);

downregulation - decreasing the expression or activity of a particular compound or effect;

15

epitope - parts of an antigen which contact the antigen binding site of the antibody or T cell receptor;

20 FcγR - Ig receptors on cell surfaces of which there are three recognized groups: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16);

heterodimer - dimeric protein consisting of 2 different protein sequences;

25 high zone tolerance - a state of unresponsiveness specific to a particular antigen that is induced upon challenge with a high concentration of said antigen;

IL-2 - refers to interleukin - 2;

30 IL-4 - refers to interleukin - 4;

immunoglobulin - a group of glycoproteins present in the serum and tissue fluids of all mammals and are located on the surface of B cells and serve as antibodies free in the blood or lymph. There are five classes of immunoglobulins: IgG (70 - 75%), IgM (10%),

IgA (15 - 20%), IgD (>1%) and IgE (found on basophils and mast cells in all individuals). IgG has four human subclasses (IgG1, IgG2, IgG3 and IgG4);

immunoglobulin backbone - refers to an immunoglobulin molecule or portion thereof wherein at least one CDR region is able to receive an inserted peptide epitope;

immunoglobulin isotype switching - stimulation of B cells to switch production from one immunoglobulin isotype to another;

incomplete Freund's adjuvant - an oil-in-water emulsion not containing mycobacterial cell wall components;

innate immunity - The innate immune system provides broad relatively nonspecific host defenses that lack antigenic specificity but have the ability to guide acquired immunity. Among the cells types involved are dendritic cells and macrophages;

intraperitoneally- within peritoneal cavity;

intravenously - within vasculature;

isoforms - different glycosylation, phosphorylation, deamidation and other posttranslational modifications of proteins;

ITAM - immunoreceptor tyrosine-based activation motifs;

ITIM - immunoreceptor tyrosine-based inhibitory motifs;

macrophages - Any mononuclear, actively phagocytic cell arising from monocytic stem cells in the bone marrow;

MHC - refers to the Major Histocompatibility Complex;

modified immune response - enhanced or diminished immune response;

monocytes - Mononuclear leukocytes found in lymph nodes, spleen, bone marrow and loose connective tissue;

murine – Of or related to a member of the rodent family Muridae, including rats and mice.

naive - non-differentiated, non-activated cell;

peptide - a compound consisting of two or more amino acids joined together by a peptide bond;

polynucleotide - a polymer of nucleotides;

professional antigen presenting cell - mature, able to present antigenic epitope;

recruitment - attraction of a cell population to inflammatory site;

secondary expansion - immune response which follows a second or subsequent encounter with a particular antigen;

self-antigens - antigens that are derived from the host;

subcutaneously - beneath the skin;

Tc1 immunity - Cytotoxic T cell type 1, CD 8+;

Th1 cells - T helper 1 cells which are involved in cell mediated inflammatory reactions, identified by production of IFN γ , TNF β and IL-2;

Th2 cells - T helper 2 cells which encourage production of antibodies and are identified
5 by production of IL-4 and IL-5;

Th3 cells - T helper regulatory cell, known to produce transforming growth factor (TGF)-beta;

10 TR1 cells - T regulatory cell, known to produce interleukin 10; and,

upregulation - enhancement of expression or activity of a particular compound or effect;

15 **Materials and Methods**

For selective *in vivo* loading of antigen presenting cell subsets and use in treatment of autoimmune disorders, the use of compounds described schematically in the Figure 1A are used: (A) representation of natural IgG (light chain – heavy chain heterodimer); (B) antigen (Ag) derived peptide inserted within CDR 3, 2, 1 or framework
20 region; (C) VH segment replaced with an antigen or fragment; and, (D) VH and CH1 segments replaced with antigen or antigen fragment. This type of molecules are engineered using methods known in the art and as stated as follows:

A. Construction of model recombinant IgG (murine).

25 Polymerase chain reaction (PCR) mutagenesis was used to replace the CDR3 region of VH chain with the stated epitopes. Briefly, a pUC19 plasmid harboring the 5.5-kb EcoRI fragment carrying the VH gene of the murine anti-arsonate antibody, 91A3, was used as template DNA in two PCRs to delete the diversity segment (D) of the complementarity-determining region 3' (CDR3) loop and inserted DNA fragments
30 encoding various antigen epitopes. These chimeric VH and as well as wild type VH genes were then ligated with Ig gamma 1 heavy chain constant region within the plasmid pSV2 Δ HgptDNSVH-hCgamma1 from which the EcoRI dansyl (dns)-conjugated VH gene was cut out. The sequences of VH and inserted epitopes were confirmed by DNA

sequencing. To express these chimeric IgGs with murine 91A3 VH-human C gamma1 heavy chain genes and a mouse-human chimeric k light chain gene, an 8-kb BamHI fragment encoding the entire murine 91A3 kappa light chain gene was subcloned into the BamHI site of pUC19 plasmid. Subsequently, a HindIII fragment with the kappa light chain promoter and the V kappa region coding sequences was cut out from this plasmid and subcloned into the HindIII site of pSV184ΔHneoDNSVk-hCk upstream of the gene encoding a human k light chain C region (Ck) from which the dns-conjugated Vk (dnsVk) had been excised. This plasmid, which will encode a murine 91A3 Vk-human Ck light chain, is designated pSV184Δhneo91A3Vk-hCk.

1. Production of recombinant murine IgG

The SP2/0 cell line (American Type Culture Collection) is used for the production of all the recombinant IgGs (rIgG) discussed in this section of the patent application. Stable expressing cell lines (i.e. transfectomas) were produced using a double transfection protocol with plasmids encoding the heavy and light chains of an anti-arsenate mouse IgG. Each transfectoma differs only in the sequence of the CDR3 region of the heavy chain. Methods for growing the cell lines as well as producing the different purified rIgG used in the experiments reported in this application are identical in all cases.

The SP2/0 transfectomas were initially grown in Quantum Yield media (BD Biosciences) supplemented with 5 % (v/v) heat-inactivated fetal bovine serum, 0.5 mg/mM gentamicin and 2.5 µg/mL Fugizone. Cultures were maintained at 37°C in a humidified CO2 incubator. Efforts were made to adapt each of the cell lines to growth in different commercially available serum-free medias (Lymphocyte Growth Media 2, Clonetics; Cell MAb Growth Media Serum Free, BD Biosciences; Animal Component Free Cell Media, BD Biosciences). Each of the serum-free medias was supplemented with antibiotics as above. Culture media containing secreted IgG was produced from each media noted above. No difference in the IgGs produced in the different medias was

observed over the course of this work (molecular weight analysis by SDS PAGE [see below], ELISPOT assays, and immune responses in mice).

The amount of secreted rIgG was quantitated using an ELISA: capture antibody was a goat anti-mouse IgG (Sigma) and secondary antibody was an anti-mouse IgG HRP conjugate (Sigma). Purified mouse IgG (Sigma) was used as a standard.

Four different methods have been used to produce media containing the different rIgGs (i.e. conditioned media, "CM"): flasks, stirred vessels, packed bed bioreactors (New Brunswick Cellagen), CELLline flasks (BD Biosciences). In the case of CM produced in flasks, the cells were fed and/or harvested twice a week and maintained at least 50% viability, but viability was generally greater than 70%. Collected media was filtered and held at 4 C. Stirred vessels (1 L) were seeded at 10^6 cells per mL in 200 mL starting volume. Media was added weekly to keep the cell number between 10^7 and 10^6 per mL until 800 mL of total volume was reached. At this point cell viability was determined (typically greater than 80%), and the run was continued until such time that the viability fell below 50%. Media was then collected and sterile filtered to remove cells and held at 4°C. For the packed bed bioreactors: each unit was seeded with approximately 10^8 cells in 400 mL of media; maintained in a CO₂ incubator at 37°C with constant stirring; media was changed every 3-4 days and CM was filtered as above; production of rIgGs in the CM was monitored with ELISA. Bioreactor runs were continued until production of rIgGs began to decline or the vessel became contaminated. The 1 L CELLline flasks were used according to manufacturer's instructions: each flask was seeded with 10^7 to 10^8 cells in a total volume 40 mL in the cell compartment; 1 L of media was added to the feed compartment; CM was harvested from the cell chamber after 2 to 3 weeks, or when viability of the cells fell below 20%.

2. Purification of murine rIgG

The rIgGs produced by the above methods were purified by one of two methods. For CM that contained FBS, an anti-mouse IgG immunoaffinity resin was used. The immunoaffinity resin was synthesized using the following protocol: 10 mL of cyanogen

bromide-activated Sepharose 4B (Sigma) was washed with 1 mM HCl as per manufacturer's instructions; 10-20 mg of goat anti-mouse IgG (Sigma) was dissolved in coupling buffer (0.1 M sodium carbonate [pH 8.4]/0.5 M NaCl) at a concentration of 2 mg/mL; the IgG solution was added to the washed resin, and the slurry was mixed end-over-end at room temperature; the extent of coupling was monitored using the Bradford assay to determine the amount of remaining soluble IgG; the coupling was quenched by addition of ethanolamine to a final concentration of 10 mM when the amount of soluble IgG was less than 10% of the starting concentration (approximately 45 minutes). The immunoaffinity resin was then washed with the following buffers: PBS, 10 mM glycine (pH 2.4), 20 mM Tris/ 1 M NaCl (pH 8.0), PBS. The resin was stored at 4°C in PBS. The protocol for purifying rIgG with this resin was initiated by passing CM through the column at 1 to 2 mL/min. The resin was then washed free of nonbound protein using the following protocol: 100 mL PBS/0.5M NaCl followed by 50 mL 1 mM Tris (pH 8). Fractions were monitored for protein using the Bradford assay. Specifically bound rIgG was eluted with a low pH buffer (5 mM glycine (pH 2.4)/0.5 M NaCl). The eluted protein was collected and held at 4°C for further processing (see below).

The rIgG produced in serum-free culture media was purified using Protein A affinity chromatography. Typically, a 5 mL rProtein A column (HiTrap rProtein A FF from Amersham Pharmacia Biotech) was equilibrated with PBS and the sample was run through the column at 2 mL/min using a FPLC unit (Pharmacia). The resin was washed free of nonspecifically bound protein with PBS, followed by 20 mM Tris (pH 8.0)/1 M NaCl, then water. The specifically bound rIgG was eluted with 1 mM glycine (pH 2.4). The eluted peak was collected and held at 4 C for further processing.

Generally, the rIgG fractions were pooled and concentrated using Centricon ultrafiltration units (Amicon) to a final concentration of 1 to 4 mg/mL (Bradford assay with IgG as standard). The concentrated fraction was then dialyzed into 1 mM glycine (pH 2.4), the final concentration determined by A_{280} using an extinction coefficient of 1.4 for a 1 mg/mL IgG solution, and aliquoted into 100 μ l fractions that were stored in the -80°C freezer. The purified rIgGs were analyzed for structural integrity and purity by SDS gel electrophoresis. The gels were stained with Coomassie blue (Pierce Chemical). In all cases the rIgGs used in the reported experiments displayed their expected molecular

weight (reduced and nonreduced) as compared to protein standards and control IgG. Generally, the purified rIgG was greater than 95% pure as determined by visual inspection of the stained bands relative to the bands of known amounts of control IgG run on the same gel.

5

B. Construction of human recombinant IgG.

The human IgG backbone was obtained from IgGA1 myeloma cell line by RT-PCR. The recombinant human IgG was cloned by inserting the stated epitopes to replace the CDR2 or CDR3 regions of the human IgG1 backbone. Briefly, T cell epitopes were created by PCR mutagenesis and subcloned into the CDR2/CDR3 region. The recombinant heavy chains were then subcloned into pMG vector (Invivogen, San Diego, CA) by BamHI and XbaI sites. The heavy chain expression was controlled by the hCMV promoter. In parallel, the human kappa light chain was subcloned into the pMG vector by StuI and NheI sites. The expression of the light chain was controlled by an EF-1 alpha and HTLV-1 LTR hybrid promoter. The double expression vector carrying both the recombinant heavy chain and light chain were then transfected into expression cell lines.

The Fc-peptides were constructed by cutting off the VH and CH1 fragment and replacing it with stated viral or tumor antigens (8-150 Aas). Briefly, the human IgG1 heavy chain was subcloned into pCDNA3 vector by EcoRI and XhoI sites. Then the stated antigens are inserted between the leader sequence and hinge region of IgG1 by PCR mutagenesis. To increase the flexibility of the fused antigens, an oligo-glycine linker (5 glycines) was added after the antigen. The expression of human IgG recombinant molecules can be performed by using either one of the strategies displayed in Figure 1B.

The human IgG backbone has been selected rationally, based on the ability to bind to FcγR, complement and cytokine activation in various states. Properties of selected human IgG backbone are shown in the Figure 1C and the sequence of the constant region of the heavy chain as well as the schematic depiction of a prospective construct, is shown in Figure 1D ("human fusion protein").

Epitopes used for model recombinant IgG are shown in Figure 1E (mouse MHC class II-restricted HA epitope and mouse MHC class I restricted NP epitope). The

nomenclature of recombinant constructs is recIgG-epitope (HA or NP)- restriction element (I-Ed or Kd, respectively). In short, they may be referred to as IgHA or IgNP. Model molecules comprising defined mouse self epitopes (MBP or PLP derived) were similarly constructed. The sequence of the variable region of the heavy chain of anti-
 5 arsonate antibody used as the backbone has been depicted in Figure 1E and the technology is well known in the art (Zaghouani et al., Science 1993 Jan 8;259(5092):224-7) the contents of which is hereby incorporated by reference.

In Figures 1E-1M, examples of antigens and epitopes (in bold) are provided that could be inserted (larger parts up to 150 AA spanning one or multiple epitopes) or
 10 attached to the backbone. Such constructs comprising the shown antigens / epitopes may be used as drugs against infectious or tumoral diseases. In Figure 1I there is the HLA-A2 anchor motif displayed, that allows the prediction of location of potentially therapeutic cytotoxic epitopes in any protein, facilitating the selection of the antigen fragment to be used in the recombinant immunoglobulin.

15 In Figure 1J, examples of "universal" T helper epitopes (Kumar et al. J Immunol 1992 Mar 1;148(5):1499-505) are provided, both dominant and promiscuous from the point of view of MHC restriction, that could be used for construction of composite molecules for the purpose of inducing or enhancing immunity to MHC class I-restricted epitopes, using compounds such as:

20 [antigen fragment]-[universal Th epitope]-Fc(IgG).

Examples of such constructs are schematically represented in Figure 1K (bottom).

In Figure 1K top, examples of human self antigens with epitopes bolded are shown, that could be used to generate recombinant IgG molecules against autoimmune / inflammatory disorders.

25 In Figure 1L and 1M other antigen sequences that could be used for the construction of above mentioned immunoglobulin constructs are shown. The antigen fragments of interest could be defined by using methods to predict MHC class I epitopes (Lim et al., Mol Immunol. 1996 Feb;33[2]:221-30).

30 C. Complete human IgG

The use of complete human IgG as a delivery vehicle for autoimmune disease-related epitopes has been shown to possess a number of distinct advantages over other methods of epitope peptide delivery. One advantage is the stealthy nature of the circulating IgG with peptide combined into the CDR ("IgG-peptide"). The use of a self-protein renders the IgG-peptide blind to the immune system of the recipient. The fact that the disease-related epitopes are inserted into the IgG CDR region(s) should in fact increase the stealth of the IgG-peptide since this region naturally carries enormous variability. Thus, the half-life of the IgG-peptide should mirror that of the endogenous circulating IgG (i.e., hours to days). The greatly extended half-life of the IgG-peptide relative to free epitope peptide alone has been shown to significantly enhance the efficacy of the delivered therapeutic.

The heavy chain human fusion protein (Fig. 1D) carrying a large piece of the targeted self-protein may not be as stealthy as IgG-peptide because it is not a protein that is normally found circulating in the blood stream. The unknown final structure of the fusion protein may be detected as foreign and stimulate an immune response. If this were to happen, the efficacy of the therapeutic would be lowered and further dosing could produce untoward side-effects due to allergic responses. Also, the undefined nature of the protein fragment could lead to further immune responses against self-epitopes that are not desired.

It has been shown that an important component of the efficacy of IgG-peptide constructs to positively impact autoimmune disease is the binding and uptake of the IgG-peptide into APCs through the Fc γ receptor interactions. Obviously, this requires that the Fc region of the Ig heavy chain of the IgG possess the appropriate conformation to be recognized by the receptor. For the IgG-peptide constructs this has been shown to be true, i.e. epitope peptides can be engineered into the CDR regions and the resulting chimeric IgG can still be processed by APC. The fusion protein may have greater difficulties, the final conformation of the human fusion protein will be greatly influenced by the antigen-containing protein fragment since it will be a significant percentage of the overall structure. Thus, it is possible that the final structural conformation of a human fusion protein will not contain an Fc region that maintains a structure that will be as readily recognized by the Fc γ receptor of APCs. Were this is the case, the human fusion protein

would not be as effective in delivering the epitope peptide(s) to the MHC II complex.

However, these problems are avoided with complete naturally occurring human IgG.

Human IgG is exemplary of the basic components of antibody structure as shown in Figure 1N. As shown in Figure 1N, Human IgG is comprised of a protein of 150 kilodaltons consisting of two identical heavy ("H") chains and two identical light ("L") chains. Each heavy chain is comprised of VH, CH1, hinge, CH2 and CH3 regions. Each light chain is comprised of CL and VL regions. Two heavy chains are coupled through two disulfide bonds on hinge regions while each light chain is coupled with a heavy chain through one disulfide bond between its CL chain and CH1 region of heavy chain to form a Y-shaped antibody complex. In solution, human immunoglobulin takes on a more globular shape.

As stated previously, the structure of human immunoglobulin is determined by its primary, secondary, tertiary and quaternary protein structure. The antigen binding sites are primarily located at the VH/VL regions. The specificity between an antigen and an immunoglobulin are determined by hypervariable regions, or complementarity-determining regions (CDRs), of the VH/VL regions. Both the heavy chain and light chain consists of three CDRs: CDR1, CDR2 and CDR3. The CDR regions are exposed on the surface of immunoglobulins and form the binding sites for the antigens (Silverton, EW et al, 1977, Kabat, EA et al, 1977). The CH2 and CH3 region of heavy chain form the Fc portion of immunoglobulin which confers the immunoglobulin biological activity including opsonization, antibody effector cell-mediated cytotoxicity (ADCC) and stability in serum. It has been shown that several types of immune cells including monocytes, neutrophils, natural killer cells and dendritic cells possess Fc receptors on their cell surface and can bind to the Fc portion on immunoglobulin to mediate its biological activities.

Three dimensional pairing of the VL and VH regions constitute the antigen-recognition portion of the "antigen combining site" ("ACS") of the IgG molecule. Because of the tetrameric nature of immunoglobulins, there are two identical antigen combining sites per molecule. The variable domains of these chains are highly heterogenous in sequence and provide the diversity for antigen combining sites to be highly specific for a large variety of antigenic structures. The heterogeneity of the

variable domains is not evenly distributed throughout the variable regions, but are located in three segments, the complementarity determining regions.

Each of the heavy chains includes three or four constant regions (designated CH₁, CH₂, CH₃ and, when appropriate, CH₄) depending on antibody class, which do not vary significantly among antibodies of a single class. The constant regions are not involved with antigen binding but are involved in "effector functions" such as binding to Fc receptors on cell surfaces such as with antigen presenting cells ("APCs").

The light chains have a single constant region (C_L). The first constant domain, C₁, is formed by the pairing of the C_L and C_{H1}. The second constant domain, C₂, is formed from the pairing of the C_{H2} regions and the third constant domain, C₃, is formed from the pairing of the C_{H3} regions.

As stated in the present application, it has been found that Ig-peptides are much more efficient in presenting antigens than free peptides and induce potent T or B cell response. This targeting to FcRs on APCs can markedly reduce the concentration of antigens required for a significant immune response. The present section is focused on the engineering strategies to manipulate human immunoglobulin for peptide delivery and their application for treatment of various diseases.

To clone the full-length heavy chain cDNA from myeloma, the mRNA was isolated from the myeloma cell line CRL 1621 (ATCC). RLM-RACE (Ambion, Austin, TX) was carried out using the primers derived from IgG1 hinge regions. 5'-RACE was done using the 5' adaptor primer and 3' hinge-specific primer (GGGCATGTGTGAGTTTTG). 3'-RACE was done using 5' hinge-specific primer (TTGTGACAAAACCTCACACA) and 3' oligo-T primer. The resulted 5'-RACE PCR product and 3'-RACE PCR product were then ligated using unique restriction site. The light chain (kappa chain) was also cloned using RLM-RACE using mRNA from the same source. The complete amino acid sequences of heavy chain and light chain of the human IgG and its domains are shown in Figure 1N – 1O. The heavy chain immunoglobulin serves as the backbone for the incorporation of peptides. The CDR2 and CDR3 regions of stated IgG1 are 16 amino acid residues and 18 amino acid residues in length respectively. The peptides can be used to replace the CDR regions.

Figure 1P shows the universal DNA cassette of immunoglobulin used for insertion of two peptides and a bridge DNA construct for manipulating of these peptides. The strategy of cloning will be to delete the DNA fragment covering CDR2 and CDR3 and the spacer regions in between using PCR mutagenesis. Specifically The N-terminal fragment between leader sequence and upstream of CDR2 is PCR amplified using primer 1: (GATCACCGGTGATGAAACACCTGTGGT) and primer 2: (TCCAATCCACTCCAGCCCCTT) and another fragment from downstream of CDR3 to the end of CH3 region is PCR amplified using primer 3: (TGGGGCCAAGGGACC) and primer 4: (GATGCTAGCTCATTTACCC GGAGA). The two DNA fragments are then ligated. This DNA cassette is subcloned into pBudCE4.1 vector from Invitrogen (Carlsbad, CA). The resulted DNA cassette lacking CDR2 and CDR3 regions serves as the universal DNA cassette for insertion of peptides into immunoglobulin. The peptides to be inserted are assembled on a different DNA vector PCR-Script (bridge vector, Stratagene, CA) in a way that two peptides are separated by the original spacer. Specifically, nucleotide sequences of peptides are used to PCR amplifying the spacer region between two CDR regions. The resulted PCR product consists of the spacer region flanked by two peptide sequences. The PCR product is then inserted into universal cassette to make the recombinant immunoglobulin with CDR2 and CDR3 replaced by peptides.

Figure 1Q shows a strategy of engineering multiple peptides. The individual peptides are linked together by PCR mutagenesis through the peptide linker. The tandemly linked peptides are then fused to the CH1 region of the heavy chain. Here peptide 1 to 4 (Pep1-4) can be the same or different peptides. The linker is oligo-glycines (up to 10 glycine residues). Specifically a DNA sequence corresponding to amino acid sequence from CH1 to CH3 is PCR amplified using primer 5 (carries the leader sequence and following CH1 sequence, (GATGCGGCCGCGTCATGAAACACCTGTGGTTCTTCCTCCTCCTGGTGGCAGC TCCAGATGGGTCCTGTCCCAG) and primer 6 (GATAGATCTTCATTTACCCGGAGA) and heavy chain cDNA as template. The resulted PCR product is subcloned into pBudCE4.1 and this construct serves as the universal cassette for inserting tandemly linked peptides. The peptide DNA is synthesized

with oligo-glycine linkers and subcloned into this universal cassette. The resulting construct carries multiple peptides which are preceded by original leader sequence for antibody secretion. This tandem linkage of peptides can enhance the binding avidity of peptide ligands to its partners if the peptides used are peptide ligands. This can also
5 enhance the immune response if the peptides used are T or B cell epitopes.

Figure 1R shows the expression strategy for engineered immunoglobulins. The pBudCE4.1 vector contains a CMV promoter and EF-1 α promoter. Both promoters are good for efficient expression of two proteins in variety of cell lines including but not limited to the NS0, SP2/0, 293, CHO and PER.C6 cell lines. The advantage of expressing
10 heavy chain and light chain on the same vector facilitate the 1:1 ratio of these two chains and subsequent assembly of immunoglobulin.

Figure 1S shows engineering of peptide ligands to bind receptors or bind to ligands which can be either agonists or antagonists. Two strategies can be used. One strategy is to replace the CDR2 and CDR3 regions with two peptides. Another is to fuse
15 tandemly linked peptide ligands to the CH1 region of heavy chain. Since the CDR2 or CDR3 regions are the regions exposed on the surface of immunoglobulin, the peptides engineered into CDR2/3 are expected to expose on the Ig surface and keep their binding properties to the receptors on cell surface or soluble factors. Likewise, recombinant immunoglobulin with multiple peptides engineered to fuse with CH1 also keep the
20 property to bind to the partners of peptides. One example is the cytokine (e.g. IL-1, TNF- α) inhibitors. IL-1 and TNF- α are important mediators of inflammatory diseases such as Rheumatoid Arthritis (RA). The blocking of IL-1 and TNF- α provide a therapy for RA. The recombinant immunoglobulins incorporated with inhibitory peptides for IL-1 and TNF- α are expected to bind these two cytokines and inhibit their activities. Flt3 ligand
25 plays an important role in the hematopoiesis by binding to Flt3 receptors on the target cells, which leads to the expansion of variety of cell lineages such as dendritic cells.

Fig. 1T shows engineering of T cell or B cell epitopes into immunoglobulin to develop T cell or B cell vaccines. The T cell epitopes can be either MHC-I restricted or MHC-II restricted epitopes. These disease-associated epitopes can be derived from
30 autoimmune, cancer or infectious disease. Figure 1T shows: 1) the construct with one T cell epitope and one B cell epitope to replace the CDR2 and CDR3 regions; 2) two T cell

epitopes can be engineered to replace CDR2 and CDR3 regions; 3) two B cell epitopes can be used to replace CDR 2 and CDR3 regions; and 4) mixed epitopes (B or T or both B and T cell epitopes) can be engineered to fuse with CH1 region of heavy chain. As shown throughout the application, the epitopes engineered into immunoglobulin are expected to be internalized into APCs through the interaction between Fc portion of Ig and Fc receptors. The peptides will be released through the processing of recombinant immunoglobulin by proteases within the APCs. These processed peptides will then be presented by MHC molecules to the cell surface. Some examples of T cell epitope for autoimmune disorders are listed in Figs. 1U and 1V. The T cell epitopes can be inserted into the immunoglobulins according to the teachings of this application.

Figures 1W – 1Z₃ is a chart of T cell epitopes/autoimmune peptides which may be inserted into the human IgG to form an IgG-peptide as taught in the present application. The chart is divided into autoimmune peptides for type I diabetes, multiple sclerosis, good pasture syndrome, primary biliary cirrhosis, pemphigus vulgaris and celiac disease. Other autoimmune peptides are within the teaching of the present invention. The table lists various T cell epitopes which can be inserted into the human IgG according to the teachings herein. The table in Figures 1W – 1Z₃ also states the relationship between HLA allele type of the human patient and T cell epitope.

RNA segments

The double stranded RNA (dsRNA) or single stranded RNA (ssRNA) segments of the present invention can be made according to the following method (and are available commercially): 1) ssRNA: The polynucleotides (polyA, polyU) are enzymatically prepared, using nucleotides and polynucleotide-phosphorylase, with no animal-sourced material entering into its preparation process. 2) dsRNA: Annealing of polyadenylic acid (polyA or pA) with polyuridylic acid (polyU or pU).

In general, the dsRNA and ssRNA of the present invention are homopolymers with, in the case of dsRNA, a single base or nucleotide (e.g., adenine) consistently forming one strand with its complement consistently forming the other strand. In the case of ssRNA, the single strand is consistently made of the same nucleotide. However, it is within the scope of the invention to use dsRNA or ssRNA compositions that are made up

of mixed nucleotides (and without or without their complements in the case of dsRNA). For example, a polyA:polyU dsRNA segment with occasional substitution by an a non-complementary nucleotide (e.g., guanine, cytosine or inosine). The dsRNA and ssRNA compositions of the present invention are comprised of the bases/nucleotides adenine (A),
5 guanine (G), cytosine (C), uracil (U) and inosine (I) and could also be comprised of a small percentage of the DNA base thymine (T). The RNA compositions in Table I and Figure 8A is descriptive of various RNA compositions used in the Examples. The RNA compositions of the present invention were prepared and purified according to Example 30.

10 The various RNA strands used in the present invention are generally between 100 - 2000 base pairs in length but may be between 1 - 20, 20 - 40, 40 - 60, 60 - 80, 80 - 100, 1 - 100, 100 - 200, 200 - 300, 300 - 400, 400 - 500, 500 - 600, 600 - 700, 800 - 900, 1000 - 1100, 1100 - 1200, 1200 - 1300, 1300 - 1400, 1400 - 1500, 1500 - 1600, 1600 - 1700, 1700 - 1800, 1800 - 1900, 1900 - 2000, 2000 - 2100, 2100 - 2200, 2300 - 2400, 2400 -
15 2500, 2500 - 3000, 3000 - 4000, 4000 - 5000, 5000 - 10,000 base pairs and greater than 10,000 base pairs in length and/or mixtures thereof.

20 **Example 1 shows that a significant factor limiting the activity of peptides that encompass T cell epitopes is the poor pharmacokinetics resulting in reduced *in vivo* loading of APC.**

Antigen presenting cells ("APCs") from 1 naïve BALB/c mouse were obtained from splenic tissue. Following washing, three million APC were incubated with 13.5nM
25 HA 110-120 peptide for 3 hours at 37°C, in 1 ml of HL-1 medium. The cells were washed, divided into three equal inoculi and injected (1/2 subcutaneously + 1/2 intraperitoneally) into 3 naïve BALB/c mice. The mice were sacrificed 2 weeks later and the immune response measured against HA 110-120 peptide, by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified
30 anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4°C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37°C. Single cell suspension was made from the

spleens, red blood cells were lysed, cells washed, counted and incubated at 5×10^5 /well together with 20 μ g /ml HA 110-120 peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37 °C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4 °C. The next day, the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). In parallel, 3 naïve BALB/c mice were each injected with 4.5nM of HA peptide in sterile PBS, half of it administered subcutaneously and half of it intraperitoneally. The mice were sacrificed 2 weeks later and the T cell response characterized as above, by ELISPOT analysis.

In Figure 2(A), the experimental protocol is described. In Figure 2(B), the results of the experiment are shown: they were expressed as number of IFN- γ , IL-2 and IL-4 spot forming colonies / spleen, after the subtraction of the background (mean $\pm$ SEM). "HA-APC" corresponds to antigen presenting cells (dendritic cells) loaded *ex vivo* prior to adoptive transfer. "HA" corresponds to peptide directly injected into animals.

The results described in the Figs. 2A - 2B show that while the injection of the peptide epitope in saline was not immunogenic, a similar dose of peptide used for *ex vivo* loading of APC effectively triggered a substantial immune response upon adoptive transfer. This shows that if directly injected, the peptide does not effectively reach APC, a prerequisite for effective induction of an immune response.

Example 2 demonstrates that incorporation of a peptide epitope within the IgG ameliorated its pharmacokinetics profile.

BALB/c *Scid* mice (3/group) were injected intravenously with 60nM of SFERFEIFPKE ("HA") [Seq. I.D. No. 5] peptide or 2.4nM of recHA (I-Ed)-IgG ("Ig-

HA") and blood was harvested at various intervals. Serum was immediately separated and promptly frozen at -70°C . Later, the serum samples were incubated with 2×10^4 cells/well/50 μl HA-specific T cell hybridoma (TcH) and 1×10^4 cells/well/50 μl M12 B cell lymphoma APC, in serum free HL-1 medium at 37°C and 5% CO_2 for 24 hours. The next day the plate was centrifuged for 15min/ 4°C /1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/ 4°C /1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 ul/well, centrifuging the plate for 3min/ 4°C /1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200 μl /well of the X-gal substrate freshly prepared as follows: 200 μl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl_2 in 1X PBS). The blue activated TcH were scored visually using the microscope.

The activation of TcH was represented as function of time post-injection. The epitope could be detected in the blood only in the case of mice injected with recHA(I-E_D)-IgG, for an interval of about one day. In contrast, the HA peptide injected as is, was not detected in the periphery despite being used in large molar excess (25 fold).

Thus, the results described in Fig. 3 show that delivery of epitope within Ig backbone considerably favored its stability in the systemic circulation.

Example 3 shows that a peptide encompassing a T cell epitope is ineffectively presented by APC to specific T cells in the presence of serum and this is corrected by incorporation of the peptide epitope within the IgG backbone.

Figure 4(A) shows the detrimental effect of serum on the presentation of a T cell epitope peptide: M12 B cell lymphoma APC were incubated with TcH in the presence of various amounts of SFERFEIFPKE (HA) peptide in serum-free HL-1 medium ("HA+HL-1") or HL-1 medium supplemented with 20% mouse serum from BALB/c *scid* mice ("HA+serum"). The number of cells incubated was 2×10^4 M12 and 1×10^4 TcH / 100 μl of HL-1 medium supplemented or not with serum. The next day the plate was centrifuged for 15min/ 4°C /1500RPM, then the supernatant was flicked, the cells were

fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰ C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl /well, centrifuging the plate for 3min/4⁰ C/1500RPM. PBS was flicked off the plate and cells were incubated

5 overnight at 37⁰ C with 200µl/well of the X-gal substrate freshly prepared as follows: 200ul of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope.

The serum negatively interfered with the formation and / or presentation of
10 immunogenic MHC-peptide complexes.

Figure 4B: the serum negatively interfered with the formation and / or presentation of immunogenic MHC-peptide complexes.

This phenomenon was further studied by sequential incubation of peptide ("HA peptide") or recHA (I-E^d)-IgG ("IgHA") first with APC or serum, followed by addition
15 after 1 hour of TcH and serum, or APC and TcH, respectively. Control corresponds to cells incubated with antigens in the absence of added serum ("Ctrl"). The number of cells incubated was 2x10⁴ M12 and 1x10⁴ TcH / 100µl of HL-1 medium supplemented or not with serum. The next day the plate was centrifuged for 15min/4⁰C/1500RPM, then the
20 supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37⁰ C with 200µl/well of the X-gal substrate
25 freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope.

The results are represented as percentage of activated T cells (beta-gal⁺ TcH) /
30 well at concentrations of 2µg/ml of recHA (I-E^d)-IgG ("IgHA") or 40µg/ ml of HA peptide (1,000 molar excess relative to the recombinant Ig). The results described in the

Fig. 4 show that pre-incubation of peptide with serum resulted in decreased TcH activation. Addition of serum after APC pulsing did not have an effect on TcH activation. In contrast, the formation of MHC-peptide complexes was not impaired by serum when the recombinant immunoglobulin carrying the peptide was used instead of the peptide alone.

Example 4 shows that incorporation of a T cell peptide epitope within an IgG backbone improves its presentation to specific T cells by APC, with a rate depending on the nature of APC.

As shown in Figure 5A, *ex vivo* formation of MHC-peptide complexes on antigen presenting cells (APCs) from spleen was measured as follows: splenic APC were isolated by magnetic sorting using anti-MHC II antibodies. Separation by using magnetic beads coupled with anti-MHC II was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and they were incubated with specific T cell hybridoma recognizing I-E^d+SFERFEIFPKE overnight, in the presence of various amounts of SFERFEIFPKE ("HA") peptide or recHA(I-E^d)-IgG ("IgHA"). Per well, 2×10^4 APC were incubated with 1×10^4 TcH. The next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl /well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200µl/well of the X-gal substrate freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium

Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope. The number of activated TcH was quantified and the results expressed as activation versus molar amount of epitope.

- 5 (B) A protocol similar to that described above has been applied to M12 B cell lymphoma APC.

Thus, the results described in the Figure 5B show that the relative efficiency of MHC-peptide complex formation greatly varied depending on the nature of antigen and APC. On a molar basis, the peptide epitope within the IgG backbone was 10 times more
10 effectively handled by MHC II+ APC from lymphoid organs and 1000 times more effectively handled by transformed B cell lymphoma cells, as compared to the free peptide itself. Thus, the cellular handling of the epitope and formation of MHC-peptide complexes subsequent to delivery within IgG, greatly varies with the nature of APC.

- 15 **Example 5 shows that FcγR-mediated delivery of a peptide encompassing a T cell epitope results in more effective cellular handling and presentation by cell populations (peripheral blood white cell) containing reduced numbers of professional APC.**

- 20 (A) To quantify the APC, peripheral blood mononuclear cells (PBMC) were separated by Ficoll gradient centrifugation from BALB/c mice and FACS analysis for expression of CD11c, CD11b and B220 was carried out. The results are represented in Figure 6A as percentage of APC and T cells in blood versus a prototype secondary lymphoid organ (spleen). The number of professional APC such as CD11c+ cells is tremendously (2 logs)
25 decreased in blood as compared to spleen. B220+ and CD11b+ cells were decreased as well (1 order of magnitude). The following materials and methods were used.

Materials:

Ficoll: Ficoll-hypaque (1.077, Amersham, cat# 17-1440-02)

- 30 Antibodies: CD11b cat#01715A, CD11c cat# 557401, B220 cat#01125A, all PE conjugated (BD PharMingen)

Flow Cytometer: FACSCalibur, Becton Dickinson

FACS Buffer: PBS, 1% FCS, 0.1% sodium azide.

Methods:

1. Animal blood was harvested and mononuclear cells were separated by Ficoll gradient separation.
2. Cells were suspended and labeled with fluorescently-tagged anti-mouse CD-11c, CD11b or B220 at 2 ug/ml for 20 minutes on ice
3. Cells were washed once and resuspended in 300 ul of FACS buffer
4. Flow cytometric analysis was carried out to determine fractions of total cell population which labeled with each specific antibody

(B) PBMC were used as APC with SFERFEIFPKE (HA)-specific TcH, in the presence of cognate peptide or recHA (I-E_d)-IgG. The cells were co-incubated for 24 hours (2×10^4 APC + 1×10^4 TcH). The next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl /well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200µl/well of the X-gal substrate freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope. The results are expressed as number of activated TcH / well, at different molar concentrations of epitope.

The results described in the Figures 6A - 6B show that the peptide epitope within IgG backbone was more effective on a molar basis (1 order of magnitude) than the peptide alone in inducing TcH activation when handled by blood-derived APC, suggesting that in suboptimal conditions associated with limiting numbers of professional APC, the Ig backbone greatly facilitates the creation of MHC-peptide complexes.

Example 6 shows that delivery of a T cell epitope within IgG backbone dramatically improves the loading and presentation of epitope by APC in the secondary (draining lymph nodes + spleen) but not central lymphoid organs. The emulsification of the peptide epitope in IFA or increase of dose 100 fold could not reproduce the same degree of loading. Thus, epitope insertion within the IgG backbone removes limiting factors associated with peptide-based strategy, that cannot be otherwise compensated by dose escalation or depot effect.

Assessment of *in vivo* formation of MHC-peptide complexes and a comparison with peptide in saline or standard oil-in-water emulsion were carried out in I-Ed⁺ BALB/c mice. BALB/c mice were treated with recHA (I-Ed)-IgG, peptide in saline or peptide emulsified in incomplete Freund's adjuvant (IFA), by subcutaneous and intraperitoneal injection (doses depicted in Figure 7B). At 24 hours, the local (mesenteric) lymphoid nodes (LN), spleen and thymus were harvested, single cell suspensions were made, red blood cells lysed from the spleens, LN and thymus were collagenase digested. All cells were washed, counted and incubated with TcH recognizing I-Ed+SFERFEIFPKE (MHC class II-HA) complexes. The number of TcH was 1×10^4 / well. The formation of such MHC – peptide complexes was evaluated by titrating the number of APC with constant number of TcH and measuring TcH activation after overnight incubation. The next day the plate was centrifuged for 15min/4⁰C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37⁰ C with 200µl/well of the X-gal substrate freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl 2 in 1X PBS). The blue activated TcH were scored visually using the microscope.

The data are expressed as TcH activation versus APC number (Figure 7A) and as estimated percentage of APC expressing MHC-peptide complexes (Figure 7B), based on *in vitro* standard curve obtained as depicted in the previous Examples, 5 and 6.

The data presented in the Figures 7A - 7B show that the use of oil-in-water adjuvant (IFA) modestly enhanced the *in vivo* formation of MHC-peptide complexes on APC of lymph nodes but not spleen or thymus. Substantial dose escalation of peptide in saline or in emulsion is not paralleled by proportional enhancement in the generation of loaded APC and/or MHC – peptide complexes on APC *in vivo*. In contrast, use of peptide within Ig backbone enhances the formation of MHC peptide complexes considerably, on APC from secondary lymphoid organs such as lymph nodes and spleen. The formation of MHC II- peptide complexes on APC from thymus remained limited, similar to that conferred by peptide alone. The enhancement factor conferred by incorporation of peptide within the IgG was unexpectedly high (approximately 2-3 orders of magnitude), indicating that other factors, in addition to cellular handling (e.g. the above described pharmacokinetics and protective effects), were involved. Even 100 fold dose escalation of peptide alone, in saline or IFA, could not restore the *in vivo* loading of APC noted with peptide within IgG backbone.

Example 7 shows that among the three major APC subsets (DC, monocytes/macrophages and B cells) that express FcγR, the CD11c+ (DC) and CD11b+ (mostly monocytes) rather than B cells are the most potent on a per cell basis in presenting the peptide epitope subsequent to *in vivo* delivery via IgG backbone. The efficiency of APC loading and resulting presentation is substantially higher than that resulting from delivery of free peptide.

In vivo formation of MHC - peptide complexes on APC has been assessed subsequent to the administration of peptide epitope within IgG backbone followed by separation of various subsets of APC.

(A) Separation by using magnetic beads coupled with anti-MHC II or anti-CD11c mAb is carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative

fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and incubated in ELISPOT plates. Usually, from the total number of approximately 90 million splenocytes separated / 1 BALB/c mouse approximately 20 millions bind to magnetic beads coupled to anti-MHC II antibody and 3 millions interact with anti-CD11c mAb. Thus, less than 20 percent of splenocytes are able to present MHC class II restricted epitopes and approximately 2-3 percent are dendritic cells (see Figure 8A). These figures were confirmed by FACS analysis using specific antibodies.

(B) The *in vivo* loading of APC and formation of MHC II - peptide complexes on MHC II+ splenocytes has been assessed comparatively in Balb/c mice injected intravenously with 0.72 uM of recHA (I-Ed)-IgG ("IgHA") or 18 uM of HA peptide. At 24 hours, MHC class II+ APC were isolated from spleen by MACS as above, and incubated with peptide specific TcH (1×10^4 /well), in dose response manner. The next day the plate was centrifuged for 15min/4°C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4°C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 µl /well, centrifuging the plate for 3min/4°C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37°C with 200µl of the X-gal substrate freshly prepared as follows: 200µl of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope.

The results are expressed in Figure 8B as number of activated TcH / well. As a control, MHC II+ APC from naive BALB/c mice were incubated *in vitro*, overnight, with an optimal concentration of HA peptide (50ug/ml), extensively washed and incubated in different numbers with TcH as above. The results show that the formation of MHC II-peptide complexes on splenic APC is at least 2 orders of magnitude more effective when the epitope is delivered within IgG backbone.

(C) A comparative assessment of the *in vivo* loading of various APC subsets after administration of recHA (I-Ed)-IgG has been carried out by magnetic separation of CD11c⁺, CD11b⁺ and CD19⁺ APC using the same protocol as above, using CD11c, CD11b and CD19 microbeads from Miltenyi Biotec. At 24 hours after intravenous injection with 0.72 μ M of recombinant immunoglobulin, the APC were isolated and incubated in a dose effect manner with a constant number of peptide specific TcH. After additional 24 hours, the assay was developed as above and results expressed as number of activated TcH / well. The results in Figure 8C show that on a per cell basis, use of peptide within IgG backbone led to predominant formation of immunogenic MHC II - peptide complexes on CD11c⁺ APC (dendritic cells), followed by CD11b⁺ monocytes and very ineffectively on CD19⁺ B cells.

(D) A comparison between the efficiency of *in vivo* formation of MHC II - peptide complexes on CD11c⁺ APC subsequent to peptide versus recombinant Ig delivery has been carried out following treatment of mice as described in the section B above. The CD11c⁺ splenic DC were isolated by MACS using CD 11c microbeads and incubated in different numbers with 1×10^4 TcH / well. Activated TcH were quantified as above and the results expressed as number of X-gal⁺ T cells / well. As a control, CD11c⁺ APC from naive mice loaded *ex vivo* with peptide were used as described in section B. The results in Figure 8D show that formation of MHC II peptide complexes was at least three orders of magnitude more effective when the peptide epitope was delivered within IgG backbone.

In conclusion, delivery of a peptide epitope within an IgG backbone resulted in more effective formation of MHC II - peptide complexes on CD11c⁺ DC. In addition, the efficiency of APC loading and formation of MHC II - peptide complexes was substantially higher when the peptide was delivered within IgG backbone. The results in Figs. 8A - 8D show that use of Fc γ R mediated delivery of peptides results in preferential formation of immunogenic MHC II - peptide complexes on CD11c⁺ and CD11b⁺ APC.

Example 8 shows a prolonged persistence *in vivo* of MHC-peptide complexes on APC (DC and monocytes) following administration via an IgG backbone.

The persistence of MHC II - peptide complexes on specific APC subsets was measured by magnetic separation of CD11c⁺ DC and CD11b⁺ monocytes at various intervals subsequent to intravenous injection of 2 μ M of recHA (I-Ed)-IgG. In brief, magnetic separation was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and incubated. Different numbers of separated APC (A - CD11b⁺ monocytes, B - CD11c⁺ dendritic cells, C - whole splenocyte population) were incubated overnight with 1x10⁴ TcH specific for the HA peptide.

As a control, APC from naive mice were used that were *in vitro* loaded with optimal amounts of HA peptide (50 μ g /ml), overnight and washed prior to incubation ("ctrl"). The next day the plate was centrifuged for 15min/4⁰C/1500RPM, then the supernatant was flicked, the cells were fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37⁰ C with 200 μ l/well of the X-gal substrate freshly prepared as follows: 200 μ l of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope and the number of activated TcH / well was plotted against the number of APC harvested at various intervals after treatment.

The results show long lasting expression of peptide onto endogenous MHC II, on both DC and monocytes. The complexes persisted between 1 and 2 weeks on these two APC subsets, in the conditions employed in this assay (strategy of APC separation and

detection of MHC II - peptides). Thus, the results in Figs. 9A - 9C show that the MHC-peptide complexes on selected APC formed subsequent to *in vivo* delivery of epitope via Ig are long-lived.

- 5 **Example 9 shows that the γ chain of the Fc receptors (I and III) is essential for effective *in vivo* loading and presentation of a T cell epitope delivered within IgG backbone, by DC and monocytes.**

10 The dependency of APC loading on the interaction with Fc γ R was studied by administration of 2 μ M of recHA(I-Ed)-IgG to BALB/c mice that lack a functional FcR gamma gene. One day after intravenous treatment, the CD11c⁺ and CD11b⁺ APC from spleen were separated by MACS. Separation by using magnetic beads coupled with anti-CD11c and anti-CD11b antibodies was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell
15 suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic
20 field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and they were incubated in different numbers with 1×10^4 TcH specific for the HA peptide, overnight. As a control, APC from FcR gamma competent BALB/c mice were used. The next day the plate was centrifuged for 15min/4⁰C/1500RPM, then the supernatant was flicked, the cells were
25 fixed with cold freshly made fixing solution (2% Formaldehyde, 0.2% Glutaraldehyde in 1X PBS) and the plate was again centrifuged for 3min/4⁰C/1500RPM. Fixing solution was flicked off the plate, cells washed once with PBS 200 μ l /well, centrifuging the plate for 3min/4⁰C/1500RPM. PBS was flicked off the plate and cells were incubated overnight at 37⁰ C with 200 μ l/well of the X-gal substrate freshly prepared as follows:
30 200 μ l of the X-gal stock solution, (40 mg/ml in DMSO) in 10ml of substrate buffer (5mM Potassium Ferrocyanide, 5mM Potassium Ferricyanide, 2mM MgCl₂ in 1X PBS). The blue activated TcH were scored visually using the microscope. The results are

expressed as number of activated TcH / well for different APC subsets: CD11c+ DC (A) and CD11b+ monocytes (B), or as control, whole splenic population (C).

The results (Fig. 10) clearly show that the formation of MHC II - peptide complexes on DC and monocytes, subsequent to IgG mediated delivery of peptide epitope, is critically dependent on ITAM+ FcγR that encompass the gamma chain. In addition, gamma chain negative FcR isoforms cannot compensate for the absence of gamma chain+ FcR isoforms, in that regard.

Example 10 shows that the efficiency of T cell activation by a peptide delivered within the IgG backbone is dependent on the expression of γ chain+ FcγR (that promote activity) and FcγRIIB (that limit the activity) on APC. In addition, this experiment shows that ITIM-bearing FcγRIIB keeps in check the immune response to a peptide delivered within IgG backbone.

The differential role of FcR gamma+ versus gamma- isoforms to the immune response triggered by peptide epitope within IgG backbone, was studied by *ex vivo* loading of APC followed by adoptive transfer. Splenocytes from wild type, FcR gamma- or FcRIIB- BALB/c mice were incubated for 3 hours at 37°C as follows: 10 million cells / 1 ml of serum free HL-1 medium were admixed with 50ug/ml of HA 110-120 peptide or 10ug/ml of recHA(I-Ed)-IgG. Subsequently, the cells were washed and adoptively transferred into naive BALB/c mice (1 million cells suspended in 200ul serum free HL-1 and divided into 2 equal inoculi administered subcutaneously and intraperitoneally). After 2 weeks, the recipient mice were sacrificed, spleens harvested and the T cell response to the HA 110-120 peptide measured by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 μg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μl/well) at 4°C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200μl/well of DMEM complete containing FBS, for an hour at 37°C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well

together with 50 µg /ml HA 110-120 peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37 °C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4 °C.

The next day plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 11 as frequency of cytokine producing (A: IL-2, B: IL-4, and C: IFN-gamma) spot forming colonies obtained by incubation with medium only, or medium supplemented with HA 110-120 peptide (10ug/ml) (mean + SEM of triplicates, corresponding to 3 mice / group).

The results (Fig. 11) show that the expression of the gamma chain of ITAM+ FcγR isoforms is necessary for the induction of T cell response to APC loaded with peptide within IgG backbone. This was not necessary for the immunogenic effect of APC pulsed with peptide. Conversely, absence of ITIM+ FcγRII results in profound increase of the T cell response to APC pulsed with recombinant IgG but not HA peptide. Together, these data show that the T cell response to recombinant IgG bearing a peptide epitope is determined by a complex interplay between ITAM+ and ITIM+ Fcγgamma receptors on APC.

Example 11 shows that unexpectedly, various subsets of APC *in vivo* loaded with epitope inserted within IgG backbone, differentially induce distinct regulatory subsets: while monocytes induce Th2 and Tr1 cells more effectively, both dendritic cells and monocytes induce Th3 cells. In addition, on a cell population level, the CD11b+ monocytes are more potent than the dendritic cells in triggering a regulatory response following IgG-mediated delivery of T cell epitope.

Four BALB/c mice were injected intravenously with 2 μ M of recHA (I-E_d)-IgG. One day later, the spleens were harvested and APC were isolated by MACS using anti-CD11c, anti-CD11b or anti-CD19 monoclonal antibodies coupled with magnetic beads. Separation by using magnetic beads coupled with anti-CD11b, anti-CD11c and anti-CD19 mAb is carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in serum free HL-1 medium as follows: 3x10⁶/ml CD11c⁺ DC, 28x10⁶/ml CD11b⁺ or 84x10⁶/ml of CD19⁺ B cells. This numerical distribution respects the proportion of the APC subsets isolated from the splenic tissue. Cells were transferred into naïve BALB/c mice by subcutaneous and intraperitoneal injection (100+100 μ l / mouse, n=2 mice / group). At 2 weeks after the adoptive transfer, mice were sacrificed and T cell response measured by ELISPOT (IL-4 and IFN- γ) or measurement of cytokine production in cell culture supernatants, by ELISA TGF- β 1 kit (R&D Systems, cat # DY240) and IL-10 kit (Biosource international, cat#KMC0104).

The results are expressed in Figure 12 as number of spot forming colonies / spleen (average of duplicates; panels A, B) or amount of cytokine measured in supernatants (pg/ml, average of duplicates; panels C, D) at various concentrations of HA peptide used for restimulation. The results (Fig. 12, panels A - D) clearly show that unexpectedly, and in contrast with the potency / cell basis (Example 8), at the organism level, the CD11b⁺ monocytes have the highest impact on the immune response to a peptide epitope delivered within the IgG backbone. Thus, the CD11b⁺ APC subset induced both Th2, Tr1 and Th3 cells. In contrast, the CD11c⁺ DC induced Th3 cells and more reduced Th2 response. Finally, despite their substantial number, the CD19⁺ B cells were poor inducers of T cell immunity to the peptide epitope within the IgG backbone. No significant Th1 responses were induced by either of the APC subsets tested.

Example 12 shows that the loading of APC *in vivo* with a peptide delivered within IgG backbone results in induction of Th2 but not Th1 immunity.

5 BALB/c mice were immunized with 100 µg of recHA (I-Ed)-IgG ("IgHA"), or a molar equivalent amount of HA peptide epitope (2µg), by subcutaneous injection and sacrificed 2 weeks later. The immune response was measured by ELISPOT analysis using splenocytes from treated mice as responders, and mitomycin-treated splenocytes from naïve mice as stimulators, as follows: the ELISPOT plates (Millipore, Molsheim,
10 France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells
15 washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml HA 110-120 peptide or just with media, to assess the background.

Stimulator cells were prepared from naïve mice as follows: single cell suspension was prepared from spleens, red blood cells were lysed, cells were washed, resuspended in HL1 complete and mitomycin treated for 30 minutes. Afterwards, cells were washed 3
20 times, counted and resuspended in serum free HL1 media. The plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

The next day, the plates were washed five times with washing buffer and
25 incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver
30 Spring, MD).

The results are expressed in Figure 13 as number of IL-4-producing (A) or IFN-γ producing (B) T cell colonies / spleen (mean ± SEM of triplicates) when splenocytes

were restimulated with 10µg/ml of HA peptide or cell culture medium alone. Thus, this Example shows that FcγR-mediated delivery of T cell epitope within recombinant Ig backbone results in Th2 rather than Th1 response.

5 **Example 13 shows that the repeated loading of APC *in vivo* with a peptide delivered within IgG backbone results in induction of Th3 and Tr1 immunity.**

BALB/c mice were immunized with 40µg of heat aggregated (15 mins at 63°C) of recHA (I-Ed)-IgG ("IgHA") administered by intranasal instillation boosted 2 weeks later
10 by subcutaneous injection with 100ug of recombinant immunoglobulin in saline. As controls, mice primed with heat aggregated IgG2b isotype control were used. After an additional 2 weeks, the mice were sacrificed and T cell response assessed by *in vitro* restimulation of splenocytes with HA peptide by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-
15 cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete, containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed,
20 cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml HA 110-120 peptide or just with media, to assess the background. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

25 The next day, plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

30 The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The TGF-beta and IL-10 production were measured by ELISA TGF-β1 kit (R&D Systems, cat # DY240) and

IL-10 kit (Biosource international, cat#KMC0104). The results are expressed as cytokine concentration (average of triplicates) after subtraction of background.

The data, as shown in Figure 14, show that mucosal priming with epitope bearing recombinant immunoglobulin resulted in differentiation of Th3 and Tr1 cells that were expanded subsequently by systemic boosting.

Example 14 shows that only a virus, but not the conventional adjuvant CFA, was able to trigger significant Th1 response to a peptide epitope inserted within the IgG backbone.

BALB/c mice were immunized intraperitoneally with 100ug of recHA (I-Ed)-IgG in saline, emulsified in Complete Freund's Adjuvant ("CFA") or with 105 TCID₅₀ of influenza virus strain WSN, that bears the HA epitope. At 2 weeks after immunization, the mice (n=3/group) were sacrificed and the T cell response to HA peptide measured by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml HA 110-120 peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day, plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are

represented as mean $\pm$ SEM of frequency of cytokine producing colonies in the spleen. The results in Fig. 15 show that a peptide epitope within the IgG backbone triggers a cellular response of Th2 profile that is enhanced but not switched by a conventional adjuvant (CFA). In contrast, the profile afforded by live virus immunization was Th1
5 biased.

**Example 15 shows that the presentation of peptide epitope subsequent to IgG mediated delivery results in a T cell response that could be further manipulated by increasing co-stimulation with anti-CD40mAb, recombinant IL-12 or synthetic
10 dsRNA.**

Dendritic cells from naive BALB/c mice were harvested by MACS from splenic cell suspensions as follows: separation by using magnetic beads coupled with anti-CD11c was carried out using magnetic cell separators and reagents from Miltenyi Biotec,
15 Germany as follows: spleens were processed to single cell suspension, red blood cells lysed, the cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative
20 fraction runs through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1 complete media and were pulsed *ex vivo* in serum free HL-1 medium for 2 hours, at a concentration of 3 million / ml, with 50ug/ml of recHA(I-Ed)-IgG alone or supplemented with 5ng/ml of recIL-12, 50ug/ml of double stranded RNAs
25 (pA:pU or pI:pC). Alternatively, the cells were incubated with recombinant Ig and wells precoated with 10ug/ml of anti-CD40 mAb. The cells were harvested, washed and adoptively transferred to naive BALB/c mice (300,000 delivered half subcutaneously and half intraperitoneally) in serum free HL-1 medium.

At 2 weeks, the mice were sacrificed and T cell responses measured against HA
30 by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C

overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 50 µg /ml HA 110-120 peptide or just with media, to assess the background. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are shown as mean + SEM (n=3) of the frequency of spot forming colonies associated with IL-2 or IL-4 production, after subtraction of the background, for each *ex vivo* stimulatory combination.

The results in Fig. 16 show that peptide presentation by APC, subsequent to loading with antigen by using recombinant IgG as delivery platform, occurs in context of limited co-stimulation. IL-12, anti-CD40 or synthetic dsRNA can all enable APC loaded with antigen via FcγR, to prime IL-2 and enhanced IL-4 producing T cell immunity against the cognate (HA) peptide.

Example 16: The activity of the long-lived IL-4 producing Th2 cells triggered by *in vivo* loading of APC with IgG-peptide is dependent on the continuous interaction with endogenous APC and requires competent CD4.

BALB/c mice were immunized with 100 ug of rechA (I-Ed)-IgG or HA peptide subcutaneously, sacrificed at 2 weeks and the T cell response measured by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml anti-IL4, from BD Pharmingen) in sterile PBS

(50 µl/well) at 4⁰ C overnight. The next day, the plate was washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed,
5 cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml HA 110-120 peptide or just with media, to assess the background. The plate was incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plate was washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

10 The next day, the plate was washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plate was then allowed to dry at room temperature for 24 hours. The data were acquired using an automated system (Navitar,
15 Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD).

(A) During the HA stimulation phase, blocking anti-CD4 or anti-CD8 mAb was added at 10ug/ml in selected wells. The results are expressed in Fig. 17A as mean+SEM of number of HA-stimulated IL-4 producing colonies per spleen, after subtraction of background (n=3 mice / group).

20 (B) Splenocytes from mice immunized with recombinant Ig as above, were incubated in elispot plate as is or after magnetic depletion of endogenous MHC II+ APC with MHC II+ from naive BALB/c mice, with medium alone or in the presence of 10ug / ml of HA peptide. Separation by using magnetic beads coupled with anti-MHC II was carried out using magnetic cell separators and reagents from Miltenyi Biotec, Germany as
25 follows: spleens were processed to single cell suspension, red blood cells lysed, then cells washed, counted and resuspended in MACS buffer (PBS supplemented with 2 mM EDTA and 0.5% BSA). Magnetically labeled cells were passed through a separation column which is placed in the magnetic field of a MACS separator. The magnetically labeled positive fraction is retained in the column while the negative fraction runs
30 through. After removal of the column from the magnetic field, the magnetically retained positive cells are eluted from the column, cells are washed, counted, resuspended in HL1

complete media and were incubated in the ELISPOT assay, protocol to follow. The ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 50 µg /ml HA 110-120 peptide or just with media, to assess the background.

The plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

The next day, the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD) and the results expressed as mean ± SEM of the frequency of IL-4 producing T cells. The results in Figs. 17A - 17B show that the activity of HA specific IL-4 producing T cells triggered by administration of recHA(I-E_d)-IgG is dependent on CD4 rather CD8. In addition, the long lived IL-4 production by primed T cells depends on stable interaction with endogenous APC.

Example 17 shows that FcγR-mediated delivery of a T cell epitope is more effective than the peptide in differentially affecting the phenotype of activated, specific T cells: dose-dependent down regulation of IL-2, IFN-γ, and IL-4, with up-regulation of IL-10 and TGF-β.

Activated SFERFEIFPKE-specific T cells were separated from BALB/c mice immunized 2 weeks previously with 100µg peptide in CFA. They were incubated with mitomycin treated splenocytes in the presence of various amounts of recHA(I-Ed)-IgG or corresponding peptide. The expansion and cytokine production (IFN-γ, IL-4, IL-2) was
5 estimated by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour, at 37⁰ C.
10 Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml HA 110-120 peptide or just with media, to assess the background.

The plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100
15 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day, the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water.
20 The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). In addition, TGF-β and IL-10 production were measured by ELISA at 48 hours after incubation using TGF-β1 kit (R&D Systems, cat # DY240) and IL-10 kit (Biosource international,
25 cat#KMC0104).The results are expressed as frequency of spot forming cells (SFC) or concentration of cytokine versus amount of antigen added *in vitro*.

The results in Fig. 18 show that the IgG mediated delivery of a T cell epitope has a profound and differential effect on the expansion and cytokine production by activated T cells: IL-2, IFN-γ and surprisingly IL-4, were down-regulated in a dose-related manner.
30 The Ig-peptide was substantially more effective in modulating the cytokine production, as compared to the peptide itself. In contrast, only the Ig-peptide turned on effectively the

production of IL-10 and TGF-beta in a dose-dependent manner. Thus, the T cell epitope in context of Ig backbone, but not separately, differentially modulated the function of activated cells.

5 **Example 18 shows that surprisingly, a peptide delivered within the IgG backbone, that is not an immune complex nor is a receptor cross-linking antibody, results in induction of a class I restricted immune response. This response had a different profile from that triggered by live virus (Tc2 type consisting in IL-4 but not IFN- γ production).**

10 BALB/c mice were injected with 50 μ g of recNP(Kd)-IgG encompassing the MHC class I-restricted peptide TYTQTRALV (Seq. I.D. No. 6) by subcutaneous injection. The mice were sacrificed 2 weeks later and peptide-specific cytokine production was measured by ELISPOT analysis as follows: the ELISPOT plates
15 (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL2 and anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37⁰ C.

20 Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with various concentrations of NP peptide. The plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% -
25 FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

30 The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 19A as total number of spot forming colonies (SFC) / spleen (mean

of $n=3$). As controls, naïve mice or mice injected intraperitoneally with 10^5 TCID₅₀ of live WSN influenza virus were used.

The results in Fig. 19A - 19B show that in contrast to viral immunization with an influenza virus strain bearing the cognate peptide, Ig-mediated peptide delivery was ineffective in triggering IFN- γ producing Tc1 cells. However, Ig-peptide administration still resulted in formation of MHC class I-peptide complexes and induced significant NP-specific MHC class I-restricted T cell immunity consisting in IL-4 producing Tc2 cells.

Example 19 shows that *in vivo* loading of selected APC with disease associated epitopes suppressed an aggravated form of autoimmunity by expanding rather than ablating, epitope-specific autoreactive T.

SJL mice were injected subcutaneously with 200 μ l of rat brain homogenate emulsified in Complete Freund's Adjuvant and boosted with 50ng of pertussis toxin at 6 hours and 2 days. The mice developed an aggravated, progressive form of paralytic disease. Half of the mice received via subcutaneous injection a combination of recombinant immunoglobulins bearing the MBP and the PLP epitopes (recMBP(I-As)-IgG; recPLP(I-As)-IgG), respectively (150 μ g/molecule, on day 8, 12, 18 after induction of disease). In Fig. 20(a), the mean clinical score for treated and non-treated mice is represented, respectively ($n=8$).

After a period of observation of 70 days, the mice were sacrificed, spleens harvested and elispot analysis carried out as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 1×10^6 /well together with 20 μ g /ml of peptides (PLP or MBP) or just with media, to assess the background.

The plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%

(ELISPOT buffer) overnight at 4 °C. The next day, the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results (Figure 20 B) were expressed as frequency of IL-4 producing T cell colonies in the absence of added PLP peptide plotted against the frequency of IFN- γ -producing T cells in condition of peptide stimulation. Mice progressing to full-blown limb paralysis (score equal to or higher than 1.5) were represented with closed symbols. Mice that did not progress to limb paralysis were represented with open symbols. In Figure 20C, the total number of IL-4 spot forming colonies / spleen (mean $\pm$ SEM) in condition of *in vitro* stimulation was represented with nil, MBP or PLP peptide. An additional control, consisting of splenocytes from mice treated with IgG2b isotype control, has been included. In parallel, *in vitro* culture was carried out in the presence of neutralizing anti-IL-4 mAb (40 μ g/ml) and the number of IFN- γ -producing T cells was represented in the panel D.

The results in Figs. 20A - D show that co-administration of MBP and PLP epitopes by using recombinant IgG significantly curbed the chronic progression of disease. The mice protected from paralysis developed unexpectedly, an enhanced reactivity to self-epitopes MBP and PLP, manifested by increased basal and peptide-stimulated IL-4 or IFN- γ production, respectively. Finally, the reactivity of IFN- γ -producing T cells is kept in check by IL-4 suggesting a complex immunomodulatory mechanism triggered by IgG-mediated delivery of epitopes.

Example 20 summarizes the impact of IgG / Fc γ R-mediated delivery of epitopes on the T cell response, based on data provided in the Examples 1-19.

First, the loading of APC T cell response to IgG-mediated delivery of T cell epitopes is controlled by two functionally opposing receptors: ITIM and ITAM Fc (gamma⁺)-bearing receptors on APCs. ITIM⁺ Fc γ RIIB limits the degree of activation of T

cells and gamma⁺ FcRs are required for effective formation of MHC-peptide complexes when epitopes are delivered via the IgG backbone. Such *in vivo* delivery of epitope results in effective formation of MHC – peptide complexes on peripheral CD11c⁺ and CD11b⁺ APCs, but not thymic APCs. However, the interplay between ITIM⁺ and ITAM⁺ FcγRs makes the nature and magnitude of resulting T cell response difficult to predict without experimentation.

The data in Fig. 21 show that IgG-delivery of peptide epitope results in exposure of T cells to peptide-loaded APC in context of limited co-stimulation, having a differential effect on naïve versus activated T cells: 1) de novo induction of Th2, Tc2, Th3, Tr1 cells; and, 2) downregulation of activated Th1, Th2 cells with stimulation of activated Tr1 and Th3 cells. The overall effect is immunomodulatory, rather than pro-inflammatory (associated with Th1 and Tc1 immunity).

Example 21. Naturally occurring dsRNA bridges the innate with adaptive immune response. Example 21 shows that natural, non-infectious double stranded RNA produced during infection with influenza virus, has substantial effects on the specific immune response to a protein antigen.

Permissive MDCK cells were infected with WSN influenza virus (10^8 TCID₅₀ / 1×10^9 cells) and after 24 hours, the cells were harvested, washed and the total RNA extracted using an RNA separation kit (Qiagen, Valencia, CA). The RNA was further purified by treatment with RNase-free DNaseI (Stratagene, San Diego, CA). The single stranded RNA in the samples was then removed by 30 minutes incubation at 37°C with 5U of S1 nuclease (Ambion, Inc., Austin-TX) / μg of RNA. The RNA was analyzed prior to and subsequent to the digestion by gel electrophoresis. The absence of infectious properties of the purified dsRNA was confirmed by standard influenza virus titration. As a control, material purified and treated similarly, from 10^9 non-infected MDCK cells was used. The concentration of nucleic acid was measured by spectrophotometry (A_{260nm}) and the absence of endotoxin confirmed by Limulus assay. The purified dsRNA and control RNA were used individually, or as a mixture with gp140 recombinant antigen (25μg of RNA and 2 μg of antigen in 25ml of sterile PBS).

After demonstrating lack of infectivity, 40µg of dsRNA or control RNA were admixed with 40µg of recombinant truncated antigen (gp140 of HIV envelope) and were administered to BALB/c mice by intranasal instillation (n=3/group). Additional controls were animals immunized with 40µg of gp140 protein in saline (n=3 / group). The mice were boosted once, at 2 weeks after priming. Blood was harvested 2 weeks after the boost, sera prepared and the antibody response against gp140 measured by ELISA. In brief, wells were coated with antigen (2µg/ml of gp140) and blocked with SeaBlock (Pierce, Rockford-IL, catalog # 37527). Serial dilutions of serum and bronchoalveolar lavage fluid were incubated for at least 2 hours at room temperature. After washing, the assay was developed with anti-mouse IgG antibody coupled with alkaline phosphatase (Sigma, cat# A7434) followed by addition of substrate (pNPP, Sigma, cat# N2765) and measurement by using an automatic microtiter plate reader (Molecular Devices, ThermoMax) equipped with SoftMax software.

In Fig. 22A, the general principle of the experiment is illustrated. In Fig. 22B, the absorption after assay development is represented, corresponding to various serum dilutions, in case of whole IgG. In Fig. 22B, the absorption at 1/50 serum dilution, in case of IgG2a and IgG1 antibody isotypes, is represented.

Overall, the data in Figs. 22A - B show that natural, non-infectious dsRNA from influenza virus-infected MDCK cells, has an unexpected enhancing effect on the adaptive response to a prototype antigen. Both IgG1 and IgG2a antibody responses were increased showing that a strong T helper1 and T helper 2 response was induced.

Example 22. Effects of selected RNA motifs on the innate immune response: heterogeneous motifs. This Example shows, unexpectedly, that different synthetic RNA motifs have a distinct effect on the adaptive specific immune response to a protein antigen.

Figure 23A shows an extensive library of synthetic RNA motifs that were grouped in pools and used for a two-tier screening process as follows:

(A) The mice were immunized intratracheally with RNA pools, followed by 2 boosts two weeks apart, carried out by intranasal instillation. The antibody response measured (Fig. 23 B) by ELISA was expressed as mean $\pm$ SEM of IgG endpoint titers (n=4/group). As controls, dose-matched OVA in sterile PBS was used, OVA with cholera toxin

subunit B (CTB) and PBS alone, respectively. In brief, wells were coated with antigen (10µg/ml of OVA) and blocked with SeaBlock (Pierce, Rockford-IL, catalog # 37527). Serial dilutions of serum and bronchoalveolar lavage fluid were incubated for at least 2 hours at room temperature. After washing, the assay was developed with anti-mouse IgG antibody coupled with alkaline phosphatase (Sigma, cat# A7434) followed by addition of substrate (pNPP, Sigma, cat# N2765) and measurement by using an automatic microtiter plate reader (Molecular Devices, ThermoMax) equipped with SoftMax software.

(B) The effect of various dsRNA motifs on the induction of antibody response to OVA: the results are expressed as in Fig. 23 C. The data are representative for two independent experiments. INSET: the ratio between mean IgG2a and IgG1 titers to OVA. For this purpose, biotin-conjugated anti-mouse IgG1 and IgG2a antibodies were used followed by incubation with streptavidin-AKP conjugate. The order from left to right is similar as in the main panel in Fig. 23C: PBS OVA, CTB OVA, pC:pG OVA, pI:pC OVA and pA:pU OVA.

(C) The magnitude and profile of T cell response induced by OVA together with various dsRNA motifs, in female C57BL/6 mice. For the measurement of cellular response, splenic cell suspensions were obtained by passing the organ through 70 micron nylon Falcon strainers (Becton Dickinson, cat# 352350) followed by lysis of red blood cells with red blood cell lysis buffer (Sigma, cat# R7757). The lymphocytes from the pulmonary associated lymphoid tissue were isolated by collagenase (Sigma, cat# C9891) digestion of lung tissue followed by Ficoll-Paque (Amersham Pharmacia, cat# 17-1440-02) gradient centrifugation. The T cell response was measured by ELISPOT analysis as follows: 96-well 45 micron mixed cellulose ester plates (Millipore, cat#MAHA S4510) were coated with 4µg/ml of rat anti-mouse anti-IFN γ , IL-2 or IL-4 monoclonal antibodies (BD-PharMingen, cat#554430, cat#18161D, cat# 554387 respectively). After blocking with 10% FCS in sterile saline for 1 hour at 37°C, spleen cell suspensions were added at 5x10⁵ cells / well, with or without antigens / peptides. For stimulation, graded amounts of antigen (OVA) were used. At 72 hours after stimulation, the assay was developed with biotinylated rat anti-mouse cytokine antibodies (BD-PharMingen) followed by

streptavidin-HRP (BioSource Int., Camarillo, CA) and insoluble AEC substrate. The results were measured using an automatic imaging system (Navitar/Micromate) equipped with multiparametric-analysis software (Image Pro, Media Cybernetics). The results are expressed in Figure 23 D as mean $\pm$ SEM of the number of IFN- γ and IL-4 spot-forming-colonies (SFC) per spleen (n=4/group). The results are representative for two independent experiments.

The results in Figs. 23B - D show that different synthetic RNAs have an enhancing effect on the B and T cell response to a prototype protein antigen. In addition, different motifs, comprising specific nucleotide combinations, have specific effects in terms of T1 versus T2 induction and subsequently, immunoglobulin isotype switching.

Example 23. Use of selected synthetic RNA motifs facilitates the induction of MHC class I-restricted Tc1 cells, producing IFN- γ .

(A) Cross-priming stimulated by dsRNA motifs was studied in BALB/c mice treated (priming plus 2 boosts) with 10 μ g of recombinant-engineered HIV gp140 antigen together with pA:pU. The response was measured by ELISPOT analysis as described in Example 22, using *in vitro* stimulation with the MHC class I-restricted cognate peptide R10K derived from the V3 domain. As a control, dose-matched gp140 antigen was used. The results are expressed in Figure 24A as mean $\pm$ SEM of the number of IFN- γ and IL-4 SFC / spleen (n=4/group).

(B) Cross-priming stimulated by dsRNA motifs was studied in C57BL/6 mice treated with 100 μ g of whole OVA together with pA:pU by ELISPOT analysis as described in Example 22, using *in vitro* stimulation with the MHC class I-restricted peptide SIINFEKL (Seq. I.D. No. 43). As a control, dose-matched OVA antigen in saline or sterile PBS was used. The results are expressed in Figure 24B as mean $\pm$ SEM of the number of IFN- γ and IL-4 SFC / spleen (n=4/group).

The results in Figures 24A - B show that a selected synthetic RNA motif was able to promote increased T cell immunity to different MHC class I-restricted peptides encompassed within larger antigens (polypeptides). This immune response comprised a Tc1 component, consisting in IFN- γ -producing MHC class I-restricted T cells.

Example 24 shows that unexpectedly, different synthetic RNA motifs bind to different receptors; in other words, there are multiple receptors that discriminate among RNA motifs.

In vitro binding of CD11b⁺ APC by fluorescently-tagged pA:pU was measured by FACS analysis. The MACS-separated APC were incubated at 4°C for 30 minutes with 10µg/ml of tagged pA:pU ([pA:pU]-F), washed and analyzed. Alternatively, APC were preincubated for 10 minutes with 20 or 100µg/ml of non-tagged pA:pU, pA or pI:pC respectively, before staining with tagged pA:pU and FACS analysis. The profiles of stained (open area), non-stained (filled area) cells and the percentage of highly stained APC were represented in each panel, with logarithmic x axis. The data are representative of two independent measurements with 10,000 events acquired for each sample.

Materials:

1. Mouse CD11b, CD11c Magnetic Separation Beads: Miltenyi Biotec, cat#130-049-601, cat#130-052-001 respectively;
2. ULYSIS Nucleic Acid Labeling Kit: Alexa 488, Molecular Probes cat#U21650;
3. RNA Motifs:
 - pA:pU, (Sigma, Lot #22K4068);
 - pI:pC, (Sigma, Lot# 52K4047);
 - pA, (Sigma, Lot#22K4022);
4. FACS Buffer: PBS, 1% FCS, 0.1% sodium azide;
5. MACs buffer: PBS, 2mM EDTA, 0.5% BSA;
6. Collagenase Buffer: 0.225mg BSA, 0.0062mg collagenase in 50ml RPMI; and,
7. 70um cell strainer: (Falcon / Becton Dickinson, cat#352350).

Methods:

I. Labeling of RNA Motifs:

1. In the following protocol, each RNA motif was tagged with the ULYSIS Alexa 488 label.

II. Splenocyte preparation:

1. Isolate splenocytes and lung cells from 4 female C57 BL/6 mice;
 - Lung cells, in contrast to splenocytes, must be minced and incubated in collagenase buffer for 30 minutes at 37°C prior to the following step;
- 5 • Pass through 70µm falcon cell strainer;
- Wash and resuspend in MACS buffer:
2. Label with either CD11b or CD11c specific MACS beads following suggested protocol;
3. Cells were then treated with:
 - 10 • Non-tagged pA, pA:pU, or pI:pC (20 or 100µg/ml) for 10 minutes at room temperature;
 - ULYSIS tagged pA or pA:pU was added at 1.5µg/tube and 10µg/tube, respectively, to match dye:dsRNA ratio of each motif.
4. Mix and incubate 30 minutes on ice.
- 15 5. Wash once and resuspend in FACS buffer

III. Flow Cytometry:

Run flow cytometric analysis to determine / compare competitive inhibition of tagged versus non-tagged RNA motifs and cell receptor binding.

20

The results in Figure 25 show that pA:pU and pI:pC bind to different cellular receptors. Since pI:pC binds to TLR3, it results that additional receptors distinct from TLR3 are involved in RNA recognition immune function.

25 **Example 25 shows that selected synthetic RNA motifs trigger *in vivo* expression of chemokine genes, of importance for immunological activity.**

Local up-regulation of chemokine gene-expression by dsRNA motifs was measured by DNA array technique using RNA from the pulmonary tissue, extracted one
 30 day after the administration via the respiratory tract. Total RNA was isolated from lungs using an RNeasy kit (Qiagen, Valencia, CA). The RNAs were further purified by treatment with RNase-free DNase I (Stratagene, San Diego, CA). DNA array was

performed by using the Nonrad-GEArray kit from SuperArray Inc. (Bethesda, MD). Briefly, cDNA probes were synthesized using MMLV reverse transcriptase with dNTP mix containing biotin-16-dUTP. The GEArray membranes were prehybridized at 68°C for 1-2 hours. The hybridization was carried out by incubation of the membranes with biotin-labeled cDNA. The hybridized membranes were washed in 2xSSC - 1% SDS twice and 0.1xSSC - 0.5% SDS twice. The membranes were further incubated with alkaline phosphatase-conjugated streptavidin (BioSource Int., Camarillo, CA) and finally developed with CDP-Star chemiluminescent substrate. The intensity of signal was measured with Image-Pro analysis system equipped with Gel-Pro software (Media Cybernetics, Silver Springs, MD).

The results are expressed as fold-increase of gene expression, over expression levels measured in the pulmonary tissue of non-treated mice. The pattern of chemokine expression triggered by dsRNAs (50 µg of pA:pU and pI:pC, respectively) was compared to that induced by 1 µg of LPS. The chemokines that selectively bind to receptors on Th1 and Th2 cells were indicated with continuous and interrupted contours, respectively.

The results in Figure 26 show that pA:pU and pI:pC trigger expression of a wide range of chemokines and that the expression pattern is motif-dependent and different from that elicited by LPS (endotoxin).

Example 26 shows that selected synthetic RNA motifs mobilize an immune defense that is capable to control infection with a pulmonary virus.

dsRNA motifs display differential ability to mobilize immune defense against influenza virus infection. C3H/HeJ mice were treated via the respiratory route with 50 µg of pI:pC, pA:pU or 50 µl of saline one day before and after pulmonary infection with a sublethal dose of influenza virus. For virus challenge, C57BL/6 and TLR4-/- C₃H/HeJ mice under Metofane anesthesia were infected with sublethal doses (10⁴ tissue culture infective doses 50% - TCID₅₀) of live WSN virus, via the nasal route. On day 5 after infection, the mice were sacrificed, lungs retrieved, homogenized and stored at -70°C.

The virus titers were measured by 48-hour incubation of serial dilutions of samples with permissive MDCK cells, followed by standard hemagglutination with chicken red blood cells (From Animal Technologies). The endpoint titers were estimated in triplicate

measurements by interpolation and expressed as TCID₅₀ / organ (means $\pm$ SEM; n=6/group; results are representative of two independent studies in C₃H/HeJ TLR-4^{-/-} and competent mice). Similar results were obtained in TLR4 competent, C57BL/6 mice.

Thus, the results depicted in Figure 27 show that the control of replication of influenza virus can be achieved by using selected synthetic RNA motifs (dsRNA1 is pA:pU and dsRNA2 is pI:pC).

Example 27 shows that co-administration of selected synthetic RNA motifs breaks tolerance to high dose standard antigen.

dsRNA motifs prevent high-zone tolerance in mice injected with human IgG. The mice (C57BL/6) were initially injected intravenously with a toleragenic dose of 200 μ g of hIgG alone (closed symbols) or together with 100 μ g of pI:pC or pA:pU (open symbols) and subsequently boosted subcutaneously with an immunogenic dose of 100 μ g of hIgG emulsified in CFA. The titer of antibodies against hIgG was measured by ELISA (as detailed in Example 23, with the difference consisting in use of 10 μ g/ml of hIgG for coating) at various intervals after the first injection. As a control, mice immunized with 100 μ g of hIgG emulsified in CFA were included and represented the maximal titer on the graph (interrupted line).

The results are represented in Figure 28 as means $\pm$ SEM of endpoint titers (n=5/group). Similar results were obtained in TLR4 deficient (C3H/HeJ) and LPS-responsive C3H/SnJ mice. Thus, the results in Figure 28 show that selected synthetic RNA motifs pI:pC and pA:pU largely prevent high zone tolerance that is usually associated with administration of large amounts of purified protein.

Example 28 shows that selected RNA motifs induce differential cytokine production by human APC.

Human THP-1 monocytic cells, following differentiation, were incubated with different concentrations of synthetic RNA (pA:pU, pI:pC or pA) for 24 hours, and the cell supernatants collected. The concentration of IL-12 and TNF- α were measured by

ELISA. The results are expressed in Figure 29 as pg/ml (concentration) for each cytokine and culture condition.

Materials:

- 5 1. THP-1 Human monocytic cell line: ATCC, cat # TIB-202;
2. IL-12 Cytokine: Human ELISA, IL-12 ultra sensitive (US) cat# KHC0123;
3. TNF alpha Cytokine: Human ELISA, TNF alpha cat# KHC3012;
4. RNA Motifs:
 - pA:pU, (Sigma, Lot #22K4068);
 - 10 • pI:pC, (Sigma, Lot # 52K4047); and,
 - pA, (Sigma, Lot #22K4022).

Method:

1. The THP-1 cells were allowed to differentiate following addition of 10ng/ml PMA in
15 media containing 10% FCS.
2. After gently washing cells and adding non-FCS containing media (HL-1), treatments (RNA motifs and controls) were added at concentrations of from 3 to 100 μ g/ml on top of adherent THP-1 cells.
3. After 24 hours incubation, cell supernatants were harvested and IL-12 and TNF alpha
20 concentrations were measured by ELISA.

The results in Fig. 29 show selected synthetic RNA motifs effect on human monocytic cells; in addition, this effect is heterogeneous, depending on the chemical structure of the motifs (nucleotide composition). Selected but not all synthetic RNA
25 motifs are able to trigger IL-12 production, an important T1 regulatory cytokine, by human monocytic cells.

Example 29 shows that two distinct synthetic RNA motifs bind to human THP-1 monocytic cells in a manner demonstrating interaction with different receptors.

30

THP-1 cells were incubated at for 15 minutes at room temperature with different amounts of non-labeled synthetic RNA. Subsequently, tagged pA:pU was added for 30 minutes at 4⁰C, cells washed and the fluorescence quantified by FACS analysis. The results are expressed in Figs. 30A - 30B as histograms corresponding to the large cell subset (A) and total cell population (B). Percentages of stained cells were represented on each Figure.

Materials:

1. ULYSIS: Nucleic acid fluorescent label (Molecular Probes, cat# U-21650).
2. RNA Motifs:
 - pA:pU, (Sigma, Lot #22K4068);
 - pI:pC, (Sigma, Lot # 52K4047);
3. Detoxi-Gel column: (Pierce, cat#20344).

Method:

Labeling of Polyadenylic-Polyuridylic Acid (pA:pU):

1. Following removal of endotoxin using a Detoxi-Gel column, pA:pU was labeled with the Alexa Fluor 488 fluorescent dye using the ULYSIS nucleic acid labeling system.
2. Briefly:
 - The pA:pU was precipitated using sodium acetate and ethanol at 70°C;
 - The pA:pU was heat denatured and labeled with the Alexa Fluor 488 reagent at 90°C; and,
 - The reaction was stopped and the labeled pA:pU was ethanol precipitated.

Cell treatment:

1. THP-1 cells were suspended at 2X10⁶ cells /ml;
2. 500l of above suspension (5X10⁴ cells) were placed in 12X75 mm tubes;
3. Non-tagged pA:pU or pI:pC were added to the THP-1 cells at a concentration of either 20 or 1000g/ml and incubated 15 minutes;

ULYSIS labeled pA:pU was added at a concentration of 100 ug/ml for 30 minutes on ice.

4. The THP-1 cells were washed once and suspended in FACS buffer followed by flowcytometric analysis to determine relative fluorescent differences between different treatment populations.

The results in Figures 30A - 30B show that non-tagged pA:pU but not non-tagged pI:pC was able to compete out the binding of tagged pA:pU to human THP-1 monocytic cells, both at the level of large cell subset and whole population.

Example 30 shows how the adjuvant synthetic RNA should be prepared and purified prior to use in its most effective format.

The bulk synthetic RNA material is obtained by standard methods of organic synthesis. Afterwards, the material is dissolved in sterile endotoxin-free saline, passed through endotoxin removal columns until the concentration of LPS is below 0.005EU/ μ g. The measurement of LPS is carried out by standard Limulus assay. Subsequently, the material is fractionated by a series of centrifugation steps through filters of defined porosity (see Fig. 31).

A useful fraction comprises synthetic RNA of less than 20 to maximum 100bp size, however, larger RNA fragments may be used. After purification, the material is measured and validated on standard assays: spectrophotometry (OD260nm); gel electrophoresis; endotoxin quantitation by Limulus assay; bioactivity on human THP-1 cells (as in Example 28).

Example 31 shows that unexpectedly, different fractions of a selected synthetic RNA compound are endowed with different biological activity, based on size.

Differentiated human THP-1 monocytic cells were incubated with different concentrations of synthetic RNA (pA:pU, fractionated as described in the Example 30) for 24 hours, and the supernatants collected. The concentration of TNF- α was measured by ELISA using BioSource International kits (Camarillo, CA). The results are expressed in Figure 32 as pg/ml (concentration) for each culture condition.

The results depicted in Fig. 32 show that lower molecular weight fractions of a selected synthetic RNA compound are endowed with higher biological activity, in terms of cytokine production, by human monocytic THP-1 cells.

5 **Example 32. Selected synthetic RNA motifs have, unexpectedly, a different immune profile in regard to generation of anti-RNA antibodies.**

BALB/c mice were immunized intraperitoneally and subcutaneously with 50 μ g + 50 μ g of hIgG and synthetic RNA (pI:pC or pA:pU) and serum samples were prepared 1
10 week later. As a control, mice injected with hIgG in saline were used. The anti-hIgG, and dsRNA IgG antibody titers against pA:pU, pI:pC, pA and hIgG were measured by ELISA. In brief, wells were coated with antigen (10 μ g/ml of hIgG or synthetic RNAs) and blocked with SeaBlock (Pierce, Rockford, IL, catalog # 37527). Serial dilutions of serum and bronchoalveolar lavage fluid were incubated for at least 2 hours at room
15 temperature. After washing, the assay was developed with anti-mouse IgG antibody coupled with alkaline phosphatase (Sigma, cat# A7434) followed by addition of substrate (pNPP, Sigma, cat# N2765) and measurement by using an automatic microtiter plate reader (Molecular Devices, ThermoMax) equipped with SoftMax software.

The results are expressed in Figure 33 as mean $\pm$ SEM of endpoint titers (n=3 /
20 group). The results in Fig. 33 show that pI:pC but not pA:pU induced antibody response against itself, with a cross-reactive component against another RNA motif.

25 **Example 33. *In vivo* loading of APC by recombinant IgG results in generation of Tc1 type of MHC class I responses only when additional conditions are satisfied.**

BALB/c mice were immunized with 50ug of recIgG-NP(Kd) subcutaneously, admixed with 50ug of selected synthetic RNA (pA:pU or pI:pC). As a control, naive mice or mice immunized with recombinant IgG only were used. At 3 weeks after immunization, the T cell response was measured by ELISPOT analysis as follows: the
30 ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C overnight. The next day, the plates were

washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with NP 147-155 peptide or just with media, to assess the background. Plates were
5 incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C.

The next day, the plates were washed five times with washing buffer and
10 incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with
15 ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The frequency of cytokine producing T cells reacting to NP peptide was measured and expressed against the amount of peptide used for stimulation. The results are expressed as means + SEM of triplicates (n=3 mice / group).

As shown previously in Figure 19, the administration of recombinant IgG bearing
20 the NP MHC class I-restricted epitope resulted in generation of Tc2 immunity but not Tc1 response, implying *in vivo* formation of class I-peptide complexes with a specific co-stimulation profile. The results in Figures 34A and 34B show that co-use of selected synthetic RNAs promoted effective induction of IL-2 and IFN-gamma subsequent to IgG mediated delivery of an MHC class I-restricted epitope (dsRNA₁ is pA:pU and dsRNA₂
25 is pI:pC).

Example 34: Effective formation of MHC class I-peptides and instruction of the resulting T cell response by simultaneous manipulation of APC loading via FcγR and activation via RNA receptors.

30 Splenic APC were isolated from naive BALBc mice and pulsed *ex vivo* overnight with 1 µg NP peptide, or 50 µg recIgG-NP (Kd) with or without 50 µg/ml selected

synthetic dsRNA (pA: pU). The cells were washed and 5×10^6 cells were administered by subcutaneous and intraperitoneal injection equal amount, to naive BALB/c mice. The response was measured 3 weeks later by ELISPOT analysis as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4 μ g/ml for anti-IL4, and 8 μ g/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 μ l/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200 μ l/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5×10^5 /well together with 30 μ g/ml, 10 μ g/ml, or 3 μ g /ml NP peptide or just with media, to assess the background. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 μ l /well of biotinylated anti-cytokine Abs, 2 μ g /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 35 as frequency of cytokine producing spot forming colonies against the concentration of peptide used for *ex vivo* stimulation (mean $\pm$ SEM, n=3 mice /group). In addition, the mean area /colony versus the concentration of peptide used for stimulation is plotted, for both IFN-gamma and IL-4 (arbitrary units).

The results in Fig 35 show that *ex vivo* APC loading by recombinant IgG is significantly more effective in formation of MHC class I-peptide complexes and generation of Tc response, compared to use of peptide itself. In addition, the mere formation of MHC class I-peptide complexes subsequent to epitope delivery via IgG / FcgammaR results in differentiation of Tc2 cells producing IL-4 but not IFN-gamma. Simultaneous treatment of APC with selected synthetic RNA results in broadening of the T cell profile, to IFN-gamma producing Tc1 cells.

Example 35 shows that co-priming with IgG-peptide together with a selected co-stimulatory motif resulted in more effective secondary expansion of MHC class I-restricted T cells subsequent of virus infection.

BALB/c mice were injected with recIgG-NP(Kd), pA:pU separately, or in combination (50 ug / injection). As a control, naive mice were used. Three weeks after treatment, the mice were infected with 104 TCID₅₀ of A/WSN/32 H1N1 influenza virus, via the respiratory tract. Four days after infection, the T cell profile in the spleen was measured by ELISPOT analysis subsequent to *ex vivo* stimulation with NP peptide as follows: the ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C. Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with 20 µg /ml NP peptide or just with media, to assess the background.

Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1% (ELISPOT buffer) overnight at 4⁰ C. The next day the plates were washed five times with washing buffer and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results are expressed in Figure 36 as frequency of NP-specific MHC class I-restricted T cells forming cytokine producing colonies (means ± SEM, n=4 mice / group).

The results in Fig. 36 show that IgG mediated delivery of a class I restricted epitope is most effective in priming class I restricted Tc1 responses when co-

administration of selected synthetic RNA was carried out. Such primed precursors were rapidly expanded subsequent to infection with influenza virus.

Example 36 shows that the most effective priming of cytotoxic lymphocytes recognizing an MHC class I-restricted epitope occurs by co-administration of selected RNA motif together with peptide epitope inserted within the IgG backbone.

BALBc mice were immunized and challenged with recIgG-NP (Kd) as in the previous Example and sacrificed 4 days after influenza virus infection. The splenocytes were prepared, suspended in HL-1 medium at 5 million / ml and co-incubated for 5 days with 10µg/ml of NP 147-155 peptide and in presence of 5U/ml of recombinant IL-2. Splenocytes from 4 mice / group were pooled and incubated in flasks.

After expansion, viable cells were recovered by Ficoll gradient centrifugation, washed and incubated for 5 hours in V-bottom plates, in various numbers, with a fixed number of sp20 target cells with or without NP peptide (20µg/ml). The supernatants were harvested after plate centrifugation, and the concentration of LDH measured by using a Promega kit (cat # G1780). The results are expressed as percent specific lysis at different E: T ratios (Effector to Target ratio).

The results in Fig. 37 show that effective priming of anti-viral cytotoxic T cells requires both effective *in vivo* loading of APC with class I restricted epitope delivered via IgG, together with appropriate instruction by selected synthetic RNA motif, namely pA:pU.

Example 37 shows that vaccination with an IgG bearing a viral MHC class I-restricted epitope, together with selected synthetic RNA motif, provided protection against infectious challenge with a prototype virus.

BALB/c mice were immunized with 50ug of recIgG-NP (Kd) together with 50ug of selected synthetic RNA (pA: pU), by subcutaneous injection. Three weeks after immunization, the mice were challenged with 10⁴ TCID 50 of infectious WSN influenza virus and sacrificed 5 days later. The pulmonary virus was titrated in lung homogenates by standard MDCK hemagglutination assay as follows: on day one MDCK cells were plated in 96 well plates at 2x10⁴ / well/ 200ul and incubated for 24 hours at 37°C, 5%

CO₂. The next day, 25 µl of the 10 fold dilutions in DMEM media of the lung homogenates were incubated in briefly trypsinized MDCK plates (1 minute) in triplicates and incubated at 37⁰ C. After one hour, 175 ul of the DMEM complete media was added and plates were incubated for 48 hours at 37⁰ C, 5% CO₂. After two days, the
5 hemagglutination-inhibition was done with chicken red blood cells incubated with the cell culture supernatants from the MDCK plate for 30 minutes at room temperature and the results were expressed as means ± SEM of total pulmonary virus (n=4 mice / group). As a control, non-immunized mice were used.

The results in Fig. 38 show that immunization with a recombinant IgG bearing a
10 viral class I restricted epitope together with selected synthetic dsRNA (pA:pU) resulted in priming of an immune response capable to limit the replication of a virus subsequent to infectious challenge.

Example 38. Figure 39 describes the tumor models used for testing the efficiency of a Ig-peptide-based molecules.
15

Balb-c mice (K^d restricted) have been used to establish a tumor model. Tumor cells (1 to 15 million in 100 µL) were typically injected in the flank to the mouse (see arrow in upper photo in Figure 39). Primary tumors (i.e. those at the sight of injection)
20 were first detected by palpating the area and then quantitated by measuring the tumor size with a caliper (see Figure 39). In one series of experiments, the mouse myeloma cell line (SP2/0), either untransfected cells or cells stable transfected expressing heterologous protein (recombinant IgG expressing different epitope peptides in the CDR3 region of the heavy chain or the complete NP protein), was used to induce tumors in the mice.
25 Expression of heterologous proteins in the SP2/0 cells provided specific tumor associated antigens (TAA) for testing various anti-tumor strategies in the immunocompetent mice. Typically, untreated mice developed palpable solid primary tumors 1 week post injection that led to morbidity and death over the next 4 weeks. Postmortem examination of the injected mice revealed metastatic lesions (see Figure 39). Sp2/0 cells were cultured from
30 primary tumor tissue as well as spleen taken from tumor-bearing mice (data not shown). SP2/0 cells were stably transfected with a recombinant IgG-expressing plasmids that were all identical except for the specific epitope sequence introduced into the CDR3

region of the heavy chain, for example, the MHC I restricted NP epitope (amino acids 147-155, see Figure 39). SP2/0 cells were also stably transfected with a plasmid containing the coding sequence for the entire NP protein of WSN virus under control of the CMV promoter. All transfected cell lines produced primary tumors over the same
5 frame as wild type SP2/0 cells.

This tumor model was extended to include an adenocarcinoma cell line (4T1, ATCC CRL-2539, K^d restricted), previously shown to induce metastatic tumors in Balb-c mice. The 4T-1 cell line was similar to that described above for the SP/0 line. Injection of 1 to 15 million 4T-1 cells into the flank of Balb-c mice produced a palpable primary
10 tumor over a time frame similar to injections of SP2/0 cells eventually leading to death. Postmortem collection of tissue from various organs showed that 4T-1 could be recovered from spleen, lungs as well as the primary tumor (not shown). 4T-1 cells were stably transfected with a NP-expressing plasmid described above. As with SP2/0 cells, transfection of the 4T-1 cell did not affect the course of tumor growth and lethality of
15 disease.

Example 39 demonstrates successful control and treatment of a tumor after clinical diagnosis, by using a tumor associate T cell epitope within a recombinant IgG together with a selected co-stimulatory RNA motif.

Balb/c mice were injected with SP2/0 cells (15 million in 100 μ L) stably expressing recombinant IgG carrying the MHC I (Kd) NP epitope peptide in the CDR3 region of the heavy chain (IgNP). At day 7 post injection all mice had palpable tumors and the mice were randomized into 3 groups: co-stimulatory motif (i.e. dsRNA
25 comprised of polymeric pApU) alone; purified IgTAA protein (IgNP); and both dsRNA pA:pU and purified IgTAA protein. The time of treatment is indicated by the arrows in Figure 40, and each injection contained 50 μ g of the indicated compound. The mice that developed metastatic disease and died are represented with a "D" in the figure.

The data show that the combination of dsRNA (co-stimulatory motif) and IgTAA
30 (IgNP) produced a dramatic protective response in mice that all had primary tumors at the start of therapy. While all mice treated with either the dsRNA or IgTAA compound alone succumbed to disease, 100% of the mice treated with both were still alive 3 weeks after

initiation of treatment and were in good clinical condition at the time of sacrifice for measurement of T cell response. These data show that *in vivo* loading of APC with TAA (accomplished by uptake of IgNP via the Fc receptor of APC) is not sufficient for a potent anti-tumor response. The tumor rejection and survival displayed by mice treated with IgNP in combination with pApU dsRNA highlights the important role co-stimulation plays in treatment of tumors with tumor-associated antigens.

In conclusion, the results in Figure 40 show that both effective *in vivo* loading of APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA motifs, are necessary and sufficient for effective control of tumor growth and induction of tumor rejection.

Example 40. This Example, in context of sublethal inoculation of tumor cells, shows that the suboptimal response to tumor antigens could be corrected by therapy with peptide epitope within an IgG backbone, together with co-stimulatory motif.

Balb/c mice were injected with SP2/0 cells stably expressing recombinant IgG (IgNP) that contains the MHC I (K^d) epitope (amino acids 147-155) of WSN virus nucleoprotein in the CDR3 of the heavy chain. The cell inoculum was 1 million cells (in 100 μ L) per mouse. The mice were observed until such time as palpable tumors were detected at the site of injection. At this point the tumors were measured and 8 mice were left untreated (control) while 6 were injected intratumorally with purified IgTAA (i.e. purified IgNP, 2 mg/kg) and dsRNA (pApU, 4 mg/kg) weekly. Weekly measurements of the tumors were taken.

Panel A of Figure 41 shows that in 6 of 8 of the control mice the induced tumor was progressive and ultimately lethal whereas 2 of the mice completely rejected the tumor spontaneously. Panel B of Figure 41 shows that the 3 weekly treatments with IgNP/dsRNA (indicated by the arrows) stimulated complete tumor rejection in 4 of the 6 mice and significant remission in another.

The results in Figure 41 shows that both effective *in vivo* loading of APC with tumor associated antigen, together with simultaneous activation by selected synthetic RNA, can trigger an effective immune response to tumor-associated antigens.

Example 41 shows that therapy of tumor-bearing mice with a tumor epitope within an IgG backbone together with co-stimulatory synthetic dsRNA results in the restoration of the activatory status of tumor infiltrating lymphocytes.

5 Two BALB/c mice were injected with 10 million sp20 transfectoma expressing the NP-K^d epitope. After tumors developed, one mouse was injected intratumorally with 50 μ g of selected dsRNA motif (pApU) plus 50 μ g of "IgNP" - recIgG-NP(K^d) in saline. The mice were sacrificed 24 hours later, tumors excised, digested with collagenase, filtered through 70 μ m filter and viable cells isolated on Ficoll gradient. Cells
10 were stained with mAbs against TCR α , CD25 or isotype control and assessed by FACS analysis. The results were expressed as histograms, with percentage stained cells indicated.

Materials:

- 15 1. SP20 cell line (ATCC);
- 2 BALB/c mice (Harland Sprague Dawley);
2. Falcon 70 micron filter(Becton Dickinson, cat# 352350);
3. Collagenase (Sigma, cat# C-9891);
4. BSA, fraction V (Sigma, cat# A-4503);
- 20 5. Collagenase buffer: 0.225gm BSA + 0.00625gm in 50 ml RPMI;
6. Ficoll-hypaque (1.077, Amersham, cat# 17-1440-02);
7. FACS Buffer:1% fetal calf serum + 0.1% azide in PBS;
8. Antibodies: All from BD Pharmingen; and,
9. Flow Cytometer: FACSCalibur (Becton Dickinson).

25

Method: Tumor cell isolation and FACS analysis:

1. Tumor was induced as stated above 6 weeks prior;
2. Tumor was isolated from BALB/c mouse;
3. Tumor was minced with sterile scissors and 10ml of collagenase buffer added;
- 30 4. Incubate 40 minutes, 37°C;
5. Force tumor through a 70 μ m Falcon filter with a 3ml syringe plunger into a 50ml tube while washing with RPMI;

6. Wash 1X and resuspend in 4 mls warm RPMI buffer;
7. With equal volume of cell suspension layered over Ficoll, centrifuge at RT, 2000 RPM, for 15 minutes;
8. Isolate layer and wash once in HL-1 buffer and resuspend in FACS buffer to
- 5 2X10⁶/ml and run flow cytometry analysis;
9. Remaining cells were used for ELISPOT analysis;
10. Cells were placed in 12X75mm tubes, 500 μ l/tube and stained with FITC labeled anti-mouse antibody, 200 μ g/ tube plus 100 μ l/tube mouse serum:
 - Isotypic Control;
 - 10 • Anti -CD40;
 - Anti -CD8;
 - Anti -CD4;
 - Anti -CD25;
 - Anti -TCR gamma delta;
 - 15 • Anti -TCR Beta;
11. Incubate 30 minutes on ice; and,
12. Wash once with FACS buffer and resuspend in 300 μ l FACS buffer.

The results in Figure 42 show that tumor infiltrating lymphocytes displaying the T cell receptor marker TCR β acquired expression of the activation marker CD25 upon treatment with recombinant immunoglobulin bearing tumor associated epitope, together with selected synthetic dsRNA motif.

Example 42 shows that successful therapy of tumor bearing mice with a peptide epitope within the IgG backbone together with a selected co-stimulatory molecule is associated with a specific differentiation pattern of Tc, comprising Tc1 in addition to Tc2.

Mice that successfully rejected the tumor following treatment with recombinant Ig carrying a tumor associated epitope together with selected synthetic dsRNA motif as explained in Example 40, were sacrificed and the T cell response against tumor associated epitope measured by ELISPOT analysis. The ELISPOT plates (Millipore,

Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with various concentrations of NP peptide. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, plates were washed 5 times with PBS-tween20 0.05% (washing buffer), and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS 0.1%(ELISPOT buffer) overnight at 4⁰ C. The next day the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water. Plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results were expressed as number (mean ± SEM) of spot forming colonies corresponding to IL-4, IL-2 and IFN-γ. As a control, non-treated mice were used, which failed to reject tumor (n=4/group).

The results in Fig. 43 show that the treated mice that successfully rejected the tumor, developed Tc1 responses against the tumor associated epitope on the therapeutic Ig, along with Tc2 immunity. In contrast, the mice that failed to reject the tumor developed only Tc2 immunity.

Example 43 shows induction of effective memory response subsequent to specific treatment of tumor bearing mice with a T cell epitope within the IgG backbone, together with a selected co-stimulatory motif.

Mice bearing sp2/0 tumors expressing the NP-K^d TAA were treated as described in the Example 40, by injection with recombinant Ig bearing TAA together with selected synthetic RNA motifs. After tumor rejection, the mice were challenged by subcutaneous

injection administered contralaterally, with 15 million SP2/0 cells expressing NP-Kd epitope. In parallel, 4 control naïve mice were similarly injected with a tumorigenic / lethal dose of same type of cells. The development and size of the tumors was monitored and represented as diameter (mm) versus time since challenge.

5 The results in Figure 44 show that successful rejection of the tumor induced by indicated treatment is followed by effective protection against subsequent challenge with the same tumor, indicating development of effective immune memory.

10 **Example 44 shows that surprisingly, the induction of tumor rejection by an IgG bearing a TAA together with a costimulator dsRNA motif, results in cross-protection against a range of tumor cell variants lacking the TAA or displaying variants of TAA.**

15 The mice protected against homologous challenge as described in Example 43, were subjected to sequential challenge with 15 million tumor cells representing the same tumor cells devoid of TAA (loss of antigen mutants) or bearing variants of TAA lacking the NP-K^d epitope. In addition, mice were challenged with a different type of tumor cell line (4T-1 adenocarcinoma) as a control, displayed in the table attached to Fig. 45A. In every case, naïve controls were included.

20 The status of T cell immunity of mice protected against multiple challenges with tumor variants, has been assessed by ELISPOT analysis using splenic cell suspensions stimulated with TAA (NP-Kd peptide), HA (MHC class II-restricted peptide), or protein extracts from cell lysates. The ELISPOT plates (Millipore, Molsheim, France) were incubated with purified anti-cytokine Abs (4ug/ml for anti-IL2 and anti-IL4, and 8 µg/ml
25 for anti-IFN gamma, from BD Pharmingen) in sterile PBS (50 µl/well) at 4⁰ C overnight. The next day, the plates were washed 2 times with DMEM media and blocked with 200µl/well of DMEM complete containing FBS, for an hour at 37⁰ C.

30 Single cell suspension was made from the spleens, red blood cells were lysed, cells washed, counted and incubated at 5x 10⁵ /well together with various concentrations of antigen. Plates were incubated 72 hours at 37⁰ C, 5% CO₂. After 3 days, the plates were washed 5 times with PBS-tween20 0.05% (washing buffer) and incubated with 100 µl /well of biotinylated anti-cytokine Abs, 2 µg /ml in PBS- tween20 0.05% - FBS

0.1%(ELISPOT buffer) overnight at 4 °C. The next day the plates were washed five times with washing buffer, and incubated for an hour with 1:1000 Streptavidin-HRP diluted in ELISPOT buffer. The reaction was developed with 3-amino-9-ethylcarbazole substrate (Sigma, St. Luis, MO) and stopped by washing the plate twice with tap water.

5 The plates were then allowed to dry at room temperature for 24 hours.

The data were acquired using an automated system (Navitar, Rochester, NY) with ImagePro-Plus) software (Media Cybernetics, Silver Spring, MD). The results were expressed as number (mean $\pm$ SEM) of spot forming colonies corresponding to IL-4, IL-2 and IFN- γ . As a control, non-treated mice that failed to reject tumor (n=4/group) were
10 used. As a control, naïve mice were included. The data are expressed as number (mean $\pm$ SEM) of cytokine producing cells / organ (n=3/group).

The results in Fig. 45A - 45B (including the table in Fig. 45 A) show that the emerging immunity, subsequent to the indicated treatment that results in tumor rejection, protects against challenge with loss of antigen variants and is associated with overall
15 expansion of cytokine producing cells. This indicates a broadening of the repertoire of anti-tumor lymphocytes, promoted by the proposed regimen, to tumor associated antigens that are not borne by the immunotherapeutic molecule.

Autoimmune Disorders

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Example 45. Use of the HLA-DR4 Transgenic mouse in T cell activation assays

The basic concept underlying the use of engineered IgGIN in autoimmune diabetes is the ability of IgGIN to bind to and be processed by APCs such that the incorporated epitopes are liberated and bound to their respective MHC II molecules.
25 Thus, it is essential to show that delivery of IgGIN to APC results in the activation of relevant T cells. The following set of experiments will address this issue.

The HLA-DR4 mouse strain (Taconic Farms Inc., Germantown, NY) was developed as means for studying HLA-DR-associated autoimmune diseases. The HLA-DR4 mouse strain is negative for the endogenous mouse MHC II and contains a mouse
30 human MHC II chimera (HLA-DRA-IE alpha and an HLA-DRB1*0401 beta). Further, the beta chain itself is chimeric in that the antigen binding domain is from DRB1*0401

while the remaining domains are from mouse ID(d)-beta chain. The GAD271-286 epitope has been shown to bind to the DR4 MHC II.

The following protocol can be used to determine if APC from the HLA-DR4 mice when challenged with human IgGIN (made according to the procedures of making a human immunoglobulin as described on pages 32 – 38):

1) Isolation of APCs. Spleens from naïve HLA-DR4 mice are harvested and processed into a single cell suspension. Briefly, spleens are minced, treated with collagenase and the red blood cells lysed in hypotonic buffer. The remaining cells are washed in HL-1 media and held for isolation of APC. A Miltenyi magnetic bead protocol is used to isolate APC. Specifically the cells are subjected to a selection of a CD11c⁺ population. The resulting cell population, primarily dendritic cells, are used as the stimulator cells in the T cell activation assay.

2) Responder cell fraction: HLA-DR4 mice will be immunized with either vehicle (PBS), IgGIN or hIgG1 (100 µg per mouse subcutaneously injected) or a molar equivalent of the GAD 271-285 epitope peptide (2 µg). Four mice per group will be immunized. Two weeks later the mice will be sacrificed. Spleens will be harvested and a single cell suspension will be prepared in the other Examples.

3) T cell activation assay. 5×10^5 cells of both stimulator and responder fractions are incubated with either media alone or media supplemented with 20 µg/mL GAD271-285 peptide. The cultures are then incubated for 72 hours. Media is then collected from the cultures and cytokine levels measured by ELISA. The cytokines tested are IFN- α , IL-4 and IL-2. Data is expressed as the mean of 4 mice $\pm$ SD. Negative controls will be minus either responder or stimulator cell fractions with and without added peptide.

This assay detects the presence of T cell subsets that specifically respond to GAD271-285. Enhanced GAD271-285-dependent cytokine secretion by responder cells derived from human IgGIN immunized mice suggested that immunization of the HLA-DR4 mice with human IgGIN result in the liberation of the incorporated epitope and presentation to T cells *in vivo*. A negative result in these experiments could have a number of causes. For one, IgGIN is a human Ig so there is expected to be an overall immune response to the backbone protein. This may obscure or interfere with the more specific response to the GAD271-285 epitope. Secondly, the processing of the human

IgGIN by mouse APC may not precisely liberate the incorporated epitope such that it can bind effectively to DR4. All work to date with Ig chimeras has examined mouse various mouse constructs processed by mouse APC.

5 **Example 46. The following experiment addresses the issue of intracellular processing of IgGIN as it relates to T cell activation.**

Spleens from the HLA DR4 transgenic mouse strain are harvested and a CD11c⁺-enriched population is isolated and is used as naïve APCs. The responder cell population is isolated from DR4 mouse spleens that are immunized 2 weeks prior with either PBS,
10 hIgG1, GAD₂₇₁₋₂₈₅ peptide or IgGIN. For the activation assay, 5×10^5 of both the APC population and the responder fraction are mixed in tissue culture wells. The wells are supplemented with GAD₂₇₁₋₂₈₅ peptide as the T cell activator. The cultures are incubated for 72 hours and the media collected. ELISA is performed on the samples for the specific cytokines shown in Figures 46A - 46B. The data, as shown in Figures 46A-46B,
15 represents the expected results of the average of 4 mice per group with a standard error of less than 10% for all groups. Background (i.e., no GAD peptide) produced an OD₄₀₅ of less than 0.05 in all cases (not shown).

The data clearly shows that the spleen cells isolated from mice immunized with IgGIN contained a significant population of T cells that were activated by the GAD₂₇₁₋₂₈₅
20 epitope peptide as shown by the robust release of both IL2 and IL4. The other immunogens produced no such response (media, hIgG1, GAD Pep). None of the immunogens resulted in a population of splenic T cells that responded by releasing IFN γ . Overall, the results support the conclusions that: 1) IgGIN immunization can result in the development of a GAD-specific T cell population; and 2) the T cells that expanded upon
25 this stimulation were of the Th2 variety (robust IL4 response, lack of IFN γ secretion).

Ex. 48. Effect of Mitomycin C on T Cell Activation

Spleen cells are harvested from HLA DR4 mice and CD11c⁺ cells are prepared as
30 previously described. The isolated cells are then plated at 5×10^4 per well and treated with one of the following immunogens: control media, hIgG1 (10 ug/well), GAD peptide (0.2 ug/well), GAD peptide (20 ug/well) or IgGIN (10 ug/well). In one set of wells, the

cells were pretreated with mitomycin C for 30 min prior to immunogen addition (mitomycin (+) in figure). The cells are incubated with their respective immunogens for 24 hours. Mitomycin is then added to the second set of treated cells and incubated for 30 min (mitomycin (-) in Figure 47). Responder CD4⁺-enriched T cells are isolated from IgGIN-immunized HLA DR4 mice and added at 1 x 10⁵ cells/well. In Figure 47, panel A, in one set of wells, [³H]thymidine is added at 1 uCi/well and the plate is incubated for 3 days. Cells are collected on filter paper and the amount of [³H] incorporated into the cells is quantitated. In Figure 47, panel B, in a second set of wells, culture media is collected after 3 days of incubation and the amount of IL2 in the media was quantitated by ELISA.

In both cases the data shows that the ability of IgGIN to stimulate T cell expansion in a splenic cell population isolated from IgGIN-immunized mice is dependent on the intracellular processing of the IgGIN by APC. This is shown clearly by the ability of mitomycin C to block T cell activation (see IgGIN Mitomycin (+) bars in both panels in Figure 47 when the CD11c⁺ cells are treated with mitomycin C prior to addition of IgGIN). It is also interesting that the minimal ability of the GAD peptide (20 ug/well) to activate T cells is not affected by mitomycin C treatment showing that its ability to bind the DR4 MHC is not dependent on an intracellular mechanism, but rather a direct binding on the extra cellular surface of the APC. Thus, the GAD epitope incorporated into the CDR3 region of the IgGIN primary structure must be proteolytically liberated and loaded onto the DR4 MHC before it can function as a T cell activator.

Example 49 - Effect of Fcγ Receptor Blockade on IgGIN-stimulated T Cell Activation

Spleen cells harvested from HLA DR4 mice and CD11c⁺ cells are prepared. The isolated cells are then plated at 5 x 10⁴ per well and treated with either control IgG or anti-Fcγ Receptor IgG for 30 min. The cells are then treated with one of the following immunogens: control media, hIgG1 (10 ug/well), GAD peptide (0.2 ug/well), GAD peptide (20 ug/well) or IgGIN (10 ug/well). The cells are incubated for 24 hours and then treated with mitomycin C for 30 min. Responder CD4⁺-enriched T cells (isolated from IgGIN-immunized HLA DR4 mice) are then added at 1 x 10⁵ cells/well. The plate is

incubated for 3 days and the media collected. The IL2 content of the media samples was quantitated by ELISA.

The data in Figure 48 shows that blockade of the APC Fcγ receptor eliminated the ability of the APCs to activate T cells. This data supports the conclusion that the loading of the DR4 MHC occurs by intracellular processing IgGIN (see Figure 48) and the mode of entry of IgGIN into the APC is via Fcγ receptor interaction.

Ex. 50. Use of human APC and human T cell clones to demonstrate IgGIN-mediated delivery of the GAD₂₇₁₋₂₈₅ epitope.

Human (DR4/DQ8 haplotype donors) blood is used to isolate an enriched dendritic cell population (> 90% pure). Cells from this stimulator group are plated at a density of 5×10^4 cells/well and treated with either: media, hIgG1 (10 ug/ml); GAD peptide (0.2 ug/ml or 20 ug/ml); or IgGIN (10 ug/ml). Equivalent wells are set-up that lack the stimulator cells ((-) APC). The plate is incubated for 24 hours. T cell responder cells (human T cell clone isolated based on its ability to respond to the GAD₂₇₁₋₂₈₅ epitope) are then added to the wells (2.5×10^4 cells/well). The plate is incubated for 24 hours and the culture media is then collected and held for analysis (data shown in panels A-C for the hIL2, IL4 and IFNγ ELISAs). Fresh culture media is added that is supplemented with 1 uCi/well of [³H]thymidine. The plate is incubated for 48 hours and the cells harvested onto filters and the amount of incorporated [³H]thymidine was determined (as shown in Figure 49B, panel D).

The data shows that in a fully human system (i.e. both stimulator and responder cells are of human origin), IgGIN is able to deliver the GAD epitope to the DR4 MHC and produce an expansion of the responder T cell population. This conclusion is supported by the stimulated secretion of both hIL2 and hIL4 and the incorporation of [³H]thymidine in the isolated cells. The lack of stimulation of hIFNγ secretion coupled with the robust hIL4 response shows that the T cell expansion was of the Th2 variety as has been previously shown in the murine models. The relatively weak stimulation of T cell expansion by the free GAD peptide shows the superior nature of delivering the epitope to the intracellular compartment as compared to the binding of free epitope peptide to the extracellularly displayed DR4 MHC complex.

Ex. 51. Use of human APC and human T cell clones to demonstrate IgGIN-mediated delivery of the Ins9-23 epitope.

5 Human (DR4/DQ8 haplotype donors) blood is used to isolate an enriched dendritic cell population (> 90% pure). Cells from this stimulator group are plated at a density of 5×10^4 cells/well and treated with either: media, hIgG1 (10 ug/ml); GAD peptide (0.2 ug/ml or 20 ug/ml); or IgGIN (10 ug/ml). Equivalent wells are set-up that lack the stimulator cells ((-) APC). The plate is incubated for 24 hours. T cell responder
10 cells (human T cell clone isolated based on its ability to respond to the Ins9-23 epitope) are then added to the wells (2.5×10^4 cells/well). The plate is incubated for 24 hours and the culture media is then collected and held for analysis (data shown in panels A-C for the hIL2, IL4 and IFN γ ELISAs). Fresh culture media is added that is supplemented with 1 uCi/well of [^{3} H]thymidine. The plate is incubated for 48 hours and the cells are then
15 harvested onto filters and the amount of incorporated [^{3} H]thymidine was determined (shown in Figure 50, panel D).

The data in Figures 50A – 50B show that in a fully human system (i.e. both stimulator and responder cells are of human origin), IgGIN is able to deliver the Ins9-23 epitope to the DR4 MHC and produce an expansion of the responder T cell population.
20 This conclusion is supported by the stimulated secretion of both hIL2 and hIL4 and the incorporation of [^{3} H]thymidine in the isolated cells. The lack of stimulation of hIFN γ secretion coupled with the robust hIL4 response shows that the T cell expansion was of the Th2 variety as has been previously shown in the murine models. The relatively weak stimulation of T cell expansion by the free Ins9-23 peptide shows the superior nature of
25 delivering the epitope to the intracellular compartment as compared to the binding of free epitope peptide to the extracellularly displayed DQ8 MHC complex.

Ex. 52. Effect of IgGIN on the progression to diabetes in DR4/RIP-B7/B6 transgenic mice

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The transgenic mouse strain DR4/RIP-B7/B6 carries the human DR4 MHC II transgene and this particular strain develops diabetes in conjunction with losing tolerance to GAD 65. To test the ability of IgGIN to restore tolerance to GAD 65 the following

experiment was designed. Two groups of 20 mice (DR4/RIP-B7/B6) are treated with either control human IgG1 or the experimental therapeutic IgGIN. Injections are IP and each dose was 5 ug/injection in sterile PBS. Injections are to begin at the point tolerance to GAD 65 is lost (insulinitis, auto antibodies to GAD 65, data not shown). The mice are injected weekly for 3 weeks. The mice are then followed for a six month period while monitoring blood glucose as an indicator of diabetes (blood glucose > 150 g/dL). As shown in Figure 51, the data show that IgGIN treatment was effective in lowering the development of diabetes in these mice by 70%. The data had a standard error of not greater than 10% (not shown).

This data is important because it strongly supports the idea that IgGIN can effectively deliver the incorporated DR4-specific GAD65 epitope to the transgenic mouse and delivery of this epitope restores a significant number of mice to GAD 65 tolerance. The overall therapeutic effect is protection from the progression to a diabetic condition.

This data and series of experiments confirm that the human IgG1 chimera, IgGIN, can function to deliver the incorporated epitope to APC and that the APC can then load the liberated epitope onto the appropriate MHC II. This data can be used to support the argument that the extensive murine data so far accumulated may be applicable to human disease thus strongly supporting the desire to move this approach into the clinic for testing on human disease.

Claims:

1. A method of tolerizing a patient to an antigen suffering from an autoimmune disease comprising:
 - 5 administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of said antigen attached to said immunoglobulin or portion thereof and administering said immunoglobulin or portion thereof to said patient.
 - 10 2. The method of claim 1 wherein said patient is human.
 3. The method of claim 1 wherein the immunoglobulin or portion thereof is human IgG.
 4. The method of claim 1 wherein the patient is human, the autoimmune disease is type
15 1 diabetes, the human immunoglobulin is human IgG and the peptide epitope is derived from GAD 65, GAD 67 or INS β .
 5. The method of claim 1 wherein the patient is human, the autoimmune disease is type
20 1 diabetes, the human immunoglobulin is human IgG and the peptide epitope is LPRLIAFTSEHS HF.
 6. The method of claim 5 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.
 - 25 7. The method of claim 1 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG and the peptide epitope is selected from the group consisting of VMNILLQYVV, MNILLQYVVKSFD, IAF TSEHS HFSLK, PRLIAFTSEHS HFSLK, LPRLIAFTSEHS HF, FFRMVISNPAATHQDIDFLI, VNFFRMVISNPAATHQD, and NFFRMVISNPAAT.

30

8. The method of claim 1 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG and at least two peptide epitope are inserted in the IgG and are selected from the group consisting of VMNILLQYVV, MNILLQYVVKSF, IAFTEHSHFSLK, PRLIAFTSEHSHFSLK,
 5 LPRLIAFTSEHSHF, FFRMVISNPAATHQDIDFLI, VNFFRMVISNPAATHQD, and NFFRMVISNPAAT.

9. The method of claims 5, 6, 7 and 8, wherein the human IgG is administered before onset of type 1 diabetes.

10. The method of claims 5, 6, 7 and 8, wherein the human IgG is administered during the pre-insulinitis stage of type 1 diabetes.

11. The method of claims 5, 6, 7 and 8, wherein the human IgG is administered after the onset of type 1 diabetes.

12. The method of claims 5, 6, 7 and 8, wherein the human IgG is administered after the patient has undergone IAA seroconversion.

13. The method of claim 1 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG and the epitope is inserted into the IgG and is selected from the group consisting of VSSQFSDAAQASPSS, SVSSQFSDAAQASPS, SSVSSQFSDAAQASP, SVSSQFSDAAQASPSSHSS, SRVSSVSSQFSDAAQASPSSHSS, SVSSQFSDAAQASPSSHSSTPSWC, VSSQFSDAAQASPSSHSSTPSWCE, SVSSQFSDAAQASPSSHSS, SRVSSVSSQFSDAAQASPSSHSS, SVSSQFSDAAQASPSSHSSTPSWC, VSSQFSDAAQASPSSHSSTPSWCE, SVSSQFSDAAQASPSSHSS, SRVSSVSSQFSDAAQASPSSHSS, SVSSQFSDAAQASPSSHSSTPSWC, AYQAEPNTCATAQ, LCAYQAEPNTCATAQG, LAKEWQALCAYQAEPNT, AYQAEPNTCATAQGEGNIK, WQALCAYQAEPNTCATAQ, KVESSPSRSDYI,

LKVESSPSRSDY, KLKVESSPSRSDYINAS, KVESSPSRSDYINASPIIEHDP, and CTVIVMLTPLVEDG.

14. A method of tolerizing a patient to an antigen suffering from multiple sclerosis comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from myelin basic protein, myelin proteolipid protein and myelin associated glycoprotein.

15. The method of tolerizing a patient to an antigen of claim 14 wherein the T cell epitope is selected from the group consisting of AVRQIFGDYKTTICGKGLSATV, FLYGALLA, GVDAQGTLSKIFKLGGRRDSRSGSPMA, SLSRFSWGA, LSRFSWGAEGQRPFGFYGG, PVVHFFKNIVT, VHFFKNIVTPRTP, KGFKGVDAQGTLSKI, LSKIFKLGGRRDSRSGSPMAR, VVHFFKNIVTPRTPPPSQGK, ASDYKSAHKGFKGVD, VLFSSDFRI, LMWAKIGPV and SLLLELEEV.

16. The method of claim 15 wherein the patient is human and the immunoglobulin is human IgG.

17. The method of claim 15 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

18. A method of tolerizing a patient to an antigen suffering from goodpasture's syndrome, comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from Collagen alpha 3(IV) Chain Precursor.

19. The method of claim 18 wherein the T cell epitope is selected from the group consisting of GSPATWTTR, TGQALASPGSCLEEFRA⁵SPF, GQALASPGSCLEEFRA⁵SPFLECH, ALASPGSCLEEFRA⁵SPF, ALASPGSCLEEFRA⁵SPFLE, LEEFRA⁵SPFLECHGRGTCN, RFTTMPFLFCNVNDVCNF, PFLFCNVNDVCNFA⁵SR, LFCNVNDVCNFA⁵SRND, SCPEGTVPLYSGFSFLFVQ, PSCPEGTVPLYSGFSFLFVQ⁵G and GTVPLYSGFSFLFVQGNQRAHG.

20. The method of claim 19 wherein the patient is human and the immunoglobulin is
10 human IgG.

21. The method of claim 19 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

15 22. A method of tolerizing a patient to an antigen suffering from primary biliary cirrhosis, comprising:
administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell
20 epitope derived from dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex precursor.

23. The method of claim 22 wherein the T cell epitope is GDLLAEIETDKATI.

25 24. The method of claim 22 wherein the patient is human and the immunoglobulin is human IgG.

25. The method of claim 22 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

30

26. A method of tolerizing a patient to an antigen suffering from pemphigus vulgaris, comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said

5 immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from desmoglein 3 precursor.

27. The method of claim 26 wherein the T cell epitope is LNSKIAFKIVSQEPA.

10 28. The method of claim 27 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

29. The method of claim 27 wherein the patient is human and the immunoglobulin is human IgG.

15

30. The method of claim 29 wherein the peptide epitope is inserted by insertion and deletion into the CDR3 region of the human IgG.

31. A method of tolerizing a patient to an antigen suffering from celiac disease,

20 comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said

immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from A-gliadin-partially deamidated peptide.

25

32. The method of claim 31 wherein the T cell epitope is QLQPFQPELPYPQPQS.

33. The method of claim 31 wherein the patient is human and the immunoglobulin is human IgG.

30

34. The method of claim 33 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

35. A method of treatment for a patient suffering from an autoimmune disease

5 comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of said antigen attached to said immunoglobulin or portion thereof and administering said immunoglobulin or portion thereof to said patient.

10

36. The method of claim 35 wherein said patient is human.

37. The method of claim 35 wherein the immunoglobulin or portion thereof is human IgG.

15

38. The method of claim 35 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG and the peptide epitope is derived from GAD 65, GAD 67 or INS β .

20 39. The method of claim 35 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG and the peptide epitope is LPRLIAFTSEHSHF.

25 40. The method of claim 35 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

41. The method of claim 35 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG and the peptide epitope is selected from the group consisting of VMNILLQYVV, MNILLQYVVKSF, IAFTSEHSHFSLK, PRLIAFTSEHSHFSLK, LPRLIAFTSEHSHF, 30 FFRMVISNPAATHQDIDFLI, VNFFRMVISNPAATHQD, and NFFRMVISNPAAT.

42. The method of claim 35 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG at least two peptide epitope are inserted in the IgG and are selected from the group consisting of VMNILLQYVV,
 5 MNILLQYVVKSFD, IAFTSEHSHFSLK, PRLIAFTSEHSHFSLK,
 LPRLIAFTSEHSHF, FFRMVISNPAATHQDIDFLI, VNFFRMVISNPAATHQD, and
 NFFRMVISNPAAT.

43. The method of claims 39, 40, 41 and 42, wherein the human IgG is administered
 10 before onset of type 1 diabetes.

44. The method of claims 39, 40, 41 and 42, wherein the human IgG is administered during the pre-insulinitis stage of type 1 diabetes.

15 45. The method of claims 39, 40, 41 and 42, wherein the human IgG is administered after the onset of type 1 diabetes.

46. The method of claims 39, 40, 41 and 42, wherein the human IgG is administered after the patient has undergone IAA seroconversion. before onset of type 1 diabetes.

20

47. The method of claim 35 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG at least two peptide epitope are inserted in the IgG and are selected from the group consisting of VSSQFSDAAQASPSS,
 SVSSQFSDAAQASPS, SSVSSQFSDAAQASP, SVSSQFSDAAQASPSSHSS,
 25 SRVSSVSSQFSDAAQASPSSHST, SVSSQFSDAAQASPSSHSTPSWC,
 VSSQFSDAAQASPSSHSTPSWCE, SVSSQFSDAAQASPSSHSS,
 SRVSSVSSQFSDAAQASPSSHST, SVSSQFSDAAQASPSSHSTPSWC,
 VSSQFSDAAQASPSSHSTPSWCE, SVSSQFSDAAQASPSSHSS,
 SRVSSVSSQFSDAAQASPSSHST, SVSSQFSDAAQASPSSHSTPSWC,
 30 AYQAEPNTCATAQ, LCAYQAEPNTCATAQG, LAKEWQALCAYQAEPNT,
 AYQAEPNTCATAQGEGNIK, WQALCAYQAEPNTCATAQ, KVESSPSRSDYI,

LKVESSPSRSDY, KLKVESSPSRSDYINAS, KVESSPSRSDYINASPIIEHDP, and CTVIVMLTPLVEDG.

48. A method of treating a patient suffering from multiple sclerosis comprising:
5 administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from myelin basic protein, myelin proteolipid protein and myelin associated glycoprotein.

10 49. The method of treatment of a patient claim 48 wherein the T cell epitope is selected from the group consisting of AVRQIFGDYKTTICGKGLSATV, FLYGALLLA, GVDAQGTLISKIFKLGGDRSRSGSPMA, SLRFSWGA, LSRFSWGAEGQRPFGYGG, PVVHFFKNIVT, VHFFKNIVTPRTP,
15 KGFKGVDAQGTLSKI, LSKIFKLGGDRSRSGSPMAR, VVHFFKNIVTPRTPPPSQGK, ASDYKSAHKGFKGVD, VLFSSDFRI, LMWAKIGPV and SLLLELEEV.

20 50. The method of claim 49 wherein the patient is human and the immunoglobulin is human IgG.

51. The method of claim 49 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

25 52. A method of treatment of a patient suffering from goodpasture's syndrome, comprising:
administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell
30 epitope derived from Collagen alpha 3(IV) Chain Precursor.

53. The method of claim 52 wherein the T cell epitope is selected from the group consisting of GSPATWTTR, TGQALASPGSCLEEFRA⁵SPF, GQALASPGSCLEEFRA⁵SPFLECH, ALASPGSCLEEFRA⁵SPF, ALASPGSCLEEFRA⁵SPFLE, LEEFRA⁵SPFLECHGRGTCN, RFTTMPFLFCNVNDVCNF, PFLFCNVNDVCNFA⁵SR, LFCNVNDVCNFA⁵SRND, SCPEGTVPLYSGFSFLFVQ, PSCPEGTVPLYSGFSFLFVQ⁵G and GTVPLYSGFSFLFVQGNQRAHG.

54. The method of claim 53 wherein the patient is human and the immunoglobulin is
10 human IgG.

55. The method of claim 53 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

15 56. A method of treating a patient suffering from primary biliary cirrhosis, comprising: administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from dihydrolipoamide acetyltransferase component of pyruvate
20 dehydrogenase complex precursor.

57. The method of claim 56 wherein the T cell epitope is GDLLAEIETDKATI.

58. The method of claim 57 wherein the patient is human and the immunoglobulin is
25 human IgG.

59. The method of claim 58 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

30 60. A method of treatment for patient suffering from pemphigus vulgaris, comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from desmoglein 3 precursor.

5

61. The method of claim 60 wherein the T cell epitope is LNSKIAFKIVSQEPA.

62. The method of claim 61 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

10

63. The method of claim 62 wherein the patient is human and the immunoglobulin is human IgG.

64. The method of claim 63 wherein the peptide epitope is inserted within the CDR3 region of the human IgG

15

65. A method of treating a patient suffering from celiac disease, comprising:
administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from A-gliadin-partially deamidated peptide.

20

66. The method of claim 65 wherein the T cell epitope is QLQPFQPELPYPQPQS.

25

67. The method of claim 66 wherein the patient is human and the immunoglobulin is human IgG.

68. The method of claim 67 wherein the peptide epitope is inserted within the CDR3 region of the human IgG.

30

69. A composition for tolerizing a patient to an antigen suffering from an autoimmune disease comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of said antigen attached to said immunoglobulin or portion thereof and administering said immunoglobulin or portion thereof to said patient.

68. The composition of claim 67 wherein the patient is human, the autoimmune disease is type 1 diabetes, the human immunoglobulin is human IgG and the peptide epitope is derived from GAD 65, GAD 67 or INS β .

69. The composition of claim 68 wherein the peptide epitope is LPRLIAFTSEHSHF.

70. The composition of claim 68 wherein the peptide epitope is selected from the group consisting of VMNILLQYVV, MNILLQYVVKSF, IAFTSEHSHFSLK, PRLIAFTSEHSHFSLK, LPRLIAFTSEHSHF, FFRMVISNPAATHQDIDFLI, VNFFRMVISNPAATHQD, and NFFRMVISNPAAT.

71. A composition for tolerizing a patient to an antigen suffering from multiple sclerosis comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said immunoglobulin has at least one peptide epitope of an antigen attached to said immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell epitope derived from myelin basic protein, myelin proteolipid protein and myelin associated glycoprotein.

72. The composition of claim 71 wherein the T cell epitope is selected from the group consisting of AVRQIFGDYKTTICGKGLSATV, FLYGALLA, GVDAQGTLSKIFKLGGRRDSRSGSPMA, SLSRFSWGA, LSRFSWGAEGQRPFGFYGG, PVVHFFKNIVT, VHFFKNIVTPRTP, KGFKGVDAQGTLSKI, LSKIFKLGGRRDSRSGSPMAR,

VVHFFKNIVTPRTPPPSQGK, ASDYKSAHKGFGKVD, VLFSSDFRI, LMWAKIGPV and SLLLELEEV.

73. A composition for tolerizing a patient to an antigen suffering from primary biliary
5 cirrhosis, comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said
immunoglobulin has at least one peptide epitope of an antigen attached to said
immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell
epitope derived from dihydrolipoamide acetyltransferase component of pyruvate
10 dehydrogenase complex precursor.

74. The composition of claim 73 wherein the T cell epitope is GDLLAEIETDKATI.

75. A composition for tolerizing a patient to an antigen suffering from pemphigus
15 vulgaris, comprising:

administering to the patient a human immunoglobulin or portion thereof wherein said
immunoglobulin has at least one peptide epitope of an antigen attached to said
immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell
epitope derived from desmoglein 3 precursor.

20 76. The composition of claim 75 wherein the T cell epitope is LNSKIAFKIVSQEPA.

77. A composition for tolerizing a patient to an antigen suffering from goodpasture's
syndrome, comprising:

25 administering to the patient a human immunoglobulin or portion thereof wherein said
immunoglobulin has at least one peptide epitope of an antigen attached to said
immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell
epitope derived from Collagen alpha 3(IV) Chain Precursor.

30 78. The composition of claim 77 wherein the T cell epitope is selected from the group
consisting of GSPATWTTR, TGQALASPGSCLEEFRAFPF,

GQALASPGSCLEEFRA SPFLECH, ALASPGSCLEEFRA SPF,
ALASPGSCLEEFRA SPFLE, LEEFRA SPFLECHGRGTCN,
RFTTMPFLFCNVNDVCNF, PFLFCNVNDVCNFA SR, LFCNVNDVCNFA SRND,
SCPEGTVPLYSGFSFLFVQ, PSCPEGTVPLYSGFSFLFVQG and
5 GTVPLYSGFSFLFVQGNQRAHG.

79. A composition for tolerizing a patient to an antigen suffering from primary biliary cirrhosis, comprising:

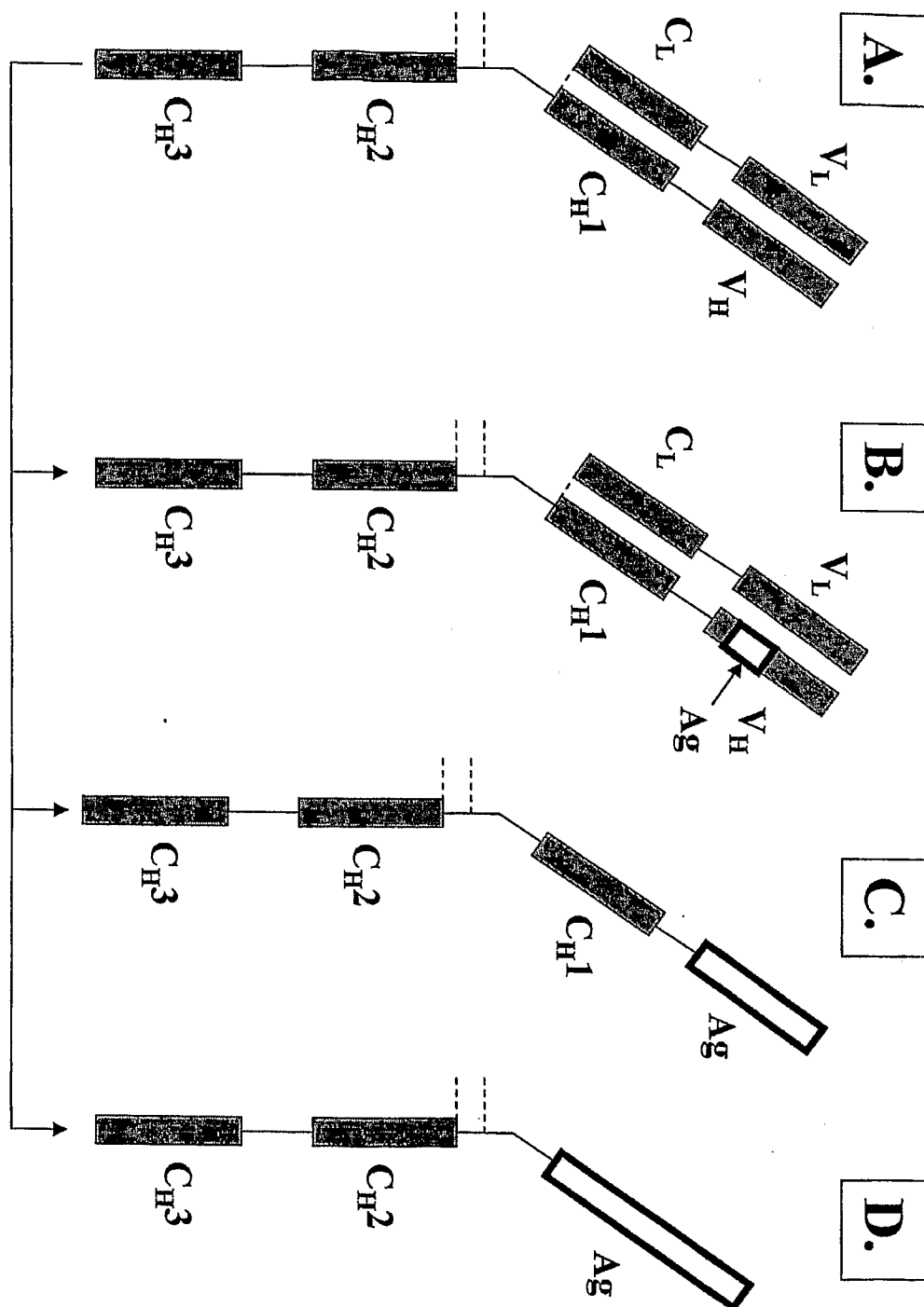
administering to the patient a human immunoglobulin or portion thereof wherein said
10 immunoglobulin has at least one peptide epitope of an antigen attached to said
immunoglobulin or portion thereof wherein said peptide epitope is selected from a T cell
epitope derived from dihydrolipoamide acetyltransferase component of pyruvate
dehydrogenase complex precursor.

15 80. The composition of claim 79 wherein the T cell epitope is GDLLAEIETDKATI.

20

25

Figure 1A:



IgG-peptides

Heavy chain

Light chain

hCMV-1A

hEF1 α

16 AA

18 AA

CDR2

CDR3

pMG

T cell epitopes

Fc-peptides

Heavy chain

hCMV-1A

pMG

VH CH₁

s.seq.

CH₂ CH₃

8-150 AA

Antigen

Figure 1C:

	Isotype	Binding to Fc γ R ¹	Complement activation ²	Induction of cytokines ³
CCL-159	γ 1	+	ND	ND
CRL-1621	γ 1	++	-/+	+
CRL-11538	γ 3	ND	ND	ND
CRL-11539	γ 3	++	++	+
HB-8636	γ 4	+	-/+	+

¹Binding to Fc γ R+ human monocytic THP-1 cells and endosomal internalization²Measurement of C4d production as is / or after heat aggregation³Induction of IL-10 and TGF- β by human PBMC or THP-1 cells exposed to adsorbed IgG

PD 02 74

Figure 1D:

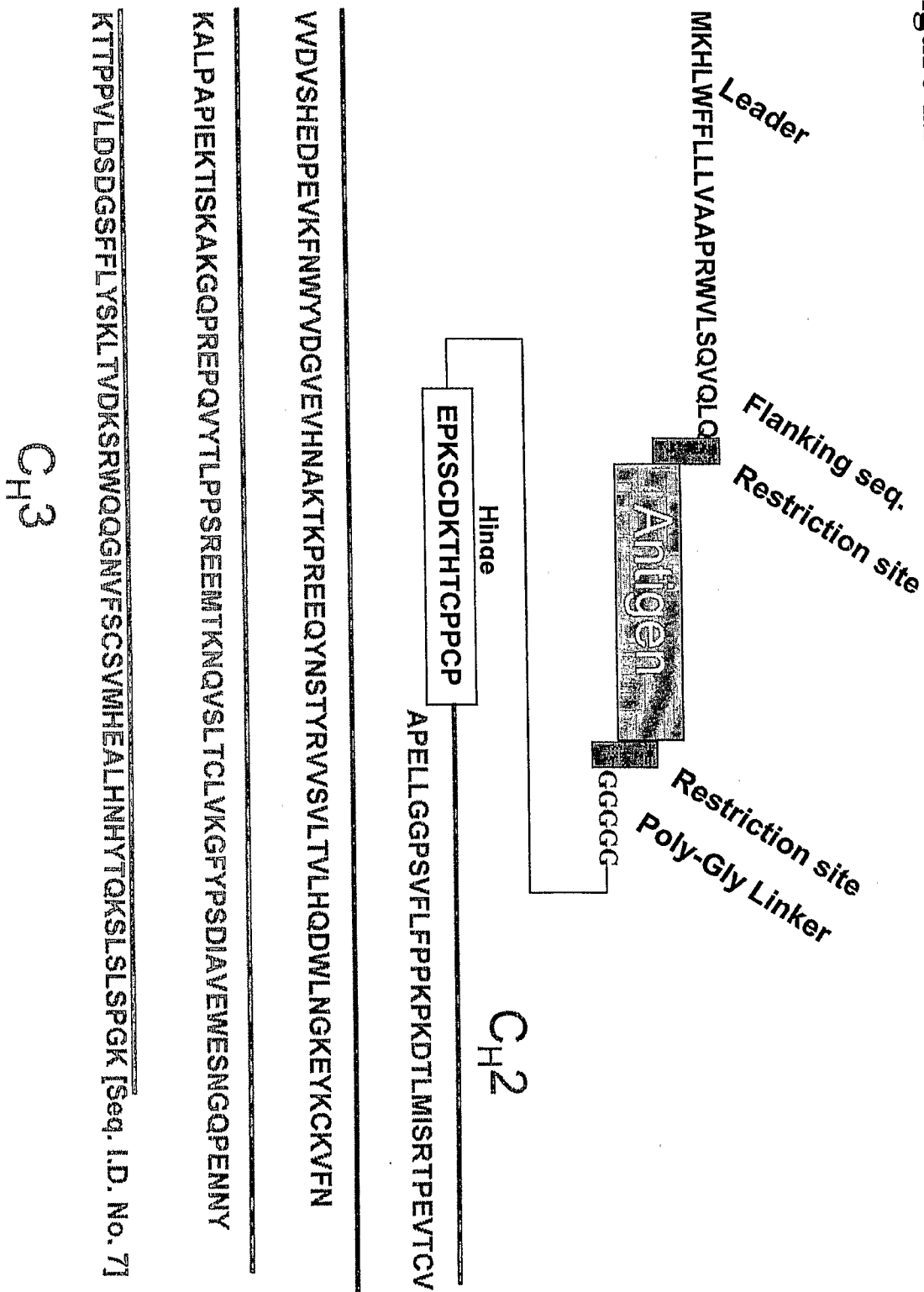


Fig. 1E: Epitopes and antigens (model)

Hemagglutinin of A/PR/8 influenza virus (precursor; HMIIV / NCBI)

I-E_d

mkanlvllc alaadadi cigyhannst divdvlekn vtrhsvll edshngklr lkgiaplqg keniagwllg npecdpllv rswsyiveip nsengicypg dldyeelr qlssvsfer feifpkessw pnhnttkgvt aacshagkss fymlwile kegsypklkn syvnnkgeve lvlwghlps nskdqniyq nenaysvvt snyntfipe iaerpkvrdq agmnywtl lkpgdtiife angliapry afalsrgfys giitsnasmh ecntkcqpl gainsslpfq nihpvitgce pkyvrsaklr mvtglmips iqsrflgai agfiegewg midgwygyhh queqsgyae dqkstqnain ginkvnsvi ekmmiqflav gketnklekr menlnkkvdd gfdiwtyna ellvllener tldhdsnvk nlyekvksq knakeigeng cfeiyhkcdn ecmesvmtgt ydypkysees klmrekvdgv klesmgryqi laiystvass lvllvslgai sfvmcsngsl qcirici [Seq. I.D. No. 8]

Nucleoprotein of A/PR/8 influenza virus (precursor; HMIIV / NCBI)

K_d

masqgtksry eqmetgery nateirasve mrvggigrfy iqmcielqis dyegrligns itermvlsa fdermkyle ehpsagkdpk ktggpiyklr dgkwmreil yldkeirriw rqaangedat aglthniwih snldatyqr tralvrtgmd prmcslmggs tiprtsgaag aavkgigtmv melimnktg indnfvrye ngrtrtiaye mnenilkgtf qtaqrammd qvresmrgn aeiedliffa rsalirgsv ahksclpaci yglvvasgyd feregyslvg idpfilqns qvtsilrtpne npvhksqliw machsaafed lvvssflrgt kvvprgqlt rgyqiasnen meindsitle lrskywait rsggntnqr asagqisvqp tfsvqnlpf eratimaft gmegrtsdm rteiimmes arpdvsvfge rgvfelsdek ahnpivpsfd msnegsyffg dnaeeydn [Seq. I.D. No. 9]

VH segment of murine anti-arsonate antibody (G1MSAA / NCBI)

evqlqsggae lvkagssvkm sckatyrtis syelywvrqa peggledley issassaypny aqkfggrvti tadesintay melslrsted tavyfcavrv isryfdgwgq gtlv [Seq. I.D. No. 10]

Fig. 1F: Epitopes and antigens

Influenza virus

M1 (PN0086/NCBI)

HLA-A2

mslltevey vlsitpsgpl kaeiaqned vfiagkntdle vlmewlktup iisptkgvl gfvfilityps erglqrrtfv qnalngndp mmdkavkly rklkreitfy gakevalsys
tegalascmgel iynrmgtvt evafglvcat ceqiadsqhr shrqmvttn plihennv lasttakame qmagssedaa eannevasqar qmvqamrtvg thpssaglk
ddlenlqay qkrmgvqlqr lk [Seq. I.D. No. 11]

M2 (PN0087/NCBI)

ZM

mslltevetp tmgweecs dssdplviaa siigllhfil wildrlffkc iyrtlkylgk rgpstegvpk smreeyrqeq qnavdvddgh fywiele

Hepatitis C virus

NS3 (AAK54587/NCBI)

apitayaqqt rgllgcitfs ltegrdmqve gevqvvsfat qstflatcing vcwvthhgag sktlagpkgp itqmytnvdq dlvgwpappg arsltpcteg ssdlylvth
advvpvrrs dsrgslspr pisylykssg eplicpsgha vgfiaavct rgvakavdv pvesmetmr s [Seq. I.D. No. 13]

Hepatitis B virus

Core antigen (AAM 18211/NCBI)

mdidpykefg asvellstfp sddffsirdl ldtasalyre alespehscsp hhtatrqail cwgelmlnat wvgsnledpa sreivvsyvn vnmglkirql lrfhiscitf
gretvleylv sfegwritpp aytrpnapi [Seq. I.D. No. 14]

Fig. 1G: Epitopes and antigens (human system)
Human Papilloma Virus

HPV 18-E7 (P06788/NCI)

HLA-A2
mhgpkatlq*d* i*vl*hlepq*ne* ipv*dl*lcheq l*sd*see*nde* i*dg*vn*h*q*h*p arraepq*rh*t m*lc*m*ck*ce*a* r*ik*l*v*vess*a* d*dl*ra*f*q*l*f l*nt*l*sf*vcpw casq*q*
[Seq. I.D. No. 15]

HPV 16-E7 (AAL96657/NCI)

HLA-A2
mh*gd*tp*l*he y*ml*d*l*q*ne*t*t* d*l*y*ce*q*l*nd s*see*de*id*g pa*g*gaep*dr*a h*yn*i*vt*f*oc*k c*ds*tr*l*evq st*h*vd*ir*l*e* d*ll*mg*tl*g*iv* cp*ic*s*q*kp [Seq. I.D. No. 16]

HLA-A2

HPV 18-E6 (CAB53096/NCI)

HLA-A2
ma*rf*edp*tr* pyk*l*p*dl*cte l*nt*slq*d*ei t*ev*y*ck*tl*ve* l*te*v*f*afk d*l*f*v*vyr*ds*i pha*ck*ci*d* f*ys*ir*el*h y*sd*svy*gd*l*l* ekl*nt*g*l*yn l*l*rc*l*rcqk
pl*np*aek*rh* l*ne*krf*h*ki a*gh*yrgq*ch*s c*en*ra*rg*erl q*rr*etq*v* [Seq. I.D. No. 17]

HLA-A2

HLA-A2

HPV 16-E6 (AAL96630/NCI)

HLA-B18
mhq*rt*am*f* d*p*qerp*ik*p h*lc*elq*ti* h*ai*l*ec*vyc kq*q*ll*re*v y*rd*g*np*y*av*c d*lc*okf*ys*ki se*vy*ryc*ys*v y*gt*ll*eq*gyn kp*l*cd*ll*irc
l*nc*qkpl*cp*e ekq*rh*l*dk*q r*fh*ni*ng*rw*t* g*rc*m*sc*rs*s* t*rr*etq*l* [Seq. I.D. No. 18]

HLA-B18

Fig. 1H: Epitopes and Antigens (human system)

Melanoma – gp100 (P40967/NCBI)

HLA-A2

HLA-A2

mdlvkrcell hlavigalla vgatkvpnnq dwlgyvsrqlr tkawmqlyp ewteaqlde wrggqvslkv sndgpitliga nasfsialnf pgsqkvlpdg qviwvnmntii ngsqvwggqp
vypqetiddac ifpddggpcps gswsqktsfv yvwktwgyw qvlggpvsgl sigtigranlg thimevtyh trgsrsvpl ahssasatit dqvplfsvsvs qlraldgenk hflmqpltf
alqlhdpsgy laeadlsytw dfgdssgtli sralvltity lepgvtaqv vllaqaipits cgsspvpgit dghrptaear nttagqvpt evvgitp gqa piaepsgtis vqvpitewis
tapvqumptae stgntpekv vsevngtlla emstpeatgm tpaevsivvl sgtaaqvtt tewvettare lpipepegpd assimstesi tsglglpldg tatitvkrq vpldcvlyty
gsfsvldiv qgiesaeilq avpsgegdaf eltvscqgl pkeacmeiss pgcqpqaql cgpvlpspac qlvlhqlkg gsgtyclnvs ladnslav stqlimpge aglgqvpliv
gilvlmavv lasilyrrl mkqdfsypql phssshwlti pificscpig enspllsqq v [Seq. I.D. No. 19]

[substitute the following sequences in place of the second epitope for agonists: **fl dqvafsv ; fl dqrvfiv ; fl flwffev**]

MART-1 (Q16655/NCI)

HLA-A2

mpredahfy gypkkghghs yttaeaaagi gilitvilgvl lligcwycrr mgyralmndk silhvtqcal ttrcpqegfd hrdsksvlqe kncepvnvna ppayeklsae qspnpysp
[Seq. I.D. No. 20]

TRP-2 (CAA0437/NCI)

msplwvgtll sclgckilpg aqgqfprvcv tvdslnkcc cpriigaesan vegsqqgrgq clevaratp wsgpyilinq ddrelwprkf hntckctgn fagyncgdck
fgwigncer kkpvrirgqi hslspqereq flgaldiack rvhpdyvitt qhwvllgpn gtaqpfaucs vydffvwilhy ysvrdtlgg ffpwlkvyvy rfviglrwq
weviscklik ratttqp [Seq. I.D. No. 21]

[alternate epitopes: **llpgrrpy r**

svydfvfw]
I

Fig. 1I: Epitopes and antigens (human system)

Carcinoma Embryonic Antigen Precursor (XP 064845/NCBI)

mdlsprwsl wrrvflmasl lacgicqasg qifitqldgi kgyrtvald kvpedvgeys wywgangsag nmiishkpps aqpgpmnytg revrnregsl lrrpalndt gnytvrvvag
netqratagwl evlelsgnlg isvnasslve nmdsvaadcl tuvnitiwyv ndvpiissdr mtispdglcl vilrvsrydr tūqcmiesfp eifgrseris llvaygpdyv llrsnpddfn
gvtæiegsq vemecicysf ldlkylhwiln gsilnfsdak mnlslaweq mgrtyrtven pvtqlinymd vtiqaphecs ssppgscfal lpasmpc [Seq. I.D. No. 22]

Her-2 (CAD28392 human Her-2/neu p/NCBI)

meaalcrwv llallppga astqvctigd mkrripase thldmlrhlv qgcqvvgvgnl eltylptnas lsflqdiqev qgyvliahnq vrvqplqrlr ivrtglqfcd
nyalavldng dplmtptvr gaspggrel qlrstetlk ggvligrnpq leyqdilwk dthknqula lthdmnr achpcspmnck gscwgesse deqslrtve aggcarkgpg
lptdcchegc aagctgpkhs dclaclhfnh sgicelhcpa lvtynidtfc smnpuegyrt fgascvtacp ynylstdvgs ctivcplhnq evtaedgtqr cekeskpccar
vcyglgmehl revravtsan igetfagckki fgsiaflpes fdgedpasnta plqpeqlqv f etleicetyl yisawpdsip dlsvrfqnlgv irgrillnga ystltqglgi swlglrstre
lsgslalhnh nthlcfvhtv pwdqlftrnh gallhtanr edecvgegla chqlcarghc wgpgrtqcvn csqflrgec veeerlqgl preyvnarhc lpcchpecqpq
ngsvctfgpe adqcvacahy kdrpfcvare psgvxpdlisy mprwktfdee gacqpcpinc thscvldldk gcpaeqrasp ltsisavvg illvvilgvy fgitikrtqq
kinkytmrri lgetelvepl tpsgampnqa qmritketel tkvkvlsgsa fgtyvkgiwi pdgenvkivp alkvhents pkankeilde ayvmagvgspp yvsrlligicl
tsivqlvtql mpygclldhv rengrlgsq dlnwcmqia kgmsyledvr lvhrdlaan vlvkspnhv k idifglarl dideteyhad ggekvpikwma lesilrrtf
hgsdvwswygv tvwelmitfga kpydgrpare **ipdlekker** lpqpictid vymimvkcwm idsecprtf elvsefsrma rdpqrftviq nedlgepaspl dsufyslle
dddmngdlvda eeylvppqgef fcpdpapagag gmvnhhrss stsgggdl lglepseea prsplapseg agsdvfdgl gmgaaqglqs lptldpsplq ryledptvpl
pseidgyvap licspppeyv ngpdrvpqpp spregplpaa rpagatlerp **ktispgekngv** vkdvafgga venpeylhpq ggaaqpnhpp pafspa fclnl yywdqdppter
gapsttkgt ptaenpeylg ldtvp [Seq. I.D. No. 23]

HLA-A2 anchor motif

FLXXXXXXXXV (X any aminoacid except C) [Seq. I.D. No. 24]

Fig. 1J: Epitopes and antigens (human system)

Tetanus toxin (AAM57257/NCBI)

kiptpmire nlymrtastl dlgegclci knstseepfq deivsyntkn kplnfmysld kiivdynlqs kitlpndtt pvlkgyrap eyksnaasti eihmiddnti yqylraqksp
tltgrmtu svddalinst kivsyyfsvi skvngqagaj lllqwwrddi ddfinessqk ttdkisdvs tivpyigpal nivkqgyegn figaletgv villeyipei tlpviaalsi aessiqkeki
iktidnfllek ryekwievyyk lvkakwlgfv ntqfqkrsyq myrsleyqv d aikkiidyey kiysgpdkqg iadeimnlkn kleeankam ininifmres srsflvngmi neakkqllef
dtqsknllmq yikanskffig itelkkiesk inkvstipr fsysknldeu vdnneedidvi lkkstihld imndiisdls gfnassvityp daqlvpging kaihlnvnes sevivhkamd ieyndmfmn
tvstfvlrvpk vsashlegyg tneysiussm kkslsisgsg wsvslkgmnl iwtlksdage vrgitfdlp dkfhaylank wvfitmndr lssanlyng vlmgsaeiig lgairedni tllkdrnmn
ngyvsidkfr ifekalnpike ieklytsyis itfirdfwgn phrydteyyl ipvassskdv qlknidymy lhnapsyng kliniyyrly ngkffikry tpmneidsfv ksgdffklyv symmehivg
ypkdgnafhn ldrilrvygn apgiplykkm eavktrdlt ysvqlklydd knaslglygt hngqigndpn rdiliasnwy fhhlkdktig cdwyfvyptide gwind
[Seq. 1.D. No. 25]

Universal T helper epitope

QYIKANSKFIGITEL [Seq. 1.D. No. 26]

HIV-1: reverse transcriptase (AAN02999/NCBI)

eiectmekeg kiskigpenp yntpvfaikk kdstkwrktiv dftelnkrtq dfwevqlgip lhpagllkkks vtrldvgday fsypldkdfr kytaftipst mnetpgriryq ynvlpqgwke
spafqssmt kilepfrfkqn peiviyqymd dlyigsdlei gphrtkieel rqlhlkwglt tpdckhqkep [Seq. 1.D. No. 27]

Universal T helper epitope

FRKQNPDIIVYQYMDLYVG [Seq. 1.D. No. 28]

HIV-1: gag (AAM98735/NCBI)

wdrthpagaq piagqirep rgsdiagttis tlqeqitwmt mnpipvgei yktrwiliijn kiwmyspvs lidikqgpke pftdyvdrtff kalraeqatq dvknwmtdtl lvqnanpdck
sitrglpgga sleemmtacq gvggspshkar vlaneansqan svmmqrsnf kgpkrivkcf ncgkeghiar ncraprkkgc wkcgqeghnqm kdeterqanf lgkiwpslshg rpgnll
[Seq. 1.D. No. 29]

Fig. 1K: Self antigen / Epitopes

Insulin precursor – human (P01308/NCBI)

DQ8

malwmrllpl lalllawgpd paaafvnhqleg shlvealy lvcgergfyy tpkttreacd lqvqvvelgg gpgagslqpl alegsldkrg lvegecstic slyqlenyen
[Seq. I.D. No. 30]

Glutamic acid decarboxylase – human GAD 65 (Q05329/NCBI)

DR4

maspgsgfws fgsedsgds enpgetarawc qvaqktfgegi gnikcallyg daekpaesgg sqppraark aacacdqkpc scskvdyvnya fihatdlipa cdgerptlaf lqdvmmllq
yvvsfstst kvldfhyrne llqeynwela dqpqnleeil mhceqtlkya ikighpyrh qstgldmvg laadwlsta ntmnfyeia pvtvlleryt lkkmreiihw pgsdgdgifs
pggaismnya mmiarfkmfp evkekgmaal prlatfseh shfsllkgaa algrtdsvi lkcdergkm ipsdleril eakqkgfvpf lvsatagtv ygaafpplav adieckykiv
mhvdaawggg llmsrkhkwyk lsgveransv twmnhkmmgv plqesallvr eeglmqncnq mhasylfqd khydlsydtg dkaqdcgrhv dvfkwlmwv akgtgfeah vdkclelaey
lwnlkureg yemvrdgkpc hmvfwyip psrltledne ernsr lskva pvikarmmey gttmvsyqpl gdkvntfmv isnpathqd idtfieeier legql
[Seq. I.D. No. 31]

DR4*

DR4** (2 epitopes)

DR4

DR4* (2 epitopes)

Example of composite self epitope Insulin-GAD (used for construction of anti-diabetes drug):

insert-cg shlvealy lvcgerg-XXXXX- prlatfseh shfsl-XXXXX-lwnlkureg yemvf-XXXXX- psrltledne ernsr-insert
[Seq. I.D. No. 32]

Example of composite non-self epitope Tetanus-gp100,MART-1, TRP-2
(used for construction of anti-melanoma drug):

insert-qyikanskfigitel-XXXXX- fldqvafsv-XXXXX- aagigiltv -XXXXX-svrdtlgg-insert [Seq. I.D. No. 33]

Fig. 1L. Additional antigens

Prostate associated PAGE-1 (Homo sapiens) AAC25990 /NCI

prostate associated PAGE-1 (Homo sapiens) AAC25990 /NCI
 prostate associated PAGE-1 (Homo sapiens) AAC25990 /NCI
 prostate associated PAGE-1 (Homo sapiens) AAC25990 /NCI

Six transmembrane epithelial antigen of the prostate (Homo sapiens) ACCESSION NP_036581 /NCI

Six transmembrane epithelial antigen of the prostate (Homo sapiens) ACCESSION NP_036581 /NCI
 Six transmembrane epithelial antigen of the prostate (Homo sapiens) ACCESSION NP_036581 /NCI
 Six transmembrane epithelial antigen of the prostate (Homo sapiens) ACCESSION NP_036581 /NCI

G antigen, family C, 1; JM27 protein; prostate-associated gene protein 4 (Homo sapiens). ACCESSION NP_008934

Mucin 1, transmembrane, (Homo sapiens). ACCESSION NP_002447

Mucin 1, transmembrane, (Homo sapiens). ACCESSION NP_002447
 Mucin 1, transmembrane, (Homo sapiens). ACCESSION NP_002447
 Mucin 1, transmembrane, (Homo sapiens). ACCESSION NP_002447

Fig. 1M. Additional antigens

MAJOR SURFACE GLYCOPROTEIN G (ATTACHMENT GLYCOPROTEIN G) OF O. RSV. ACCESSION G86695

nsrtttttf kllkkaas k/vfngsd ykrltvlqrnsdntf lvtlttdi ystgylak pmplldt qqrtslpdrtmrsh spqfshp ftaadef nyrltklqgylgpphchtkqss
qsrpsdqdsdflp/vprnagds adslarps ellkdctf tpkddqnt tkqlkesh trtslrky lkttrtlpgh glstdtrk nshvqph fta

Attachment glycoprotein (Human respiratory syncytial virus). ACCESSION AAM82069

ctdtkrtlp/vltqpdglstnselt sqdlldt fpaeslpp tkklrtrf tqdalkdt kqqrkqrk pmtdfaf rlyaslanpdaodr lprtkqgyl tkplkqdl krlkqddqqlkda/rf
kdekdlsf tktrtrfll trrtlqph tsdqgppnh

Glycoprotein G (Human herpesvirus 1). ACCESSION AAN04791

ttptpdlgeesssgyphgtfeggyt rdlpdygdpdaedk dqrmpas pprtspprpsrpkpdt llgddrpd ttf //

Immediate early protein ICP 47 (human herpesvirus 1). ACCESSION AAG3134

msvderatd flt

Neurovirulence factor (ICP 34.5) (Human herpesvirus 2). ACCESSION BAA23428

nsrttggtr gtrtpgypaaprrypovqrqtpadqnyqys glaesqaussllmvlv pqrtdtdty qy rtttaqrnsppsggk
qqrtrpdaaqrqrpk ergqplprrtrtrftrf ekdlsfr rpprpprdqrgvdsprvqtrlvawdcalargswaraidrtrmcare
algudpaagaaac dtefyyeeesumng sauggrav

Fig. 1N - Heavy Chain of IgG1:

MKHLWFLLVAAPRWVLSQVQLQQWGAGLVKPSSETLSLTCAVFGGSFSGYYWSWIRQPPGK
 Leader sequence CDR1
GLEWIGEINHSGSTNYKTSLSKSRVTISLDTSKNLFSLKLSSVTAADNAVYYCARGLLRGGWNDV
 CDR2 (16 AAs) CDR3 (18 AAs)
DYYYGMDVWGQGTDDTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS
GALTSGVHTFPAVLQSSGLYLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVPEPKSCDKTH
 CH1 Hinge
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVYVDVSHEDPEVKFNWYVDGVEVHNAKT
KPREEQYNSTYRVVSVLTVLHODWLNGLKEYKCKVFNKALPAPIEKTISKAKGQPREPQVYTLPPS
 CH2
 REEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSR
 CH3
 WQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Fig. 1O - Light chain sequence of IgG1:

MDMRVPAQLLGLLLLWLRGARCDIHMTQSPSSLSASVGDRVTITCRASQSTGSLLNWYQQK
 PGKAPKLLIYAASSLQNGVPSRFSGSGSGTDFTLTISLQPEDFATYFCQQSYSTPLTFGGGTK
 VEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQ
 DSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Fig. 1P

Strategy for engineering of immunoglobulin cassette for insertion of two peptides

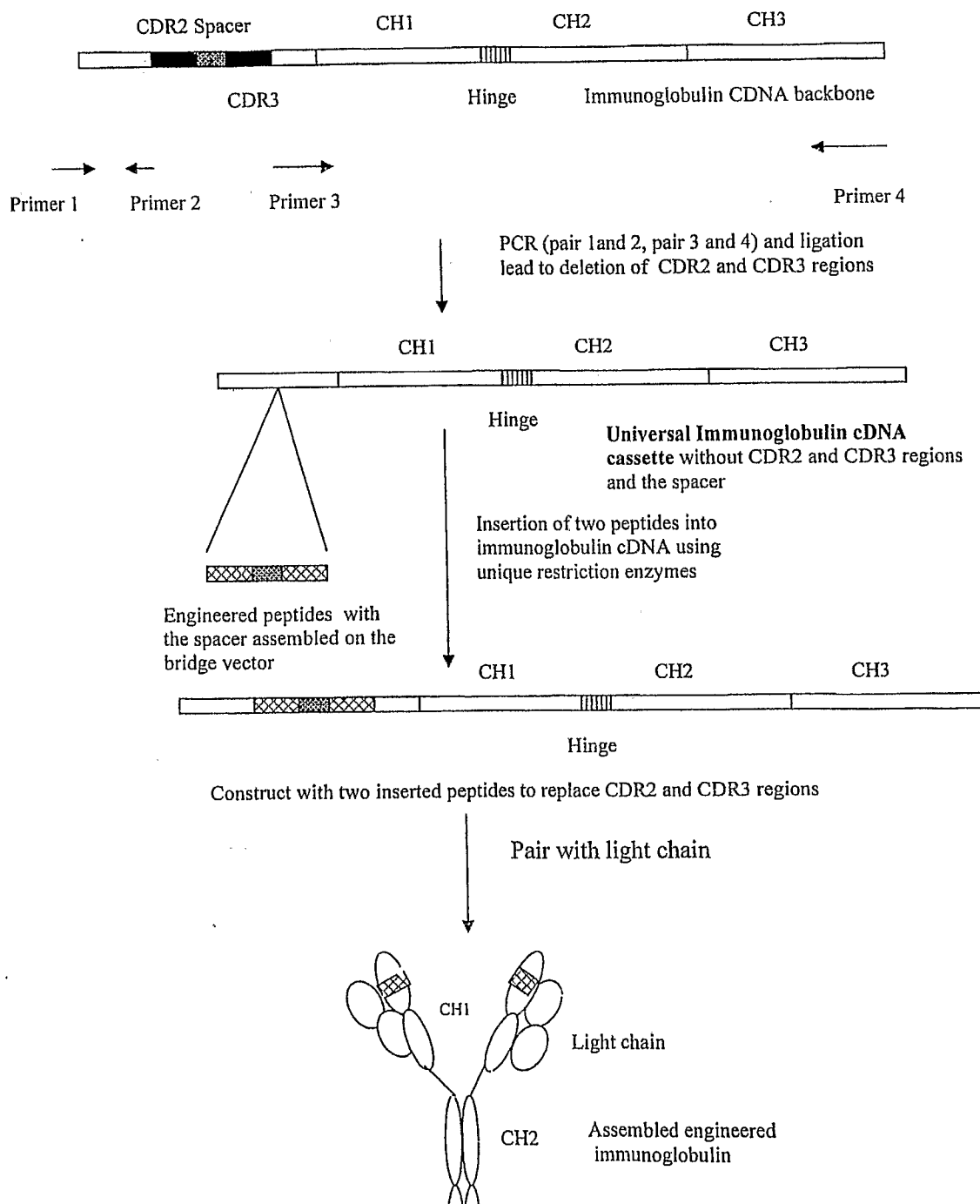


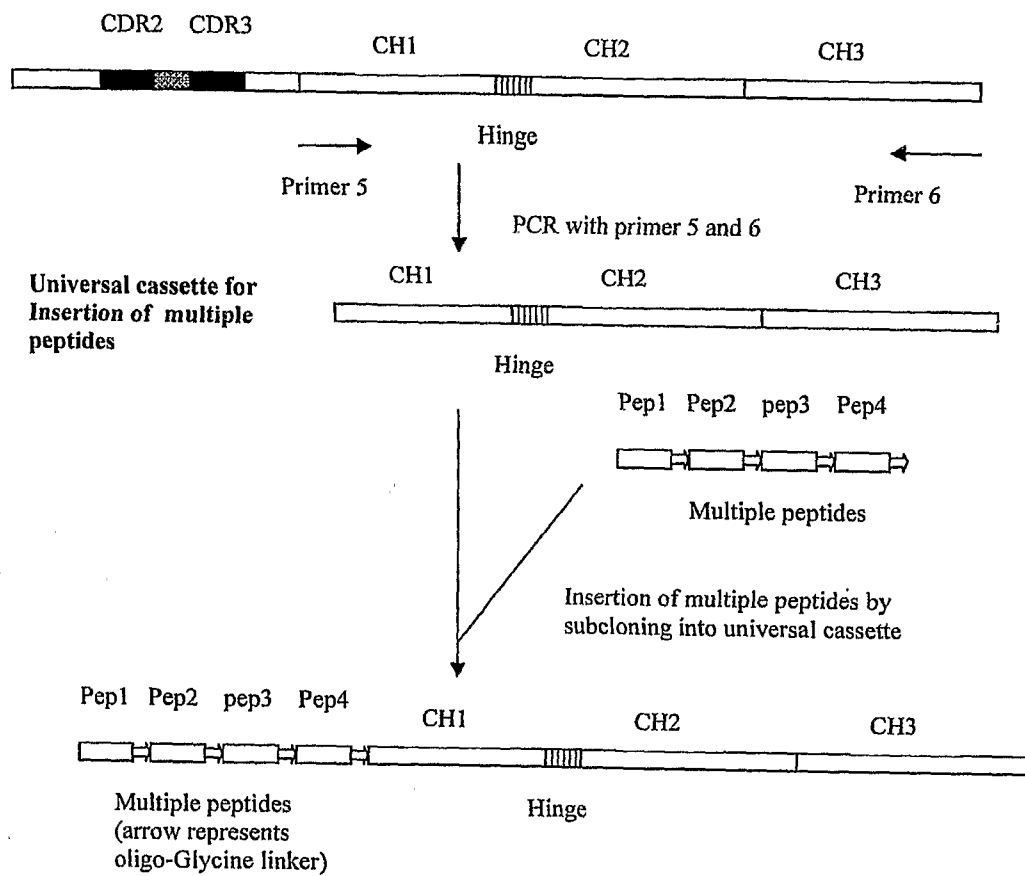
FIG. 1Q Strategy for engineering of multiple peptides

Fig. 1R

Fig. 4. Expression of engineered immunoglobulins in various cells with double expression vector pBudCE4.1

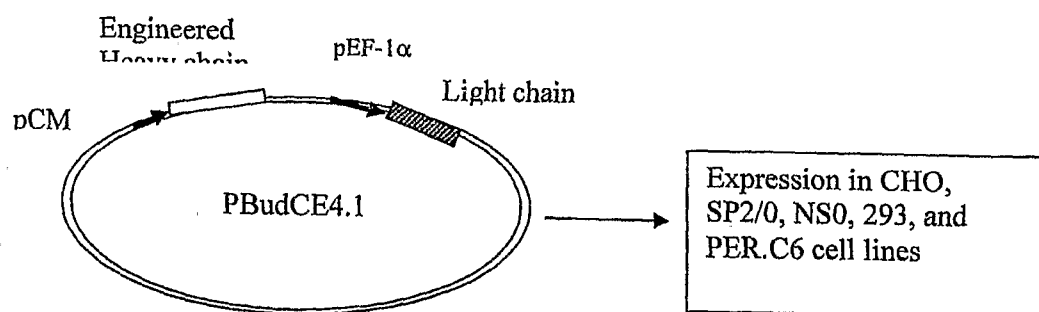


Fig. 1S

Engineered immunoglobulin to deliver peptide ligands

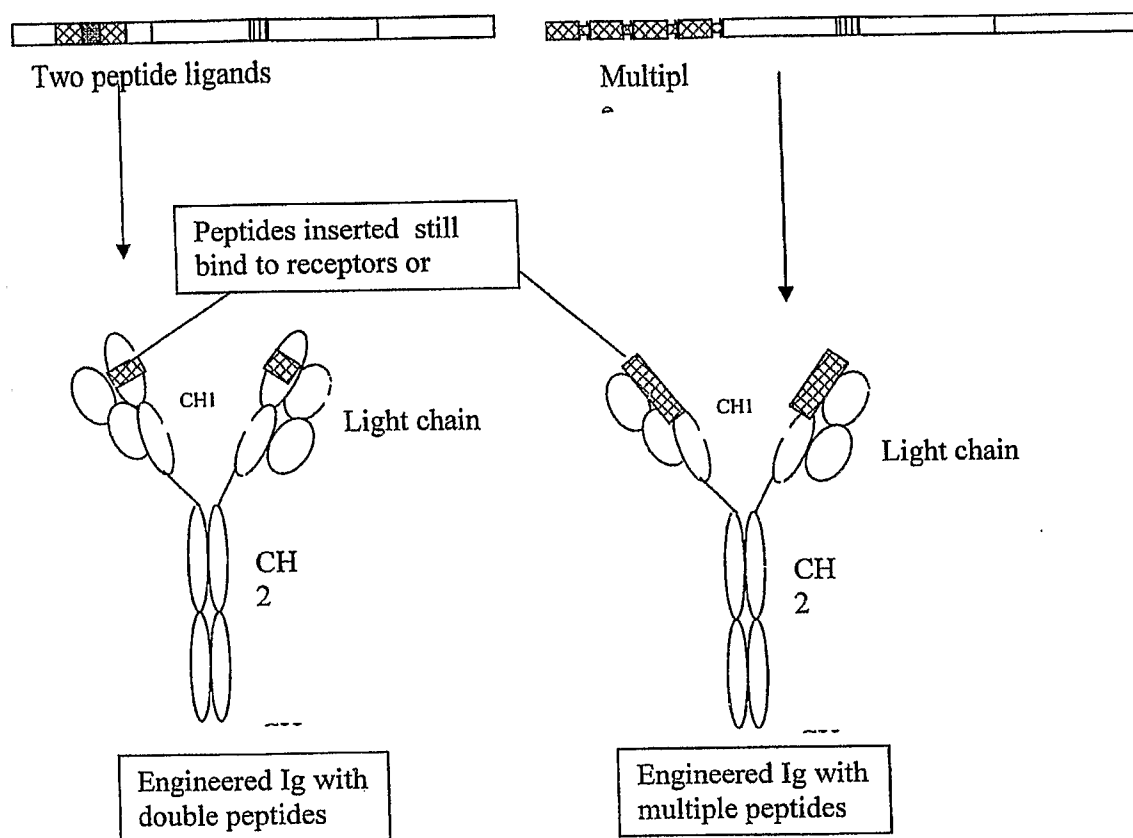
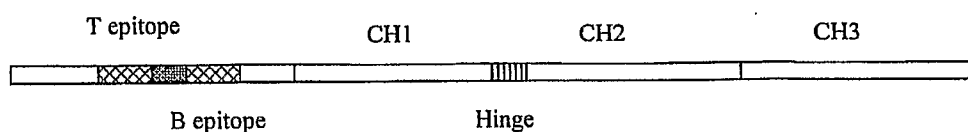
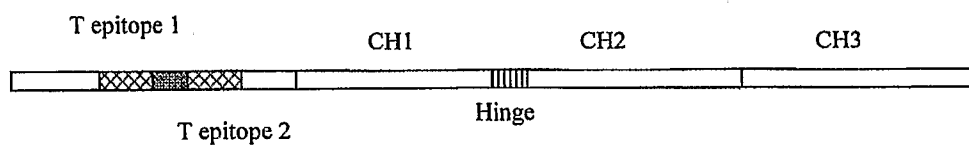


Fig. 1T Engineered immunoglobulin to deliver peptide ligands

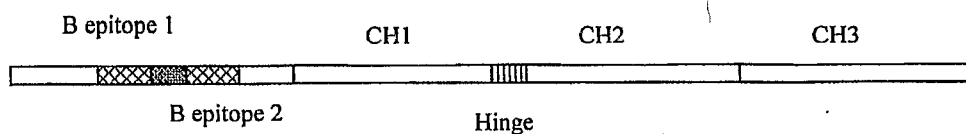
A construct with both T and B cell epitopes:



A construct with two T cell epitopes:



A construct with two B cell epitopes:



A construct with mixed multiple epitopes: Peptide 1 to 4 are either T cell epitopes only or B cell epitopes only, or both B and T cell epitopes:

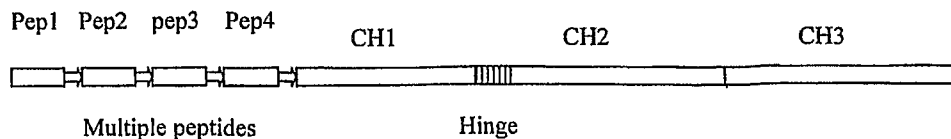


FIG. IU

Examples of T or B cell epitopes can be used for vaccine development:

1. Diabetes I (MHC-II restricted T cell epitopes)

GAD65 (AA 271-285): PRLIAFTSEHSHFSL

Prepro-insulin (AA 32-46): CGSHLVEALYLVCGERG

IA-2 (AA 805-820)

2. Solid tumors (MHC-I restricted T cell epitopes)

Breast Cancer	Her-2	GVGSPYVSRLLGICL
		PAREIPDLLEKGERI
Melanoma	gp100	ITDQVPFSV
	MART-1	AAGIGILTV
	TRP-2	YMDGTMSQ
	Melan-A26-35	ELAGIGILTV

3. Leukemia (MHC-I restricted T cell epitopes)

hTERT	VYAETKHFL
	VYGFVRACL
BCL-ABL (CML)	KQSSKALQR
TEL-AML (ALL)	RIAECLGM
AML-ETO (MTG8) (AML)	PFLKANLPLL
Survivin	LMLGEFLKL (sur1)
	ELTLGEFKL (sur2)

4. Other diseases including infectious and inflammatory diseases

HIV envelope protein V3 peptides containing PND domain

Chlamydia MOMP peptides containing exposed VDI-IV domains (Cheng, 1992)

IgE mediated asthma: IgE CH4 peptides including KTKGSGFFVF(Seq ID No: 79)

Fig. 1V

Inhibitory peptides for human cytokine IL-1:

- 1) VITFFSL; and,
- 2) NENRENH

Inhibitory peptides for human cytokine TNF- α :

- 1) QVYFGIIA; and,
- 2) PFYKQWL

Human growth factor FLT3 ligand: amino acid residues 1-235.

GPCR receptor ligands such as amylin: KCNTATCATQRLANFLVHS
SNNFGAIL SSTNVGSNTY

FIG. 1W

Autoimmune Peptides -
Diabetes

Name/Descriptions	Position	Peptide	HLA	Disease	Misc.
T cell Epitopes					
GAD 65	114	VMNILLQYVV	A*020101	Diabetes	J Exp Med 1995 181(5):1923-7
GAD 65	115	MNILLQYVVKSF	DRB1*040101	Diabetes	J Clin Invest 1996 98(11):2597-603
GAD 65	274	IAFTSEHSHFSLK	DRB1*040101	Diabetes	J Clin Invest 1996 98(11):2597-603
GAD 65	271	PRLIAFTSEHSHFSLK	DRB1*040101	Diabetes	J. Exp. Med. 1994 180:173-181.
GAD 65	270	LPRLIAFTSEHSHF	DRB1*040101	Diabetes	J Clin Invest 1997 99(10):2406-15
GAD 65	556	FFRMVISNPAATHQDIDFLI	DRB1*040101	Diabetes	J Clin Invest 1997;99(10):2405-15
GAD 65	554	VNFFRMVISNPAATHQD	DRB1*010101 DRB1*040101	Diabetes	Proc Natl Acad Sci U S A. 2001 98(4):1763-8.
GAD 67	564	NFFRMVISNPAAT	DRB1*010101 DRB1*040101	Diabetes	Proc Natl Acad Sci U S A. 2001 98(4):1763-8.
Insulin	9	SHLVEALYLVCGERG	DRB1*0401 DQB1*0302	Diabetes	Proc. Natl. Acad. Sci. USA Vol. 93, 956-960, 1996
Naturally Processed					
Protein-tyrosine phosphatase-like N precursor	657	VSSQFSDAAQASPSS	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	656	SVSSQFSDAAQASPS	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	655	SSVSSQFSDAAQASP	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	656	SVSSQFSDAAQASPSSHSS	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	652	SRVSSVSSQFSDAAQASPSSHSST	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.

Protein-tyrosine phosphatase-like N precursor	656	SVSSQFSDAAQASPSSHSTPSWC	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	657	VSSQFSDAAQASPSSHSTPSWCE	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	656	SVSSQFSDAAQASPSSHSS	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	652	SRVSSVSSQFSDAAQASPSSHST	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	656	SVSSQFSDAAQASPSSHSTPSWC	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	718	AYQAEPNTCATAQ	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	716	LCAYQAEPNTCATAQG	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	709	LAKEWQALCAYQAEPNT	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	718	AYQAEPNTCATAQGEGNIK	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	713	WQALCAYQAEPNTCATAQ	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449-57.
Protein-tyrosine phosphatase-like N precursor	754	KVESSPSRSDYI	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	753	LKVESSPSRSDY	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	752	KLKVESSPSRSDYINAS	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	754	KVESSPSRSDYINASPIEHDP	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	802	GCTVIVMLTPLVED	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	803	CTVIVMLTPLVEDG	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	800	ESGCTVIVMLTPLVEDG	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449

Protein-tyrosine phosphatase-like N precursor	797	MVWESGCTVIVMLTPL	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	800	ESGCTVIVMLTPLVEDGV	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	795	WQMWWESGCTVIVMLT	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	793	DFWQMWWESGCTVIVMLT	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	794	FWQMWWESGCTVIVMLTPLV	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	861	TQETRILTQFHF	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	855	YLKNVQTQETRTL	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	859	VQTQETRILTQFHF	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	855	YLKNVQTQETRILTQ	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	857	KNVQTQETRILTQFH	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	853	SFYLNKNVQTQETRILTQFH	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	957	DQFEFALTAVAAEE	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	957	DQFEFALTAVAAEEVNAI	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	959	FEFALTAVAAEEVNAILKA	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	955	SKDQFEFALTAVAAEEVNA	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	955	SKDQFEFALTAVAAEEVNAILK	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	854	FYLNKNVQTQETRILTQFHF	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449

Protein-tyrosine phosphatase-like N precursor	797	MVWESGCTVIVMLTPLVEDGV	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	709	LAKEWQALCAYQAEPNTCATAQGE	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449
Protein-tyrosine phosphatase-like N precursor	654	VSSVSSQFSDAAQASPSSHSS	DRB1*040101	Diabetes	J Clin Invest. 1999 104(10):1449

FIG. 1X **Autoimmune Peptides**
Multiple Sclerosis

Name/Descriptions	Position	Peptide	HLA	Disease	Misc.
T cell Epitopes					
Myelin Proteolipid Protein (PLP)	95	AVRQIFGDYKTTICGKGLSATV	DRB1*150201	MS	J.Neuroimmunol. 1998, 84(2):172-8.
Myelin Proteolipid Protein (PLP)	79	FLYGALLA	A*020101	MS	Proc Natl Acad Sci USA 1994 91(23):10859-63
T cell Epitopes					
Myelin Basic Protein (MBP)	169	GVDAQGTLSKIFKLGGDRSRGSPMA	DRB1*150101	MS	Cell. 1995 80(5):695-705.
Myelin Basic Protein	136	SLSRFSWGA	A*020101	MS	Proc Natl Acad Sci USA 1994 91(23):10859-63
Myelin Basic Protein	137	LSRFSWGAEGQRPGFGYGG	DRB1*040101	MS	J Clin Invest 1997 100(2):339-49
Myelin Basic Protein	111	PVVHFFKNIVT	DRB1*150101	MS	J Exp Med 1994 179(1):279-90
Myelin Basic Protein	113	VHFFKNIVTPRTP	DRB5*010101	MS	J Exp Med 1991 173(1):19-24
Myelin Basic Protein	165	KGFKGVDAQGTLSKI	DRB5*010101	MS	J Immunol 1994 153(4):1665-73
Naturally Processed					
Myelin Basic Protein	176	LSKIFKLGGDRSRGSPMAR	DRB1*010101	MS	Eur J Immunol 1997 (4):941-51
Myelin Basic Protein	112	VVHFFKNIVTPRTPPPSQGK	DRB1*0404	MS	J Immunol 1992 148(5):1359-66
Myelin Basic Protein	157	ASDYKSAHKGFKGVD	DRB5*010101	MS	J Immunol 1994 153(4):1665-73

Name/Descriptions	Position	Peptide	HLA	Disease	Misc.
T-Cell Epitopes					
Myelin-associated Glycoprotein (MAG)	556	VLFSDFRI	A*020101	MS	Proc Natl Acad Sci USA 1994 91(23):10859-63
Myelin-associated Glycoprotein (MAG)	509	LMWAKIGPV	A*020101	MS	Proc Natl Acad Sci USA 1994 91(23):10859-63
Myelin-associated Glycoprotein (MAG)	287	SLLLEEEV	A*020101	MS	Proc Natl Acad Sci USA 1994 91(23):10859-63

FIG. 1Y

Autoimmune Peptides Goodpasture Syndrome

Name/Descriptions	Position	Peptide	HLA	Disease	Misc.
T cell Epitopes					
Collagen alpha 3(IV) Chain Precursor	1436	GSPATWTTR	A*110101	GS	Kidney Int 1996 49(4):1127-33
Naturally Processed					
Collagen alpha 3(IV) Chain Precursor	1594	TGQALASPGSCLEEFRASPF	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440-7.
Collagen alpha 3(IV) Chain Precursor	1595	GQALASPGSCLEEFRASPFLECH	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1597	ALASPGSCLEEFRASPF	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1597	ALASPGSCLEEFRASPFLE	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1605	LEEFRASPFLECHGRGTON	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1496	RFTTMPFLFCNVNDVCNF	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440

Collagen alpha 3(IV) Chain Precursor	1501	PFLFCNVNDVCNFAFR	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1503	LFCNVNDVCNFAFRND	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1459	SCPEGTVPPLYSFGSFLFVQ	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1458	PSCPEGTVPPLYSFGSFLFVQG	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440
Collagen alpha 3(IV) Chain Precursor	1463	GTVPLYSFGSFLFVQGNQRAHG	DRB1*150101	GS	J Biol Chem. 1998 273(19):11440

FIG. 1Z₁

Autoimmune Peptides Primary Biliary Cirrhosis

Name/Descriptions	Position	Peptide	HLA	Disease	Misc.
		T Cell Epitopes			
Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex precursor	216	GDLLAEIETDKATI	DRB4*010101	PBC	J Exp Med 1995 181(5):1835-45

FIG. 1Z₂

Autoimmune Peptides Pemphigus Vulgaris

Name/Descriptions	Position	Peptide	HLA	Disease	Misc.
		T Cell Epitopes			
Desmoglein 3 precursor (human)	190	LNSKIAFKIVSQEPA	HLA-DRB1*0402	Pemphigus	Proc Natl Acad Sci USA 5;92(25):11935-9

FIG. 1Z₃

Autoimmune Peptides Celiac Disease

Name/Descriptions	Position	Peptide	HLA	Disease	Misc.
		T cell Epitopes			
A-gliadin - partially deamidated peptide	57	QLQPFPPQPELPYPQPQS	DQA1*0501 DQB1*0201	Celiac Disease	Nat Med. 2000 6(3):337-42.

Figures 2A-2B:

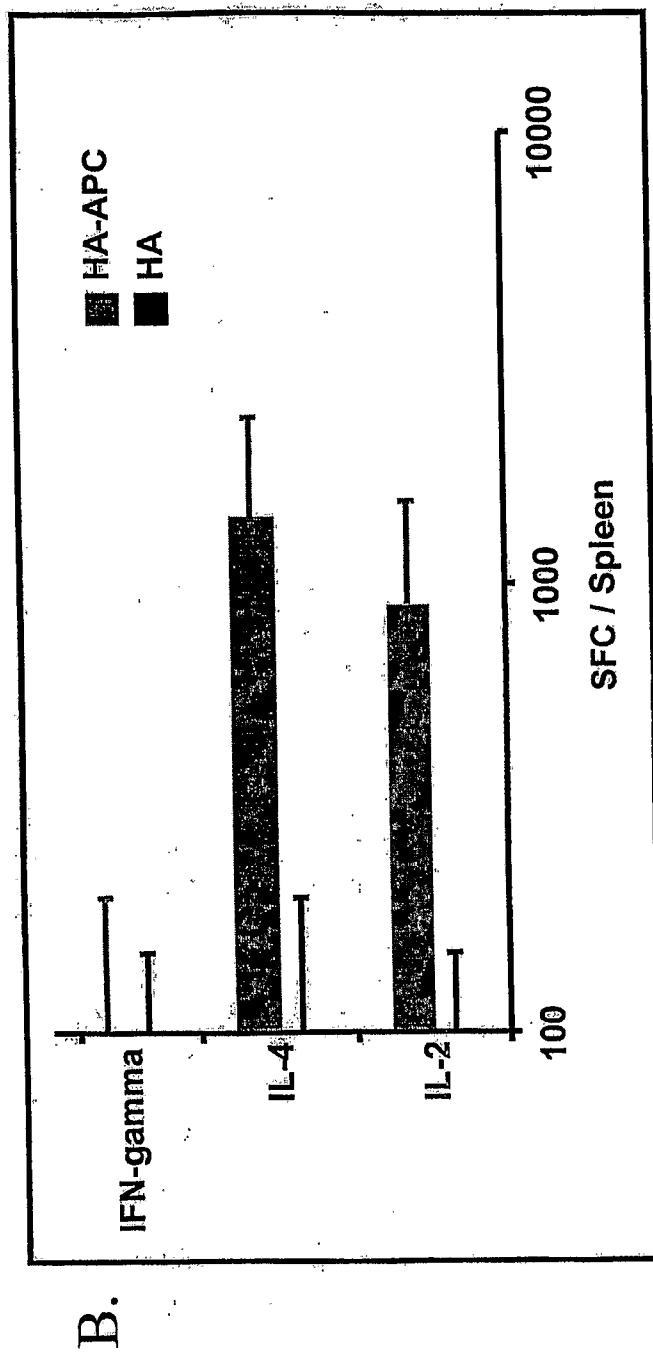
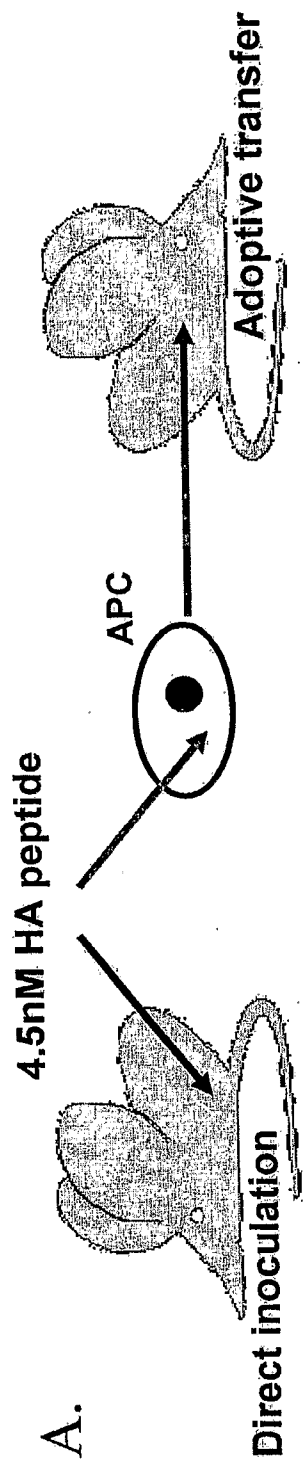
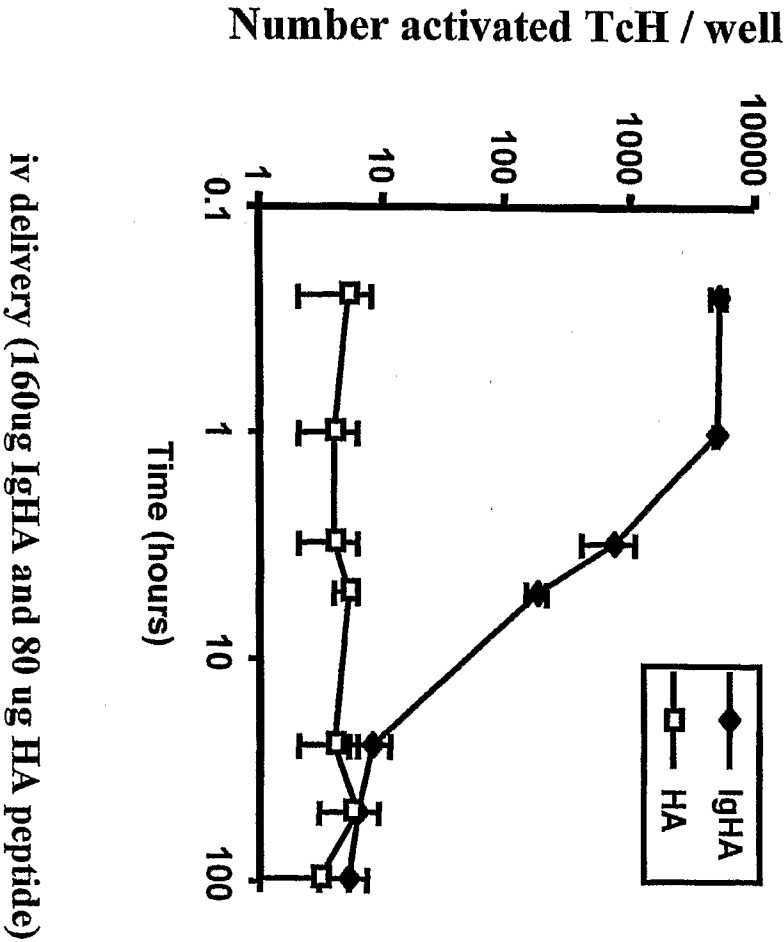
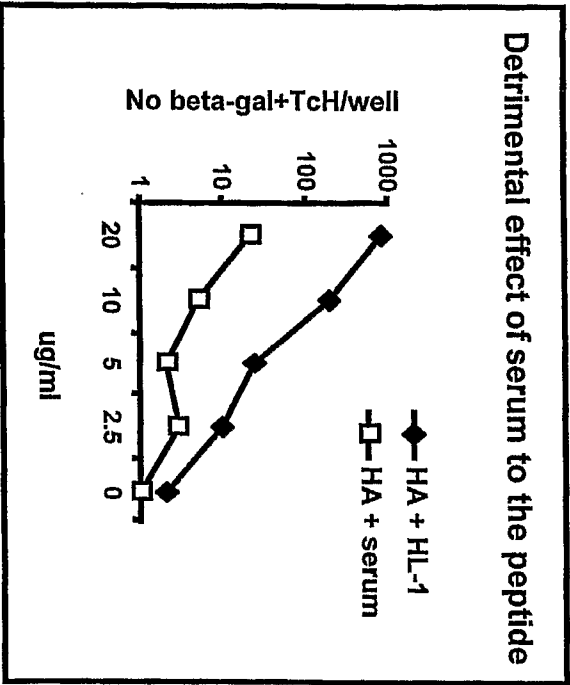


Figure 3:

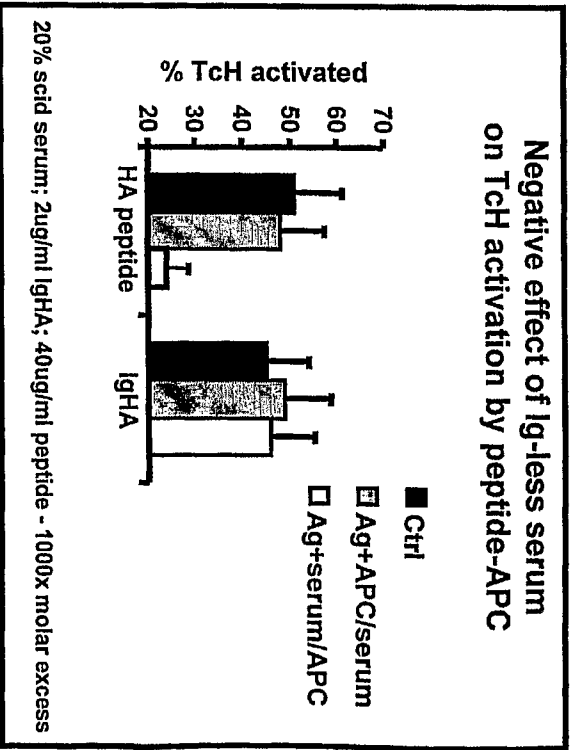


Figures 4A-4B:

A.

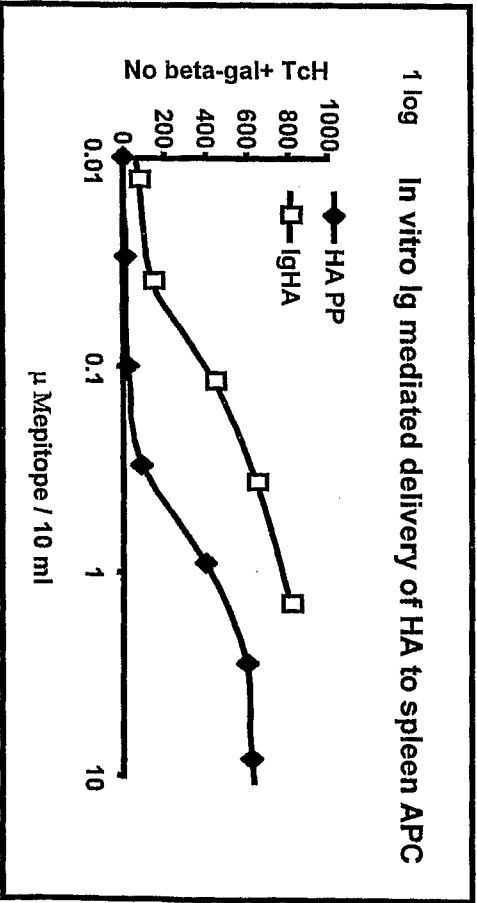


B.

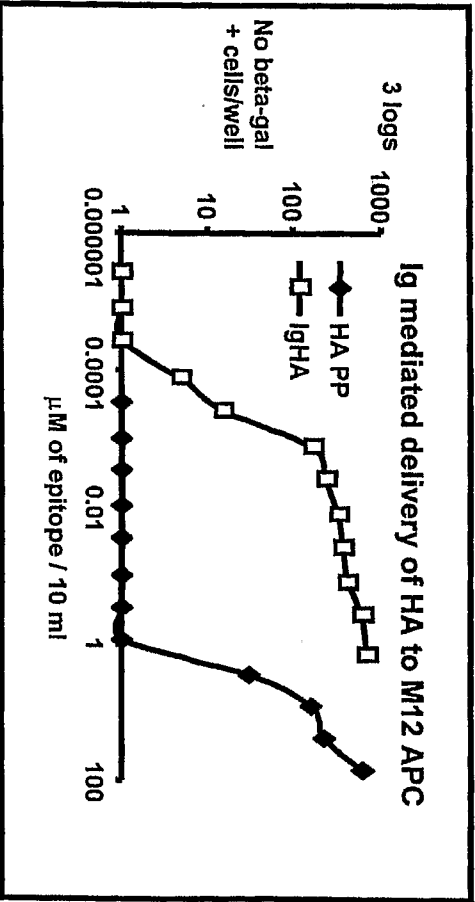


Figures 5A-5B:

A.

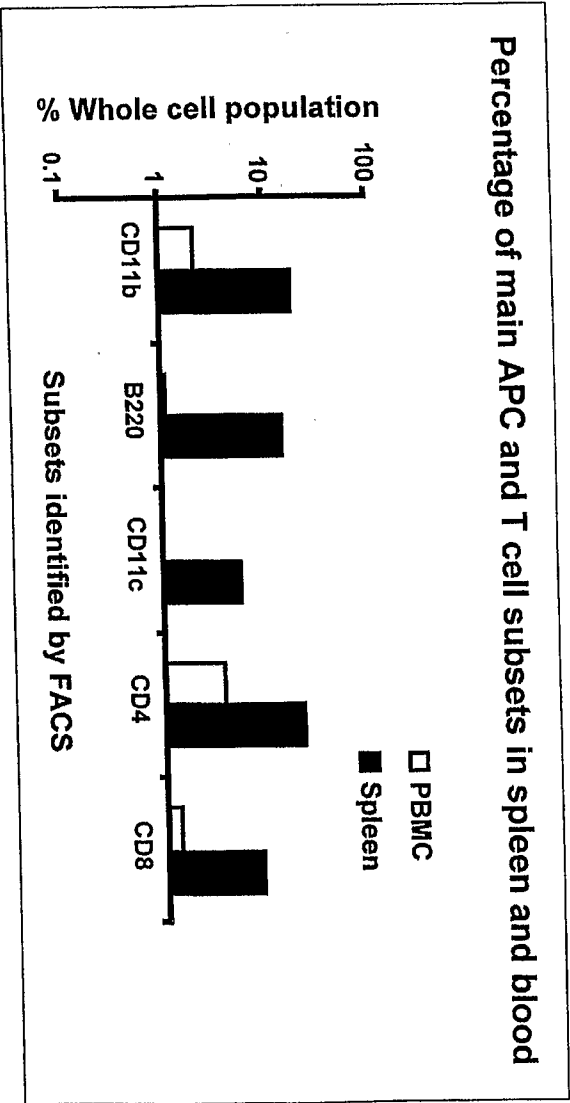


B.



Figures 6A-6B:

A.



B.

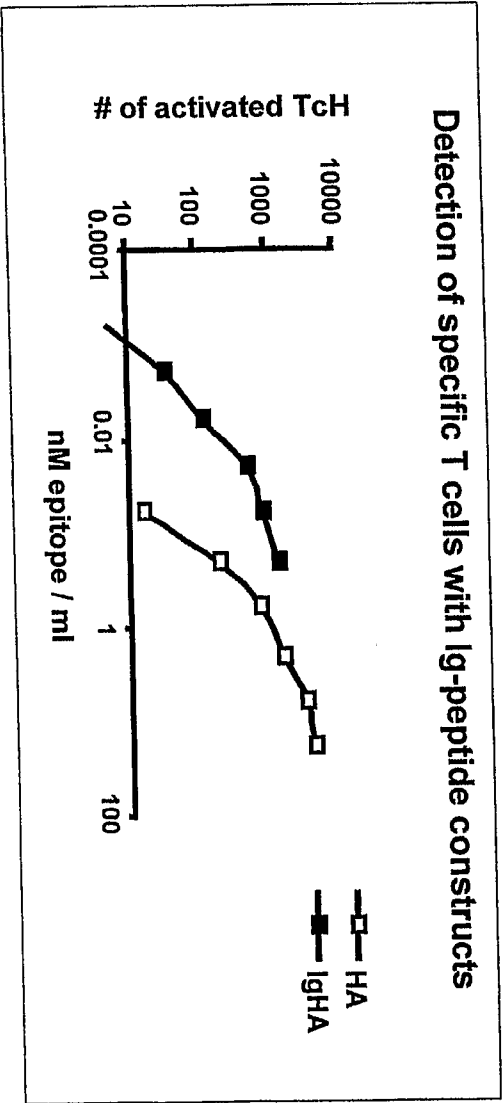


Figure 7A:

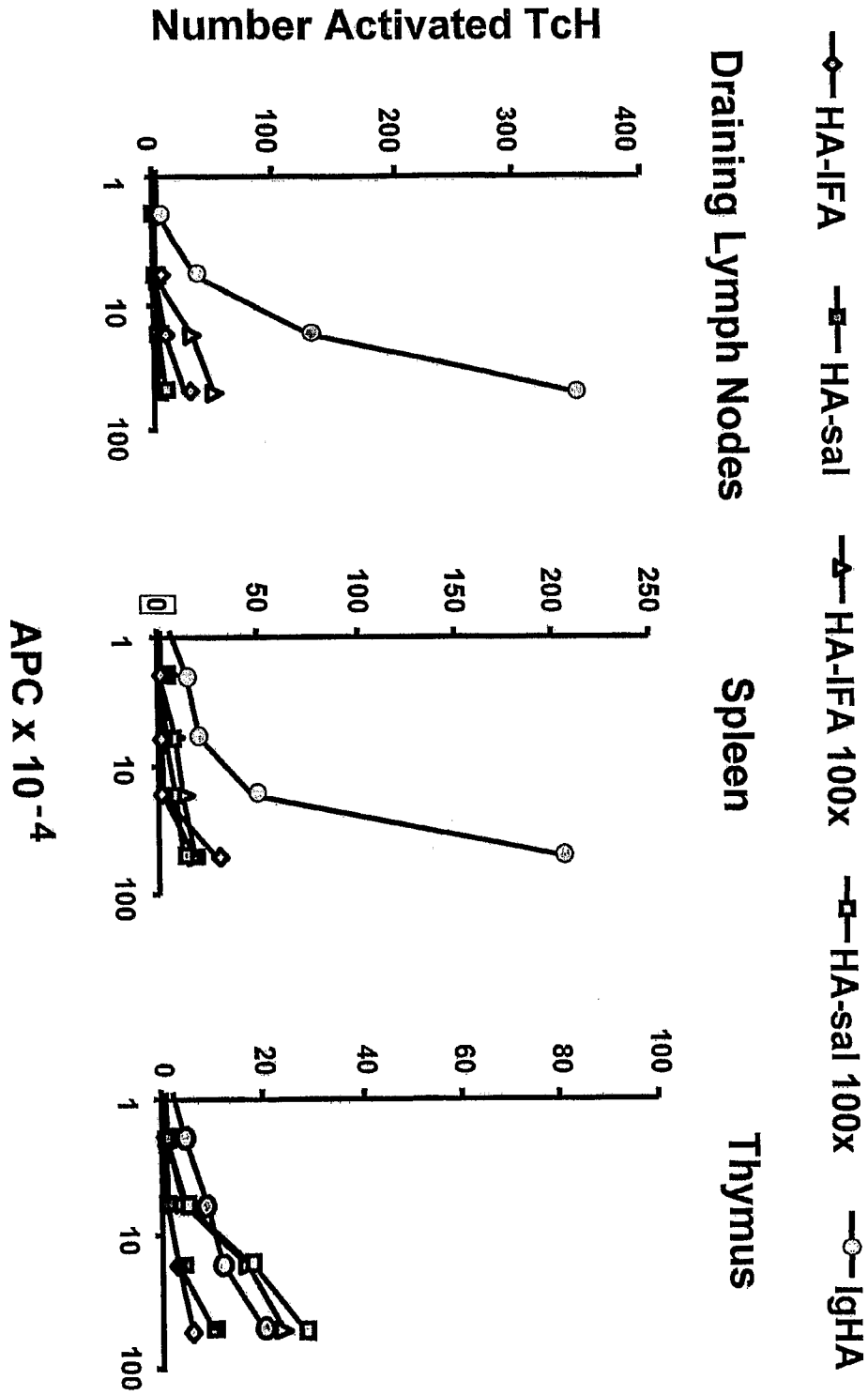


Figure 7B:

Superior in vivo loading of APC by Ig-peptide technology

	Spleen	Local lymph nodes	Thymus
HA -saline (4.5nM)	0.12-0.4*	<0.04	0.12
HA-saline (450nM)	0.4-1.2	0.12-0.4	1.1-3.5
HA-IFA (4.5nM)	0.12-0.4	0.04-0.12	0.04-0.12
HA-IFA (450nM)	0.4-1.2	3.5-10.0	1.1-3.5
IgHA (0.45nM)	1.2-3.5	0.4-1.2	1.1-3.5
IgHA (4.5nM)	10.0	10.0-33.3	1.1-3.5

% of APC that are able to present peptide in given condition

Figures 8A-8D:

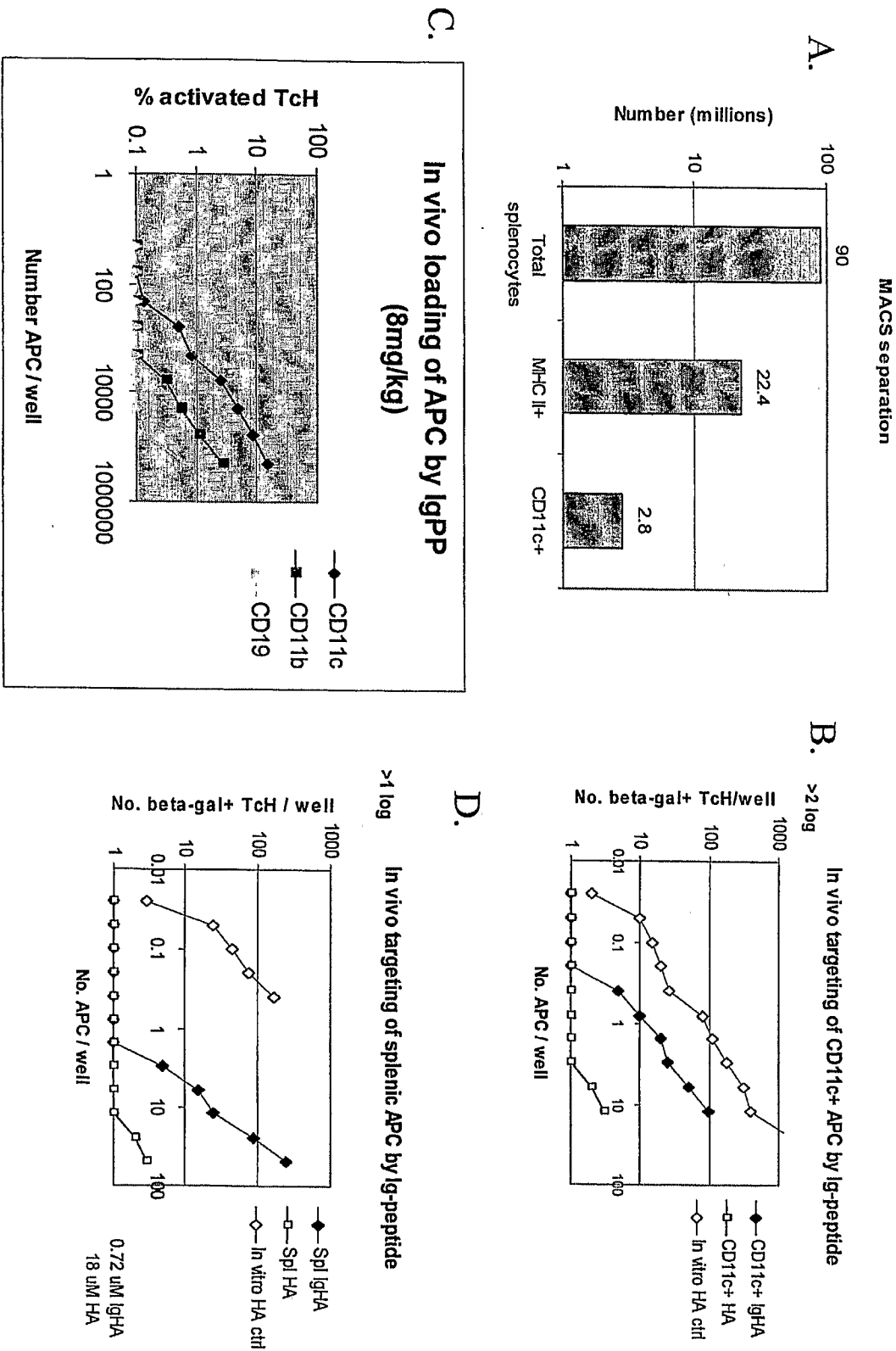
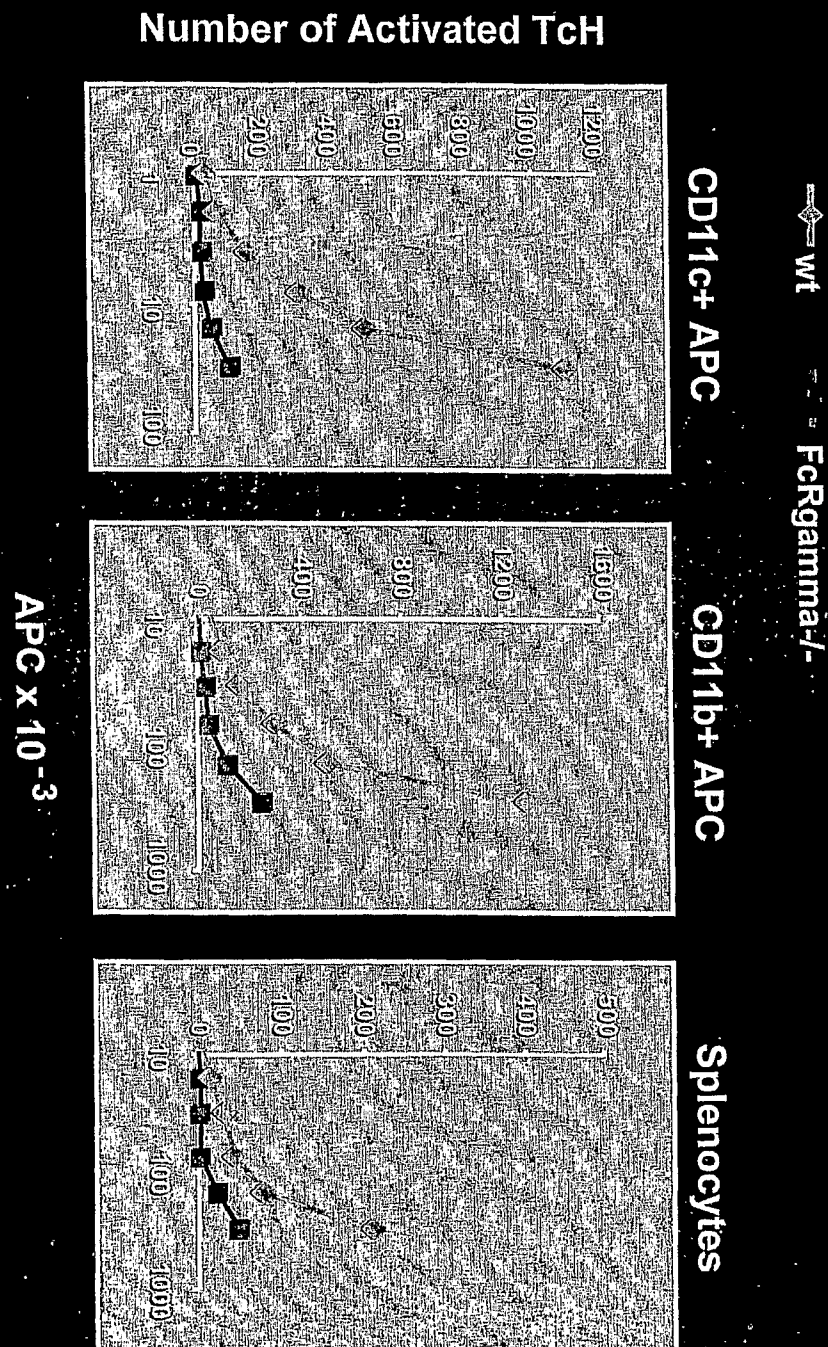
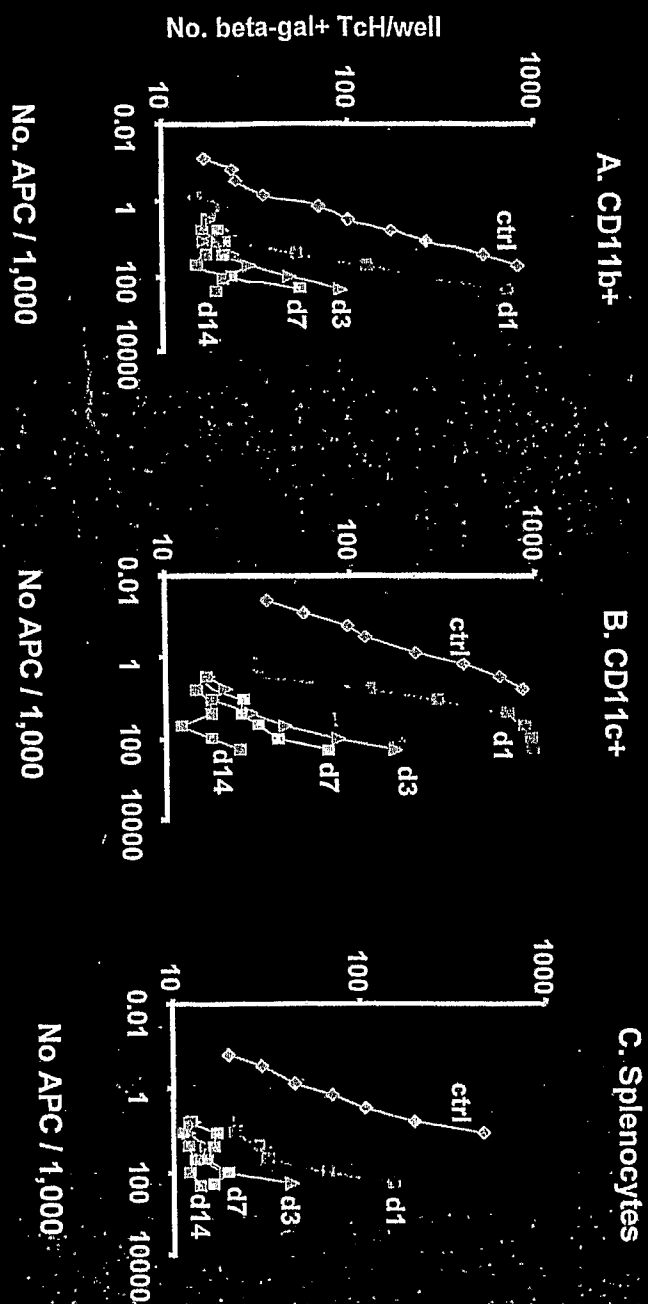


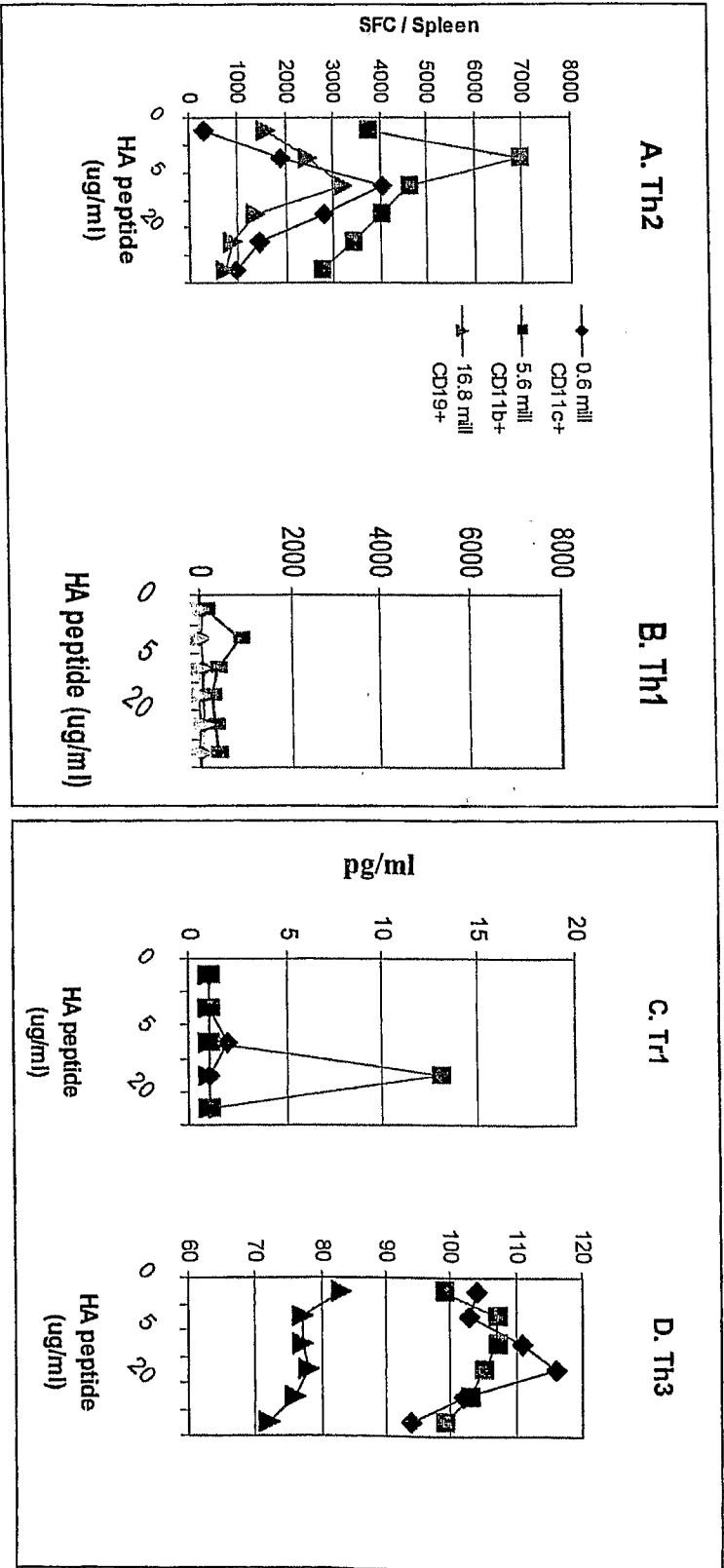
Figure 10:



Figures 9A-9C:



Figures 12A-12D:



Figures 13A-13B:

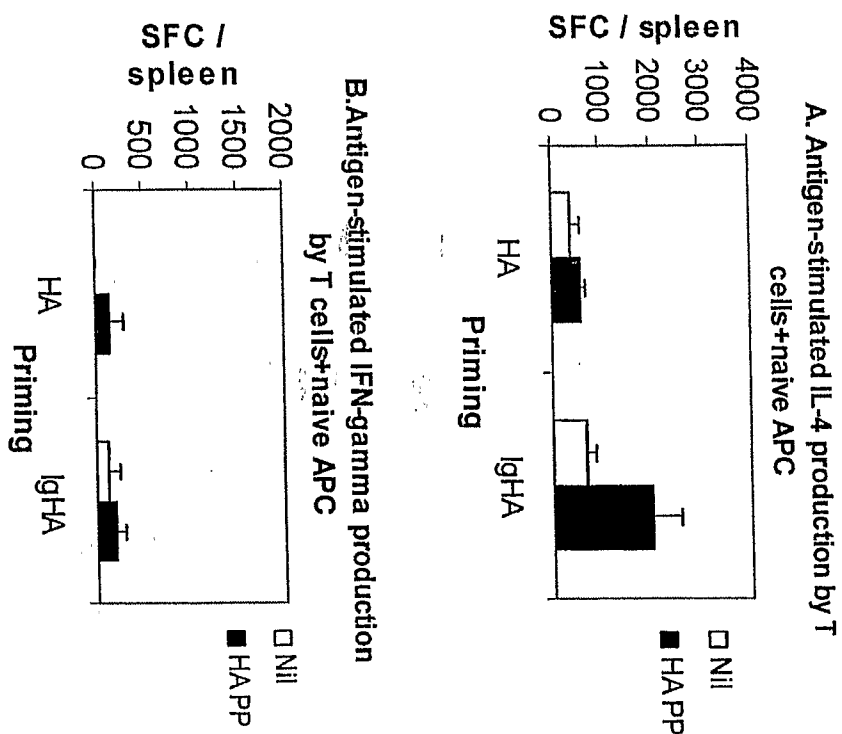


Figure 14:

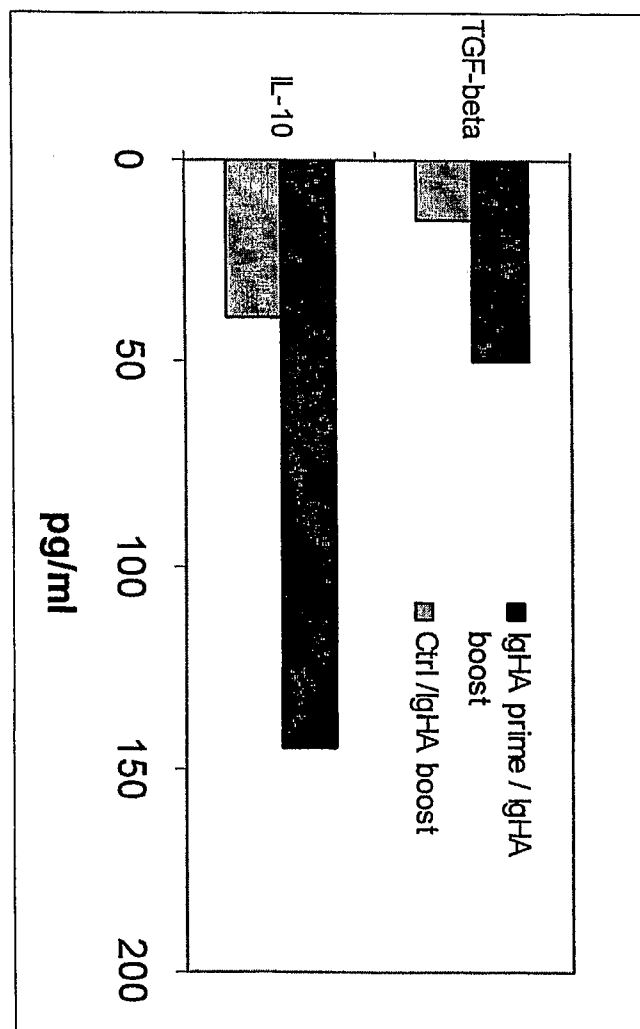


Figure 15:

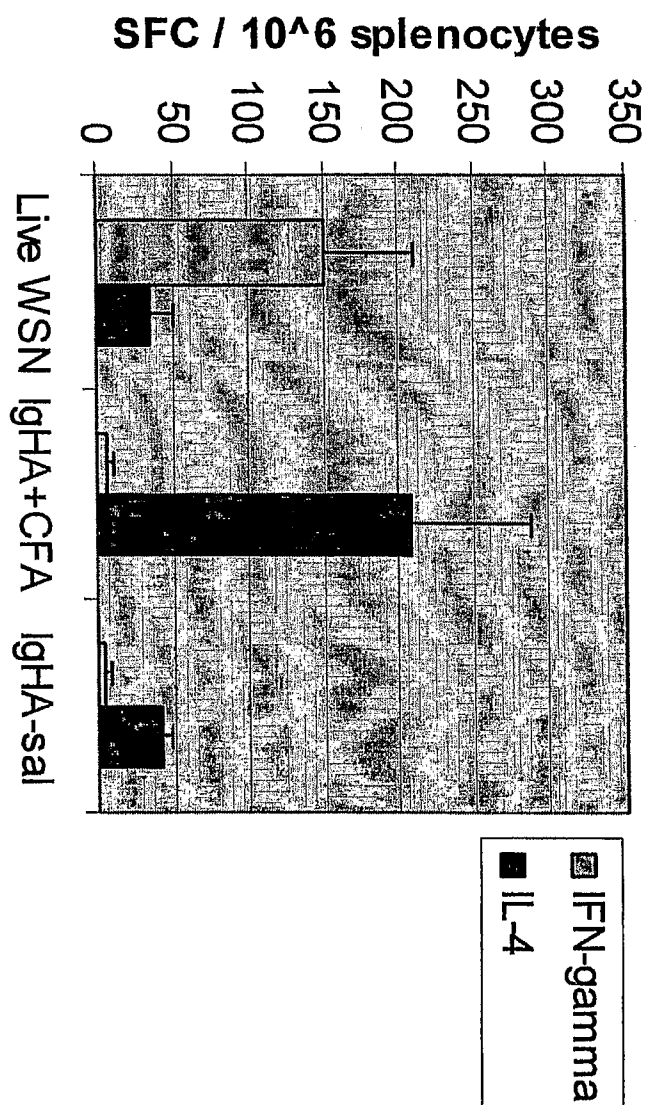
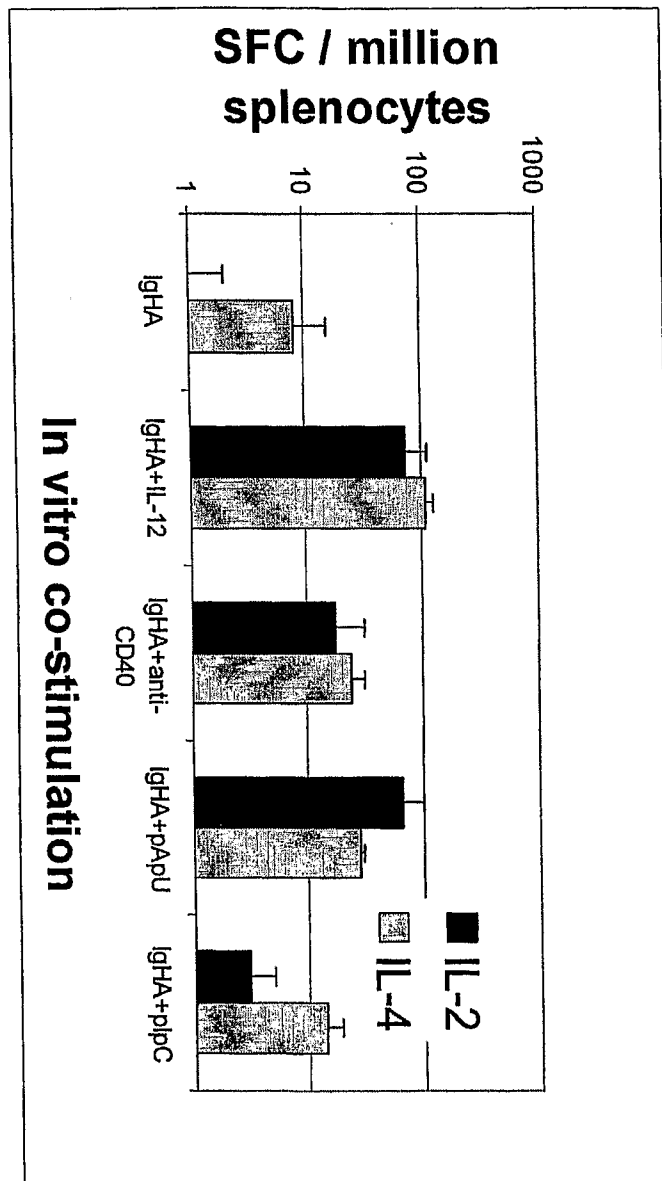
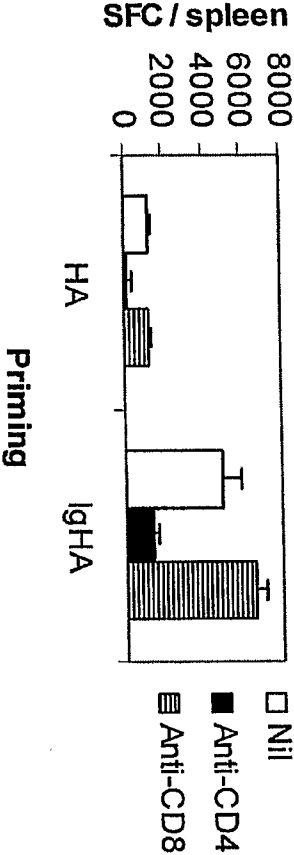


Figure 16:



Figures 17A-17B:

A. Ongoing IL-4 production is CD4 dependent



B. Dependency of IL-4 production on endogenous APC

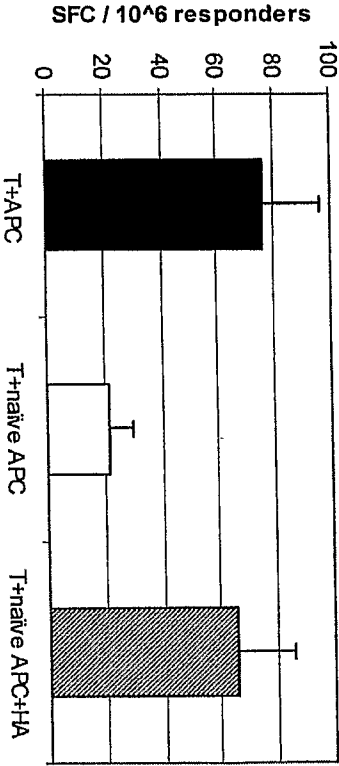


Figure 18:

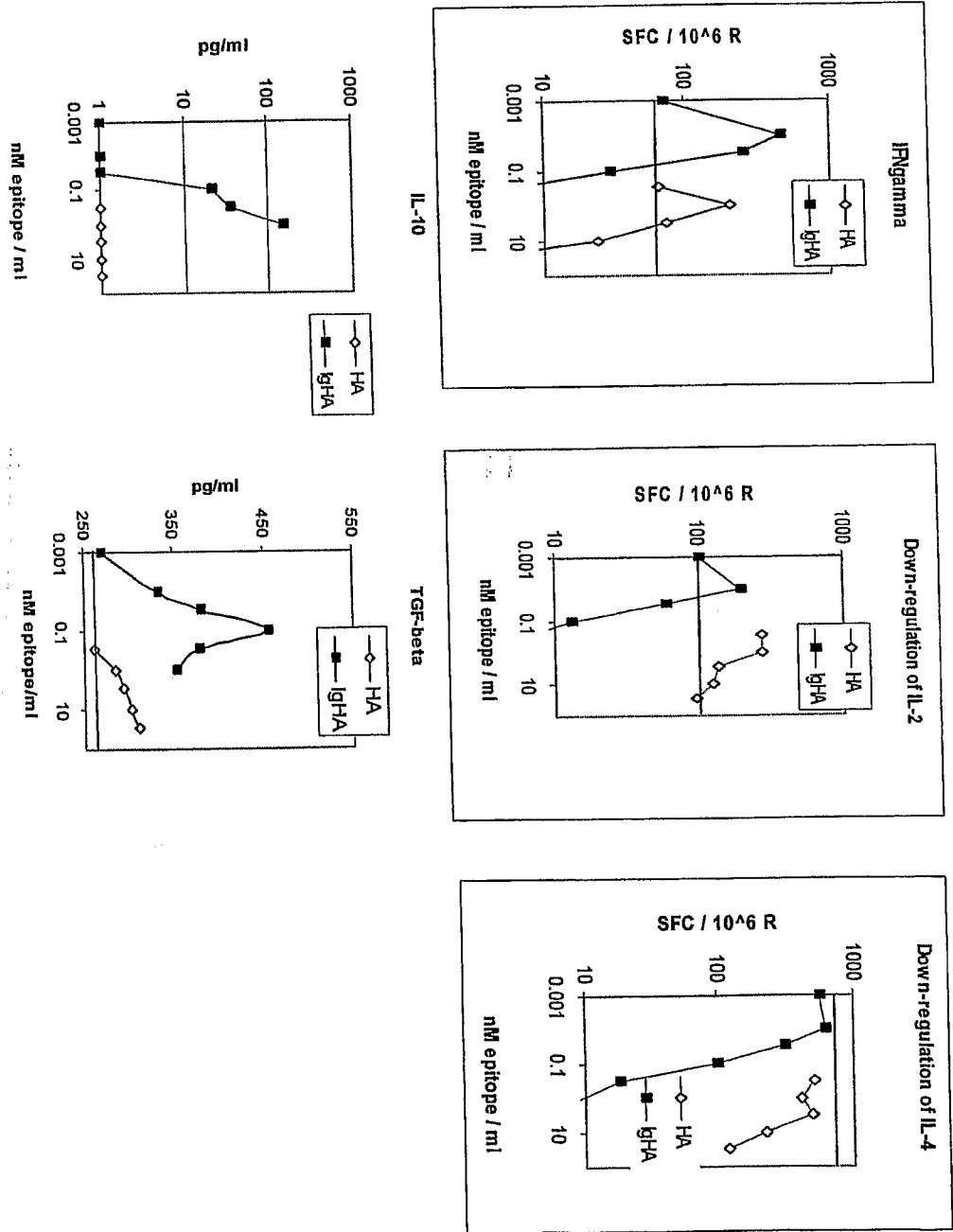


Fig. 19A: The Default Pathway: Induction of Tc2 Immunity

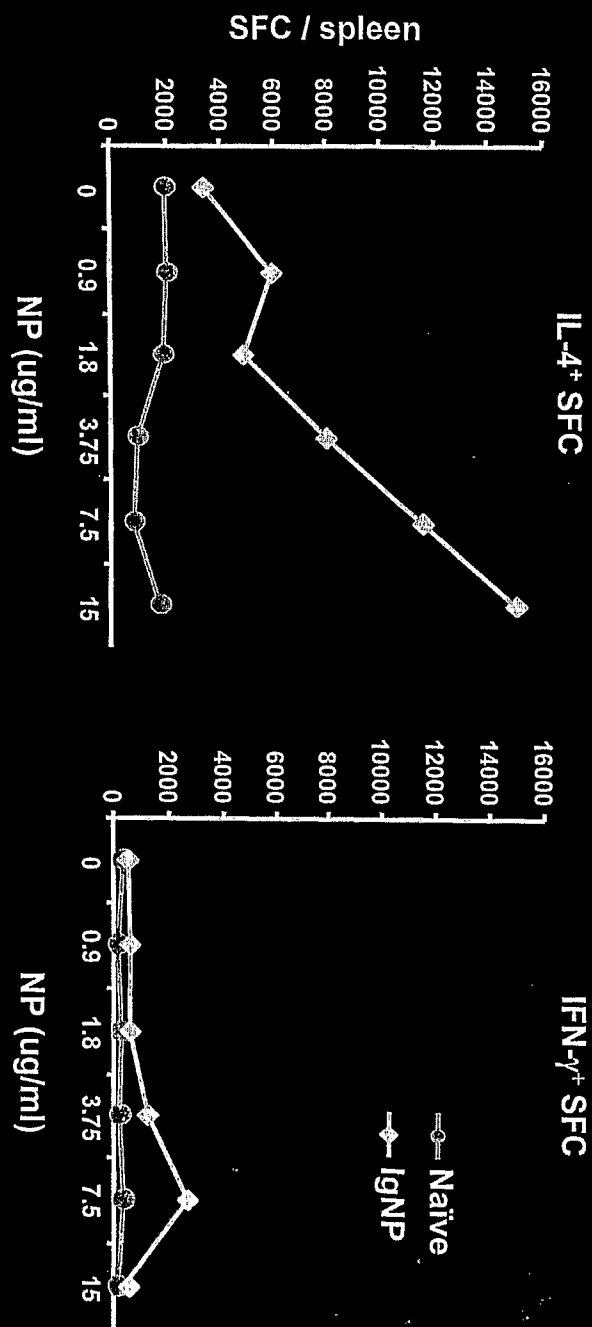
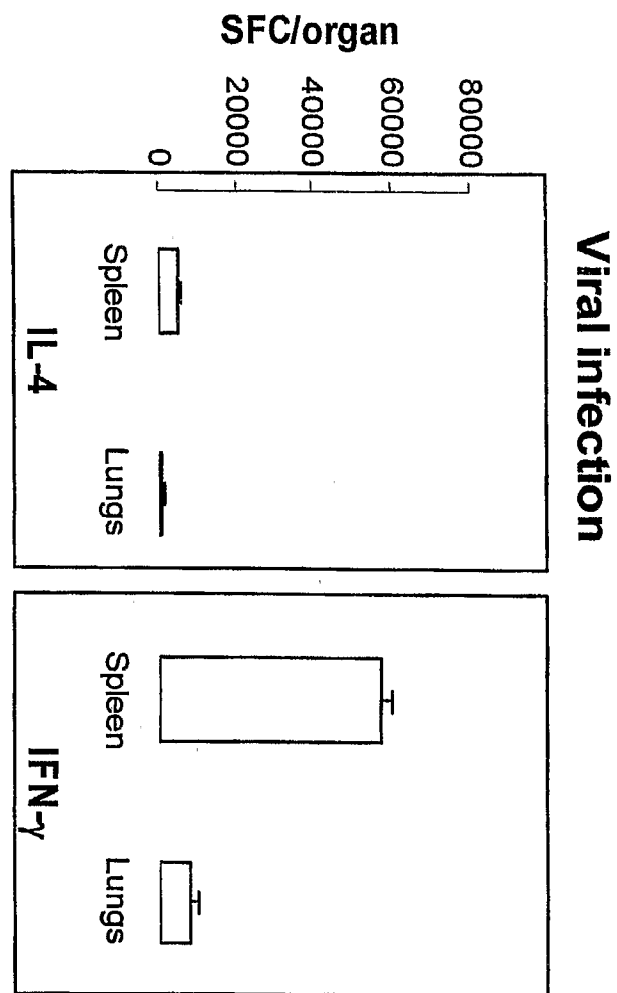
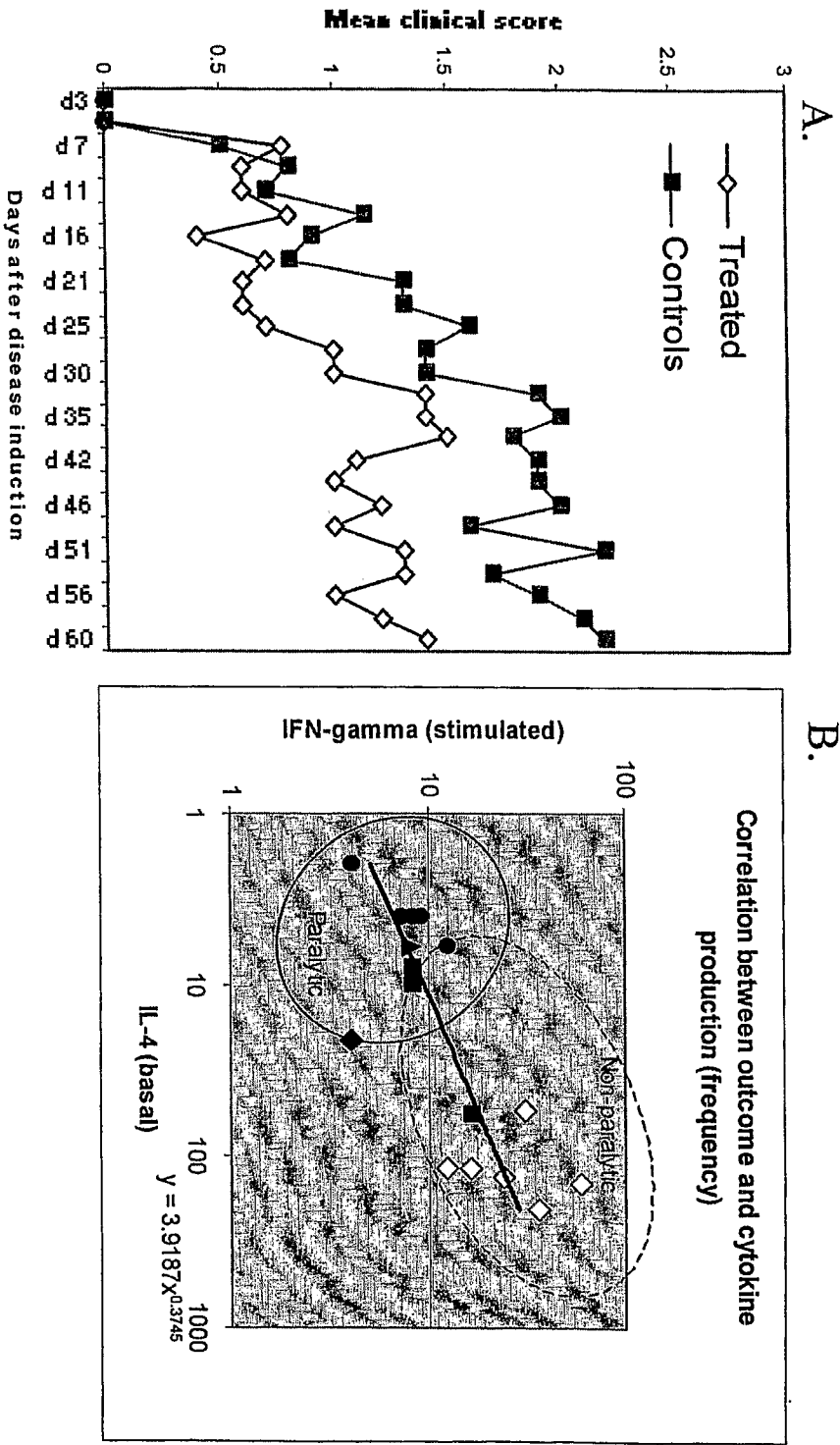


Fig. 19B.

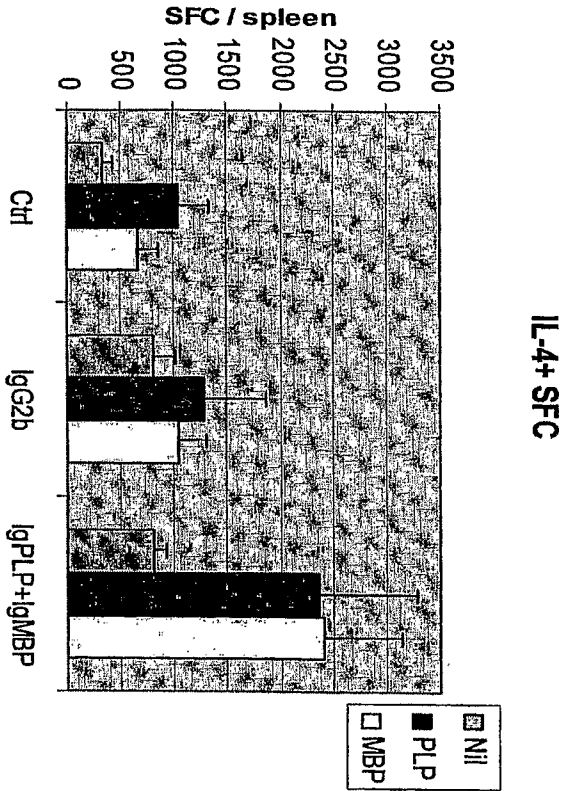


Figures 20A-20B:



Figures 20C-20D:

C.



D.

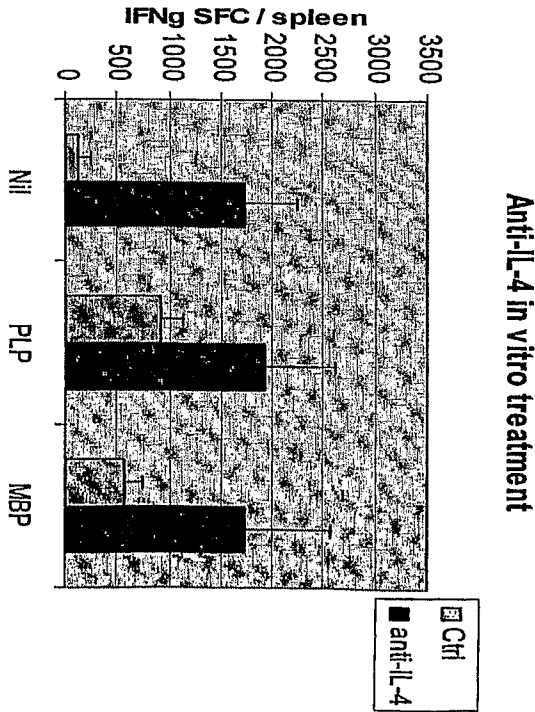


Figure 21:

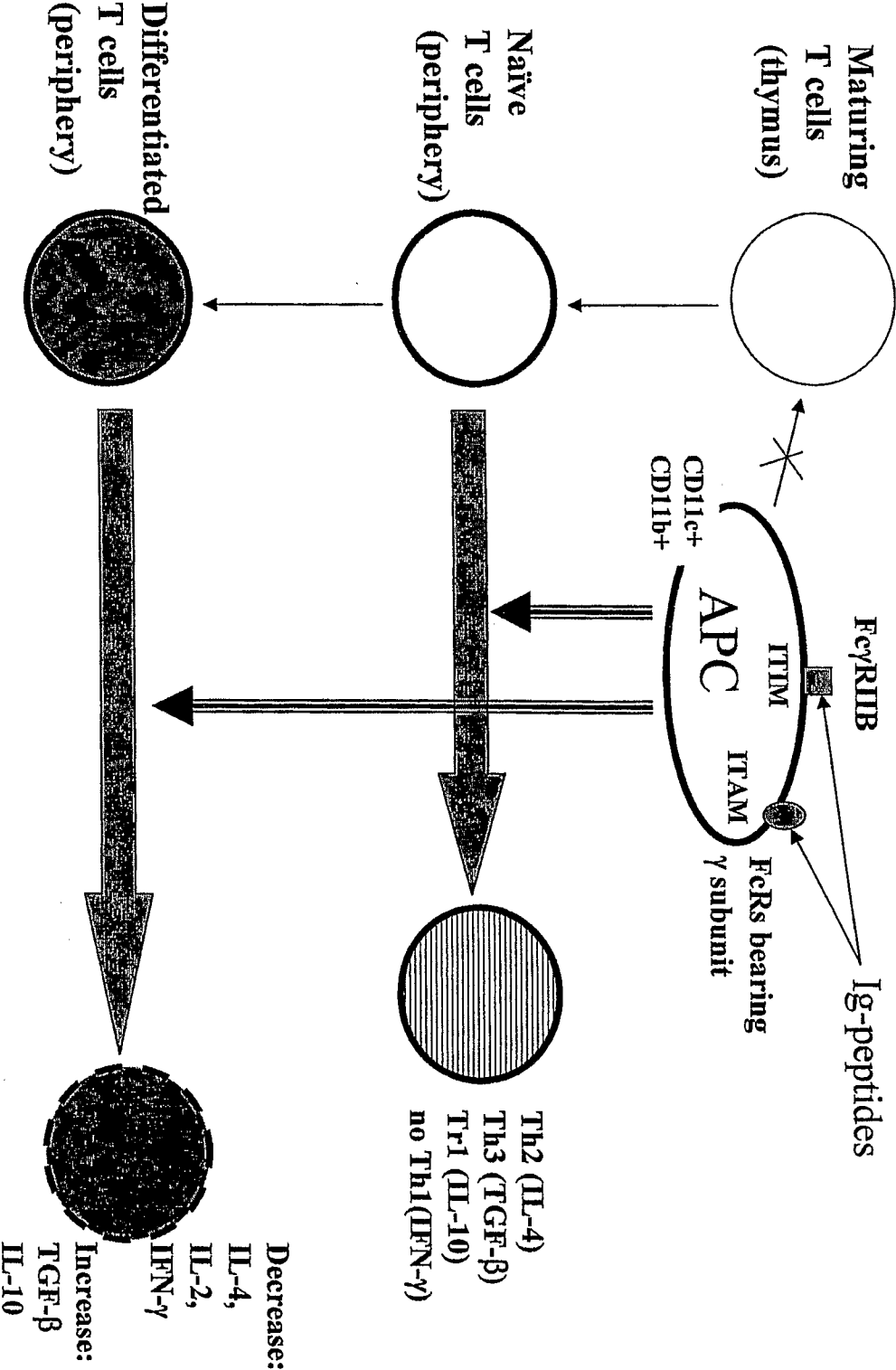


Figure 22:

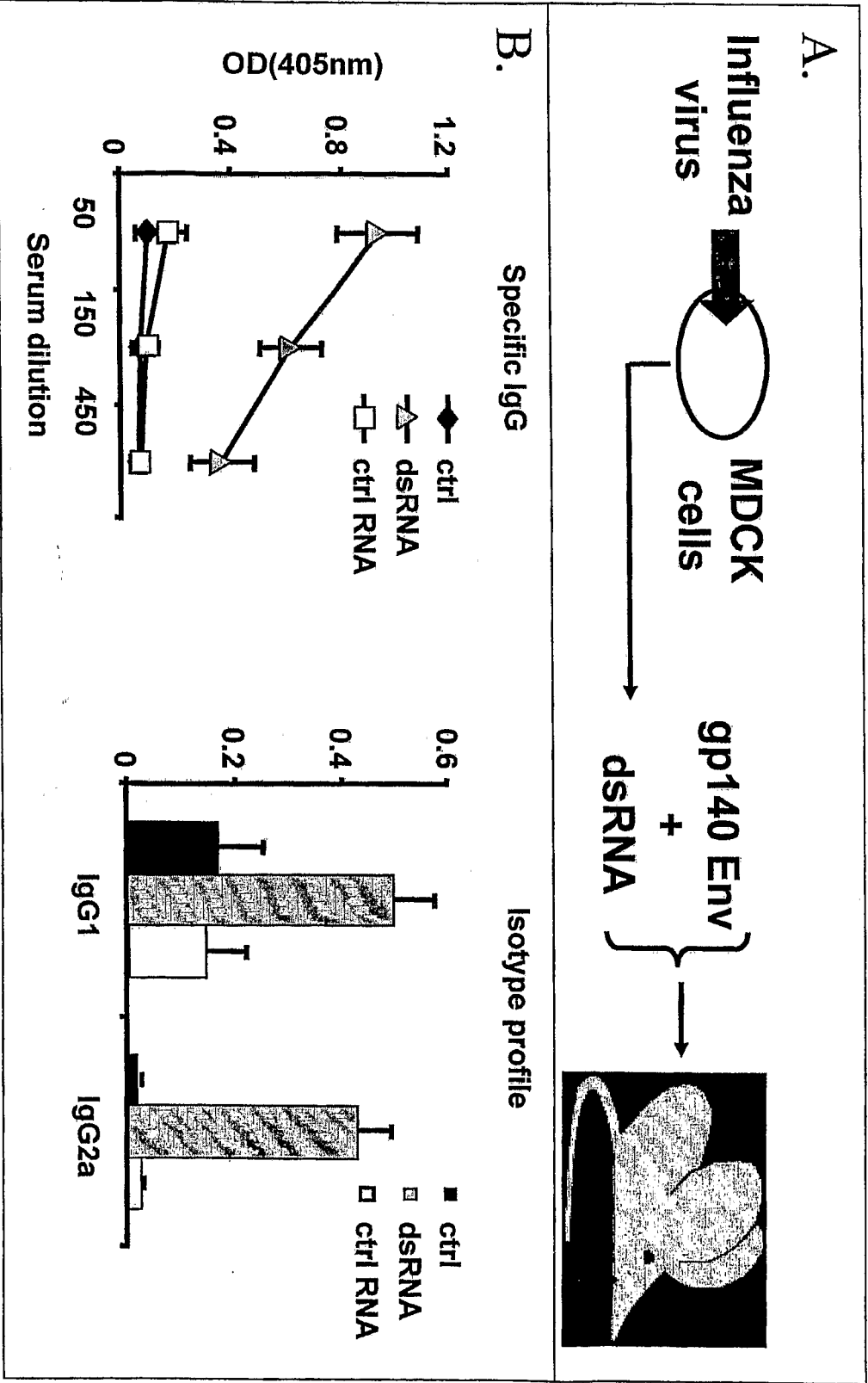
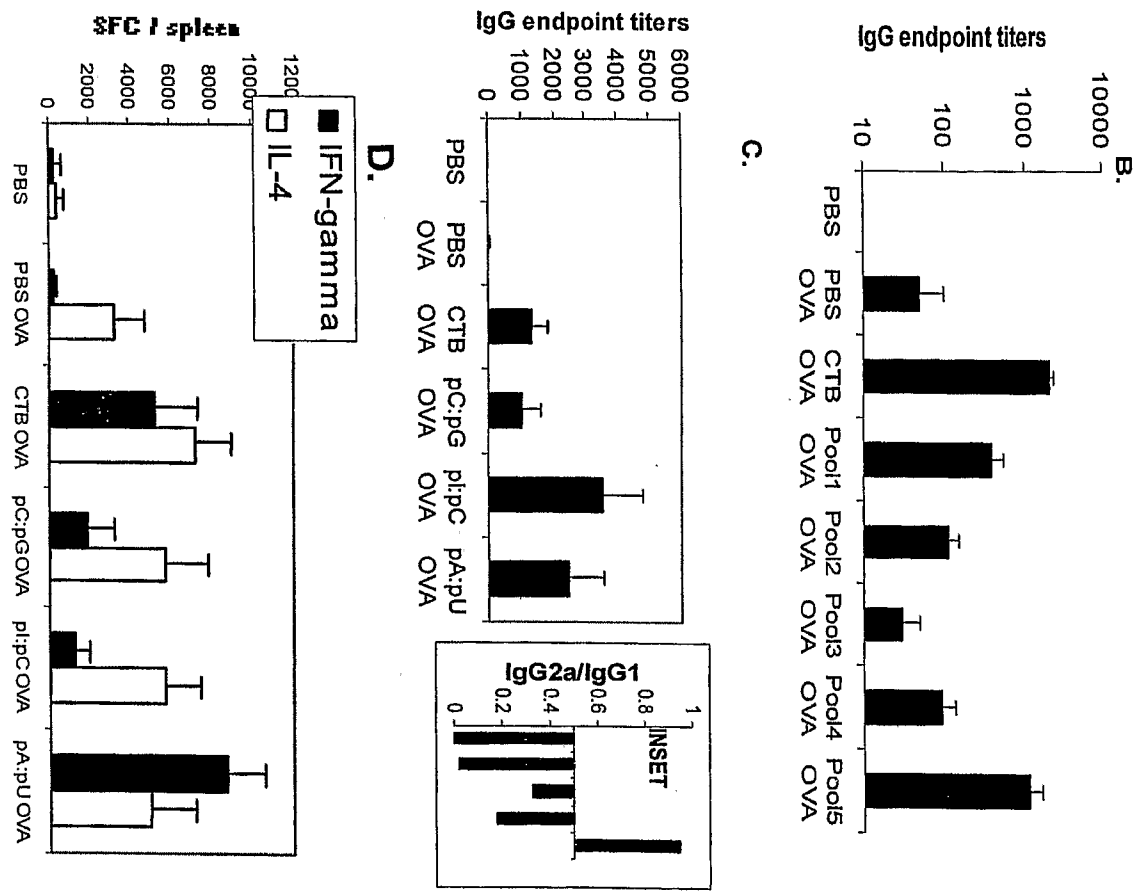


Figure 23A:

Nucleotide composition of synthetic RNA compounds used for screening of immunomodulatory motifs.

Category of RNA ^A	Nucleotide composition	
Single-stranded RNA	Group 1 (Pool 1)	p ^B (A); p(C); p(G); p(U); p(U)
	Group 2 (Pool 2)	p(G,U); p(C,U); p(A,C); p(U,U)
	Group 3 (Pool 3)	p(C,D); p(A,U); p(A,G)
	Group 4 (Pool 4)	p(A,C,G); p(A,C,U); p(A,G,U)
Double-stranded RNA	Group 5 (Pool 5)	pC:pG; pA:pU; pI:pC

Figures 23B-23D:



Figures 24A-24B:

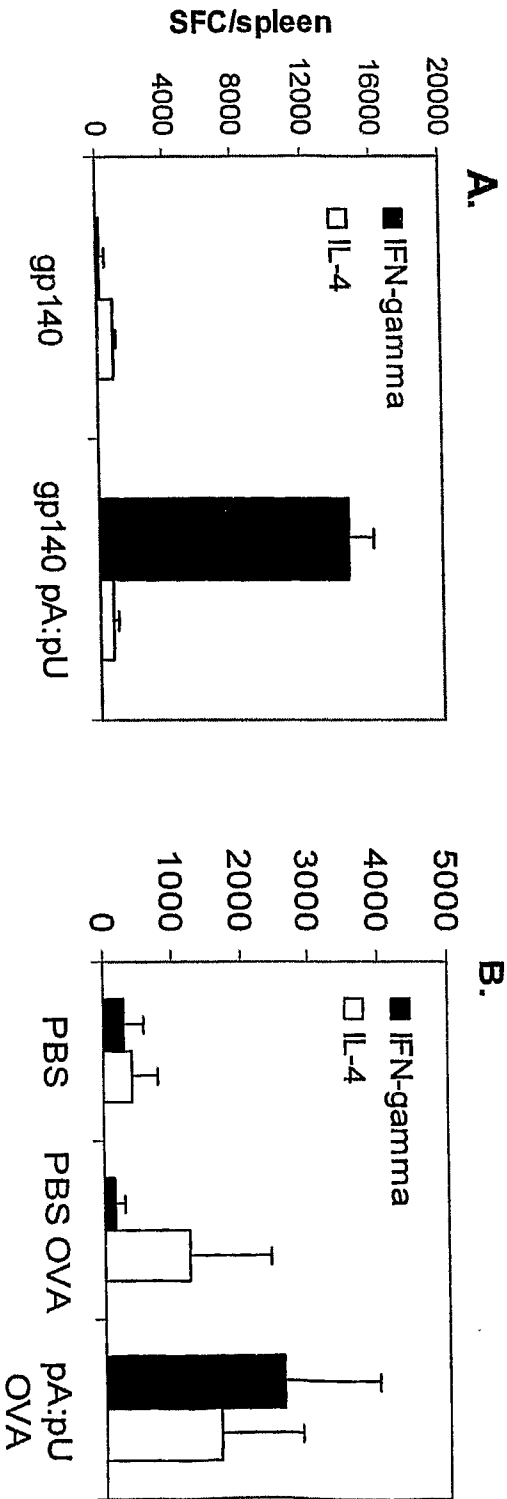


Figure 25:

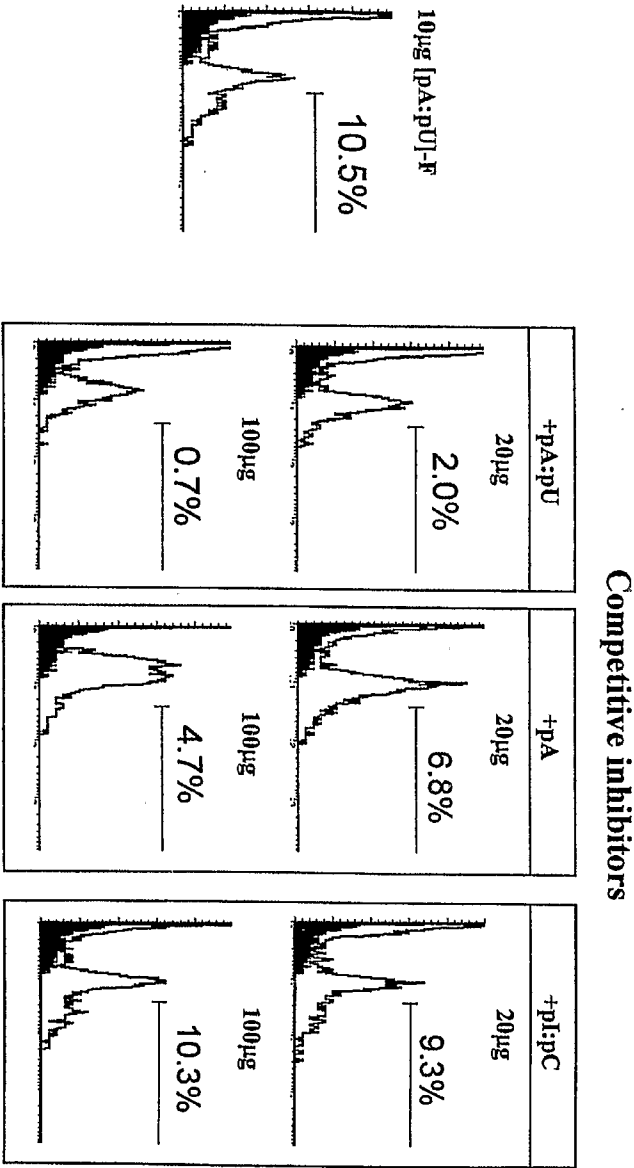


Figure 26:

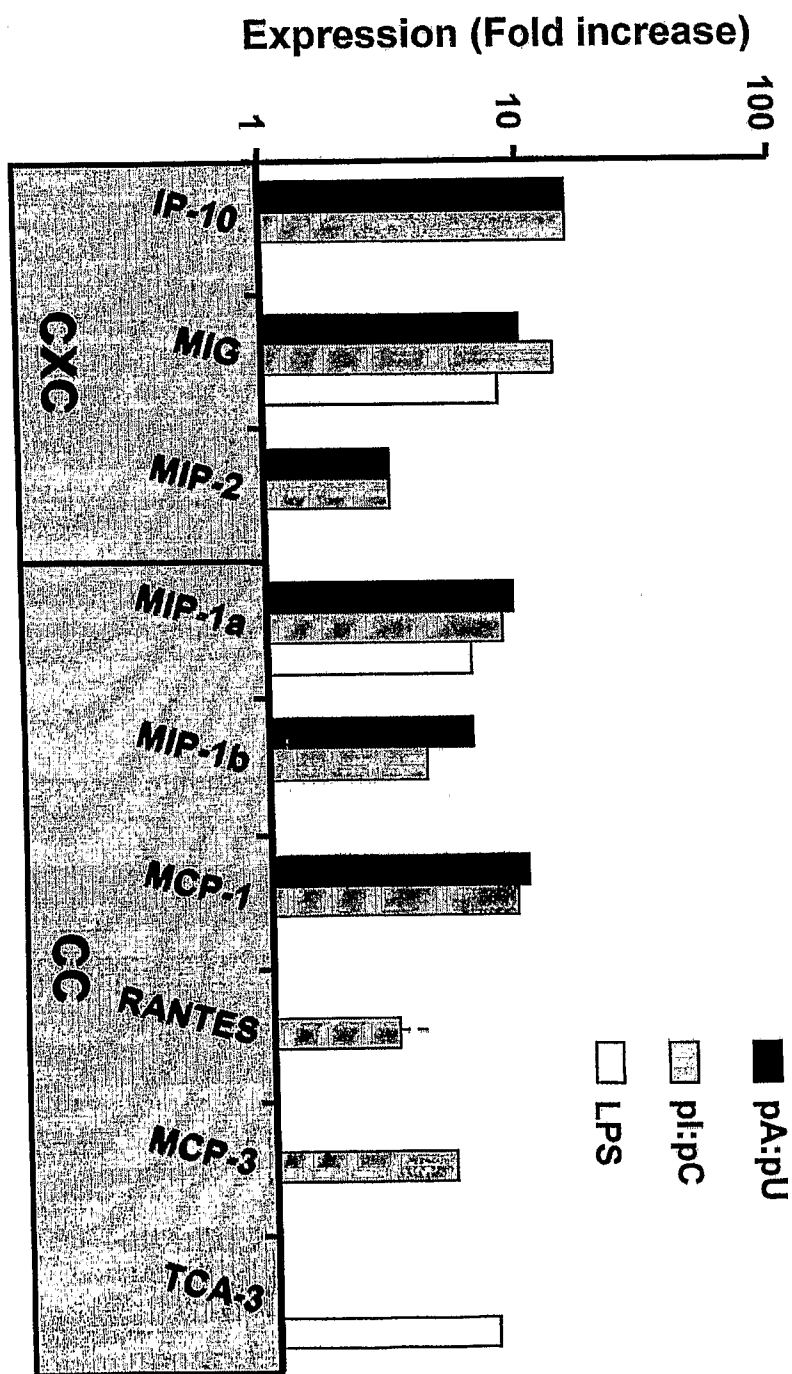


Fig. 27
Potent Anti-Viral Activity
of Selected dsRNA Motifs

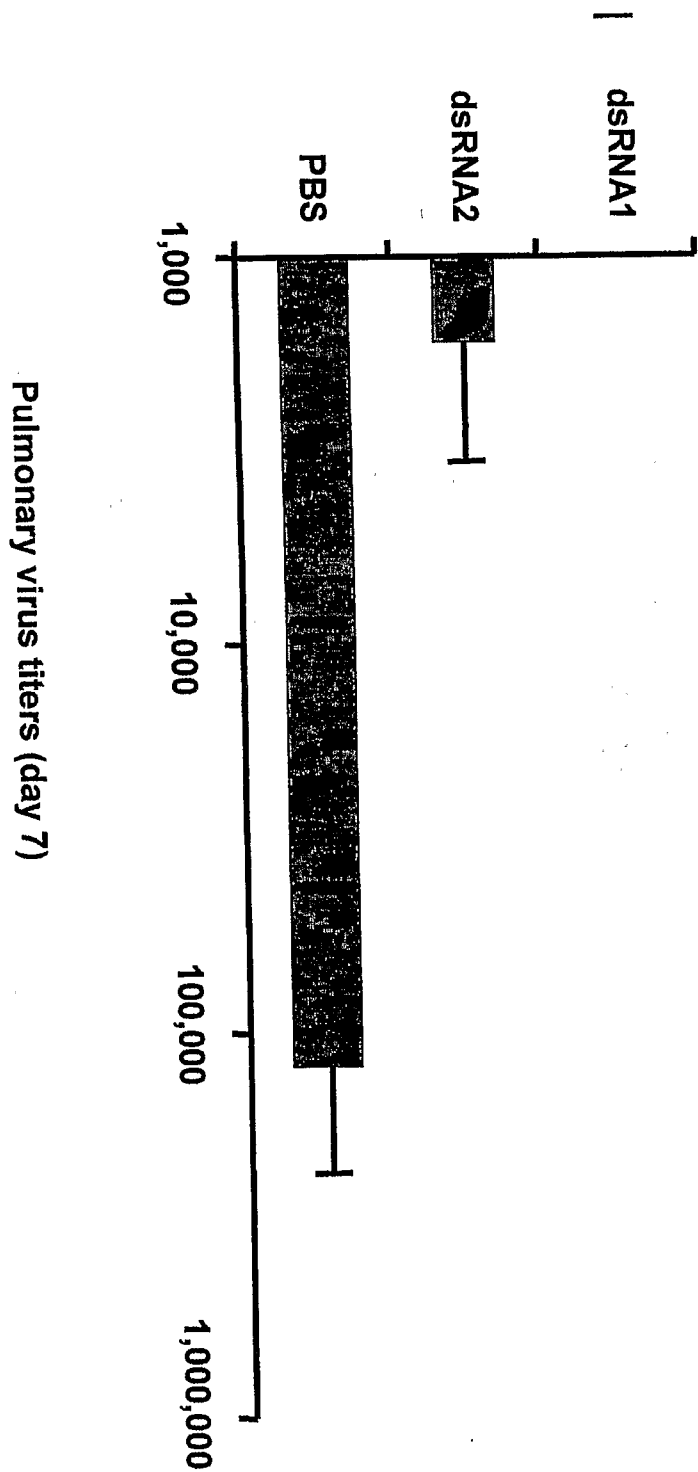


Figure 28:

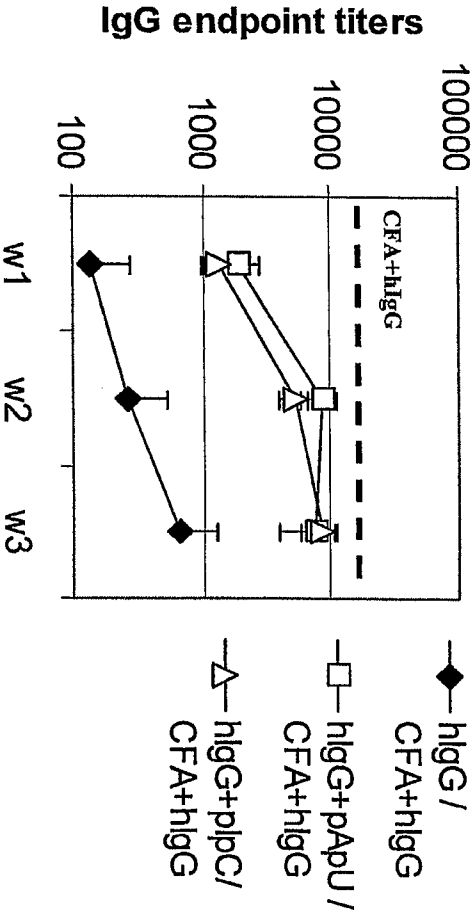


Figure 29:

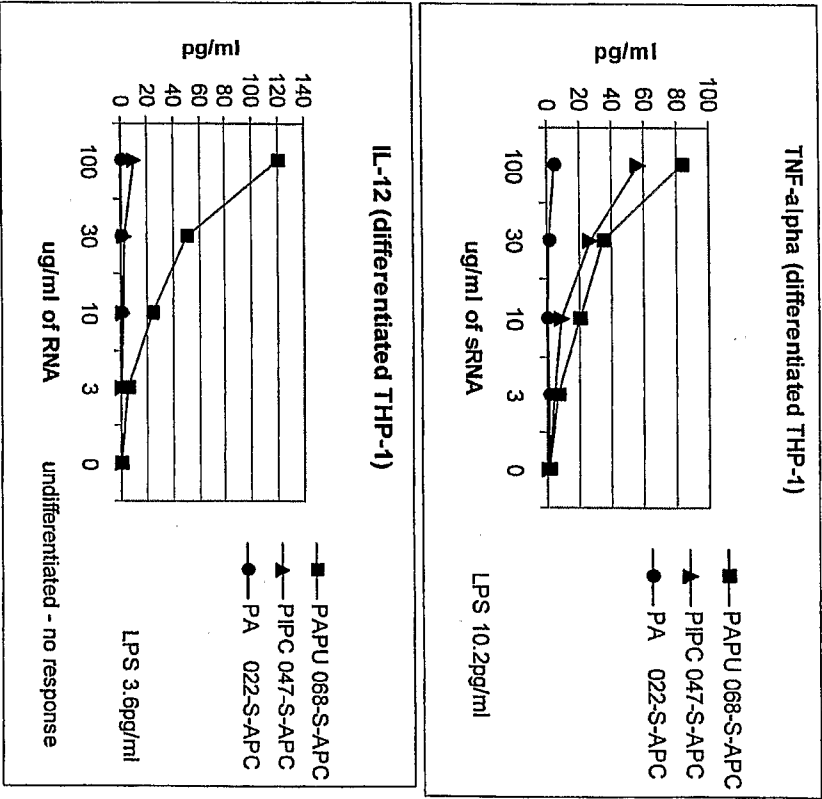


Figure 30A: Binding (FACS) to human cells

Large Cell Population

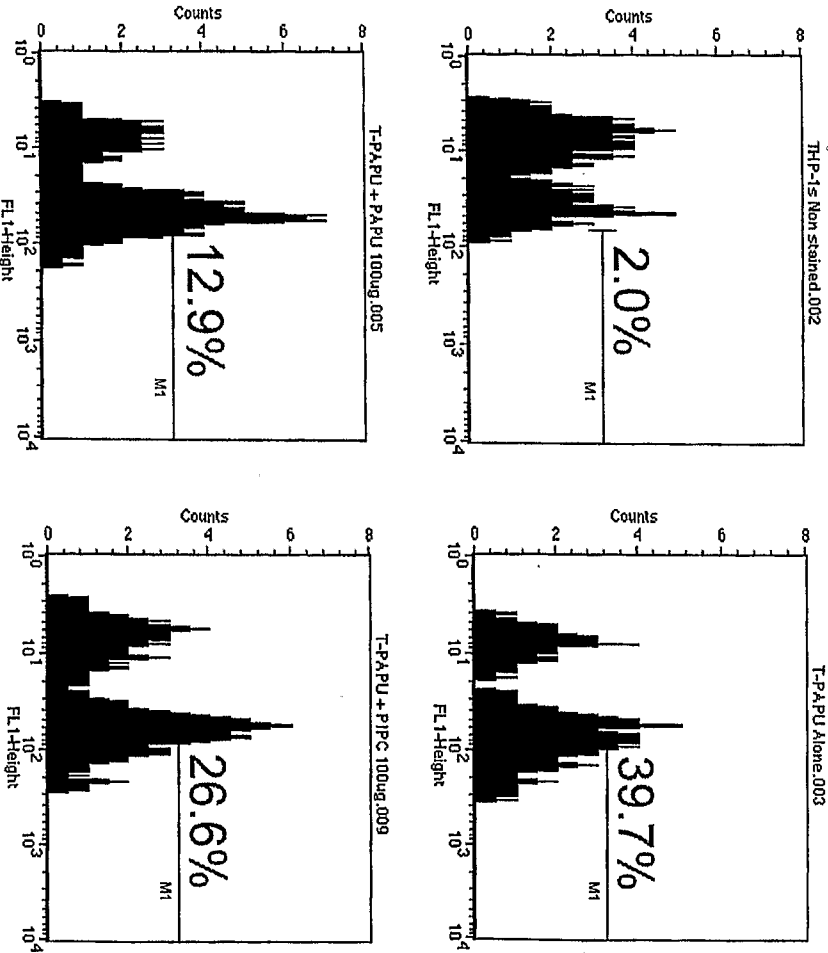


Figure 30B: Binding (FACS) to human cells
Total Cell Population

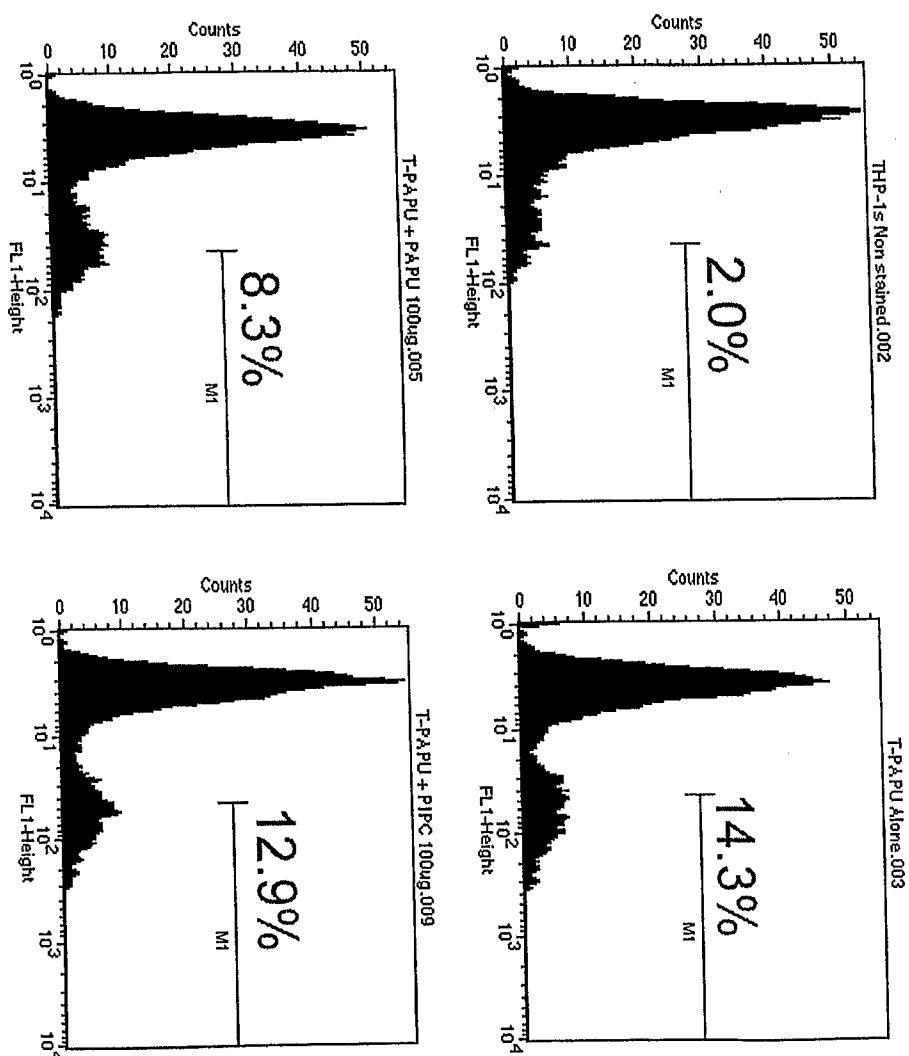


Figure 31: Fractionation of adjuvant dsRNA

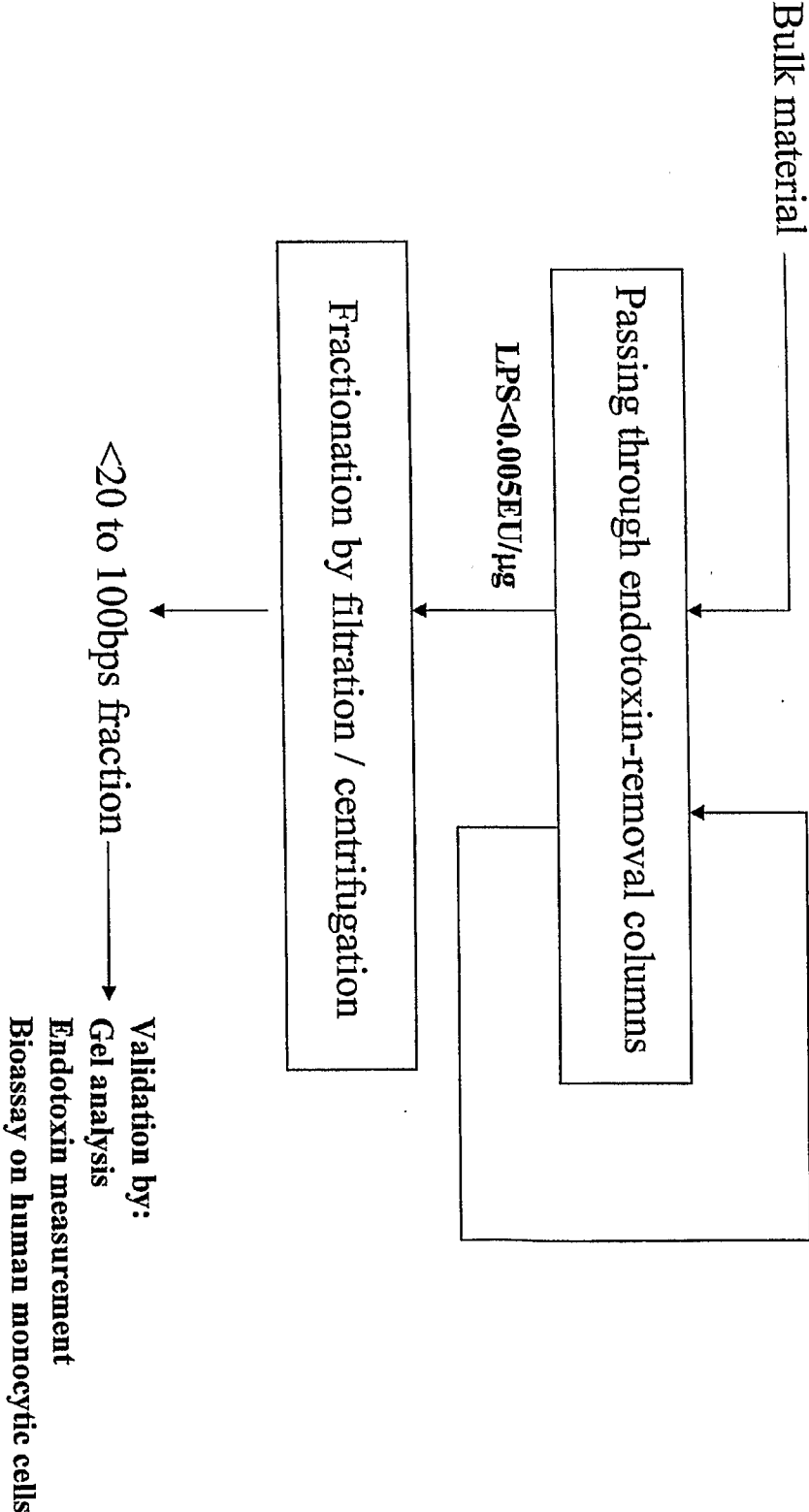
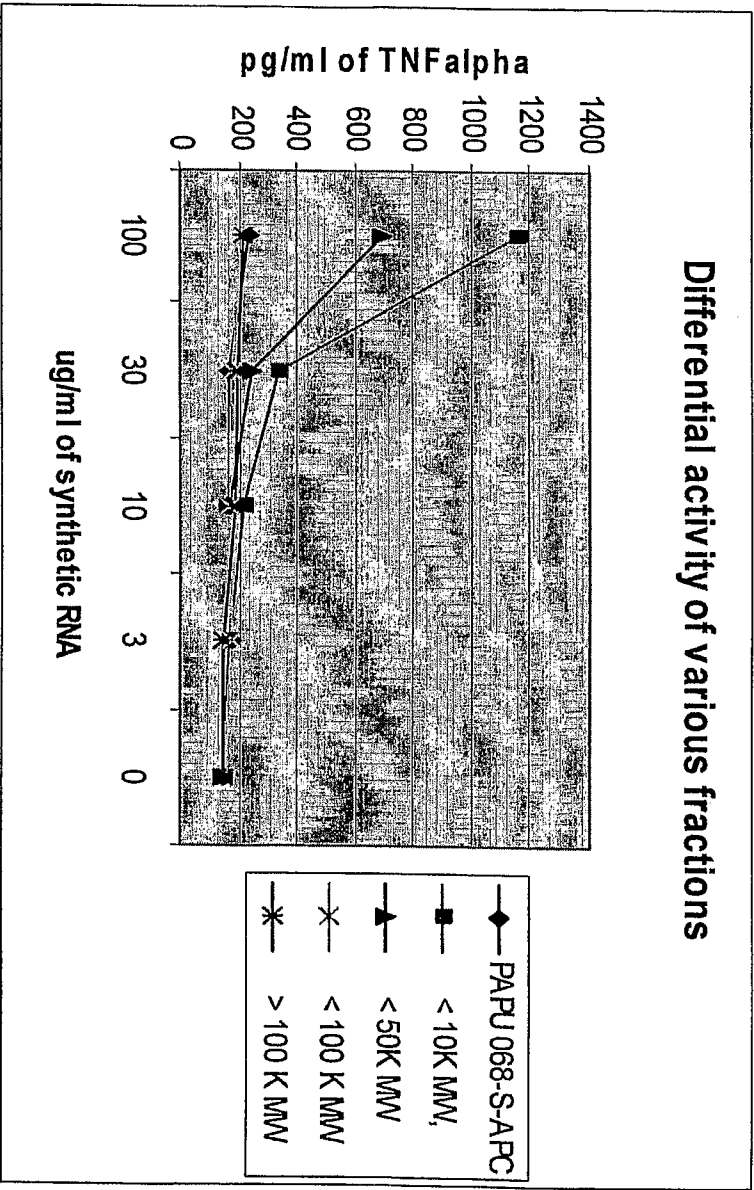
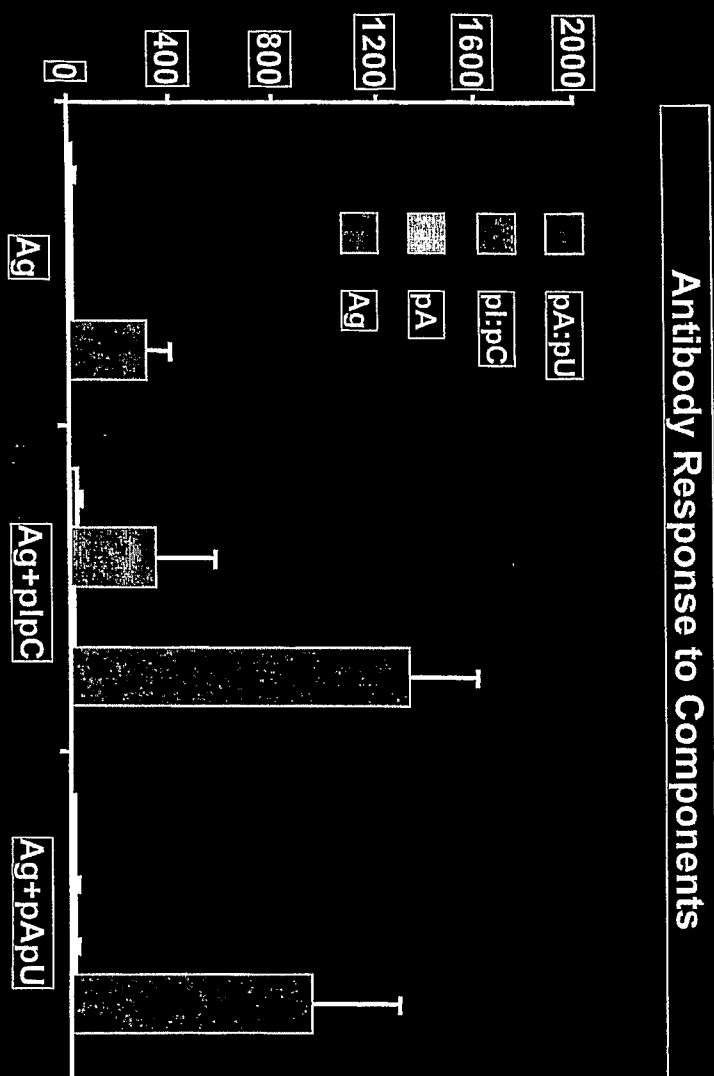


Figure 32: Activity of different fractions



Amounts of endotoxin similar to contamination result in < 160pg/ml TNF-alpha

Fig. 33: Lack of Immunogenicity of Synthetic dsRNA
Comprising Naturally Occurring Nucleotides



Figures 34A-34B:

Frequency of NP-specific T cells

A. IFN- γ

B. IL-2

Naïve IgNP+dsRNA₂

IgNP

IgNP+dsRNA₁

SFC / 10⁶ R

NP peptide (ug/ml)

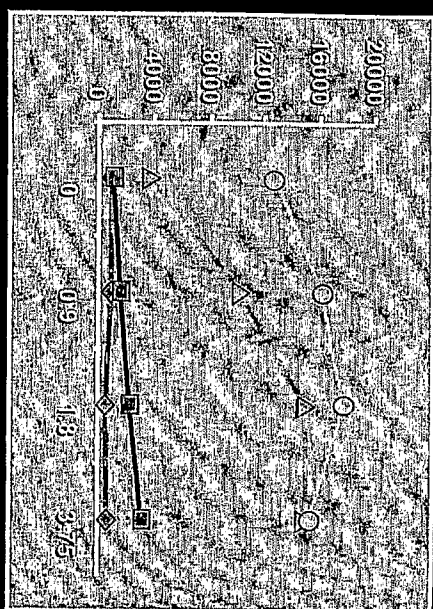
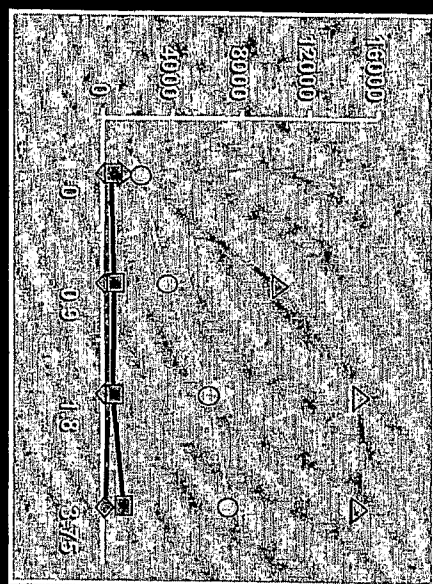


Figure 35:

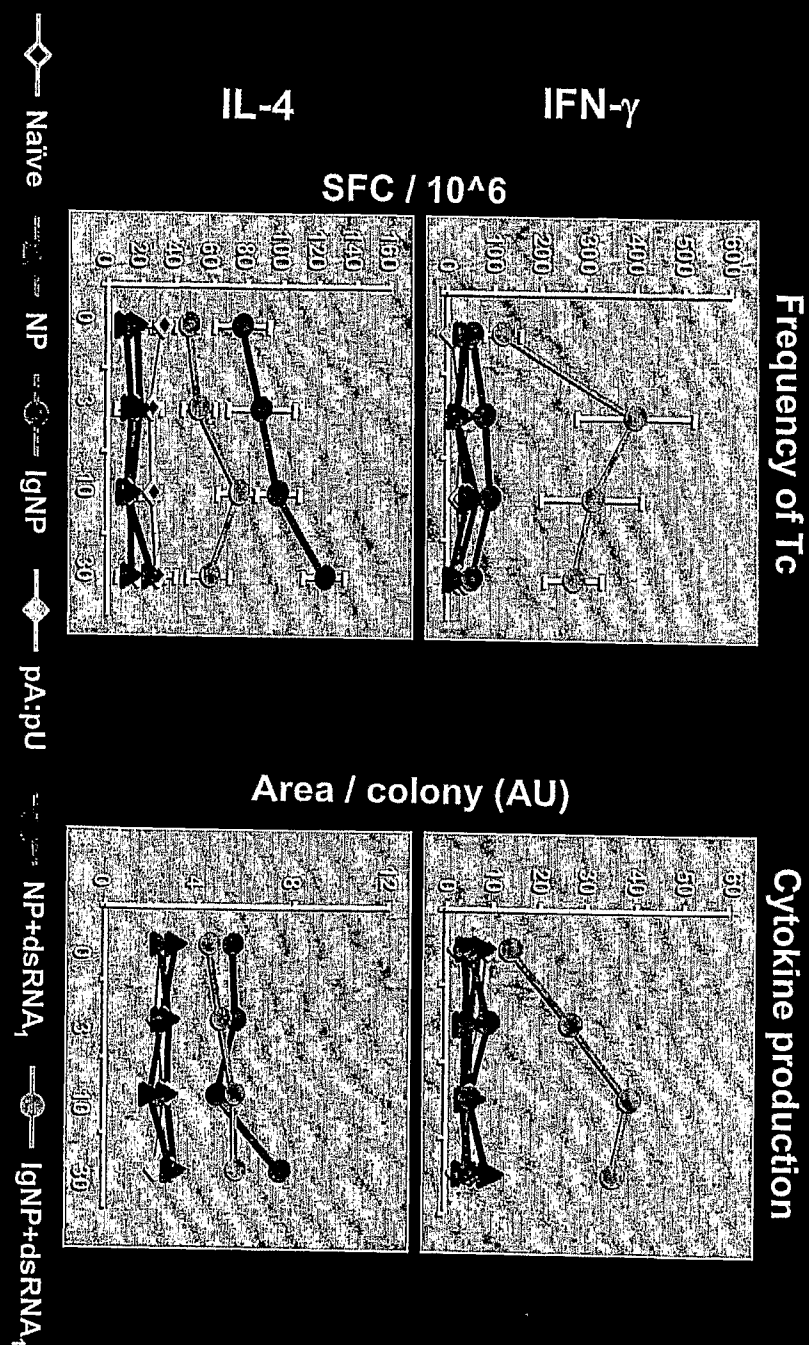


Figure 36:

Frequency of IFN- γ ⁺ SFC

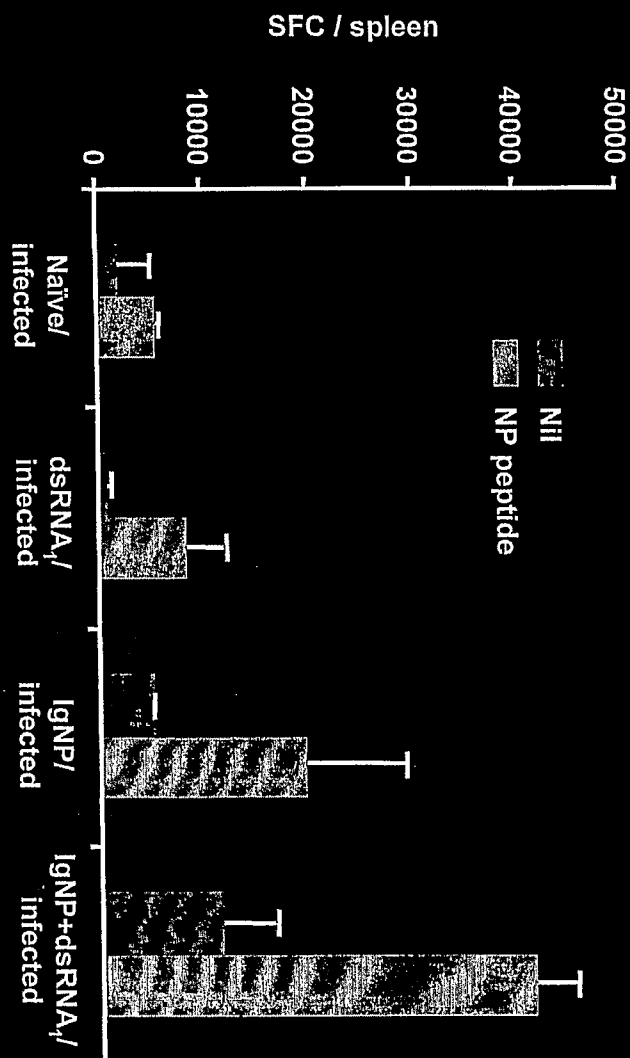


Figure 37:

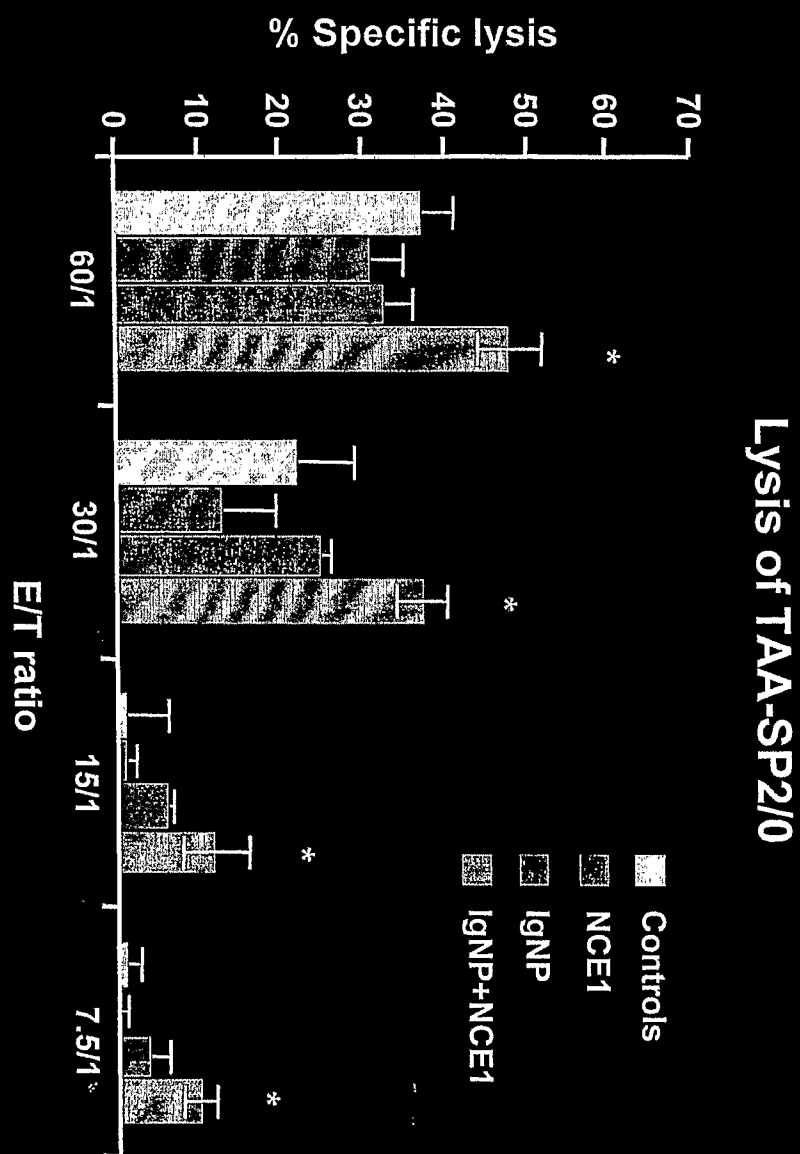
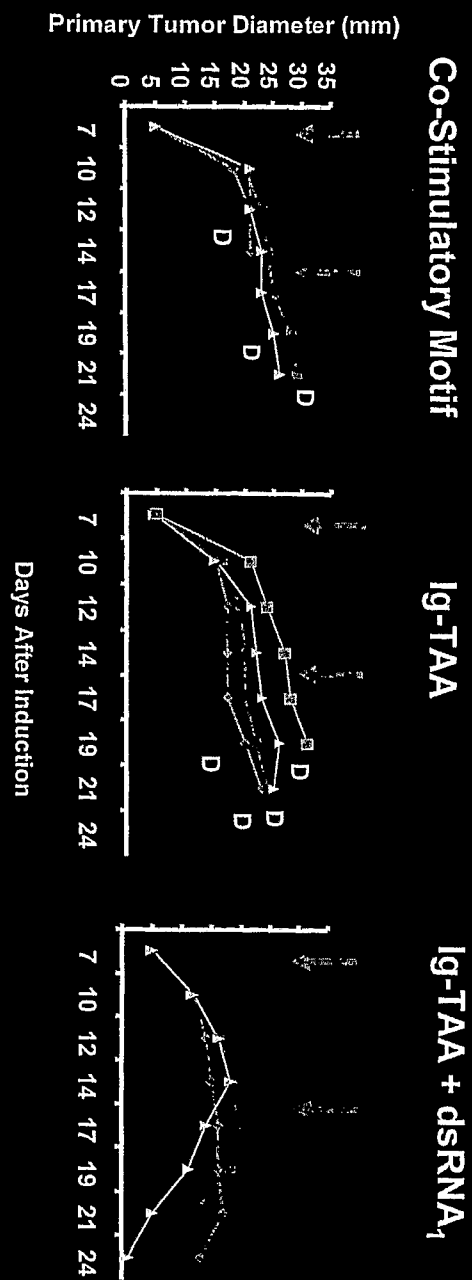


Figure 40:



Figures 41A-41B:

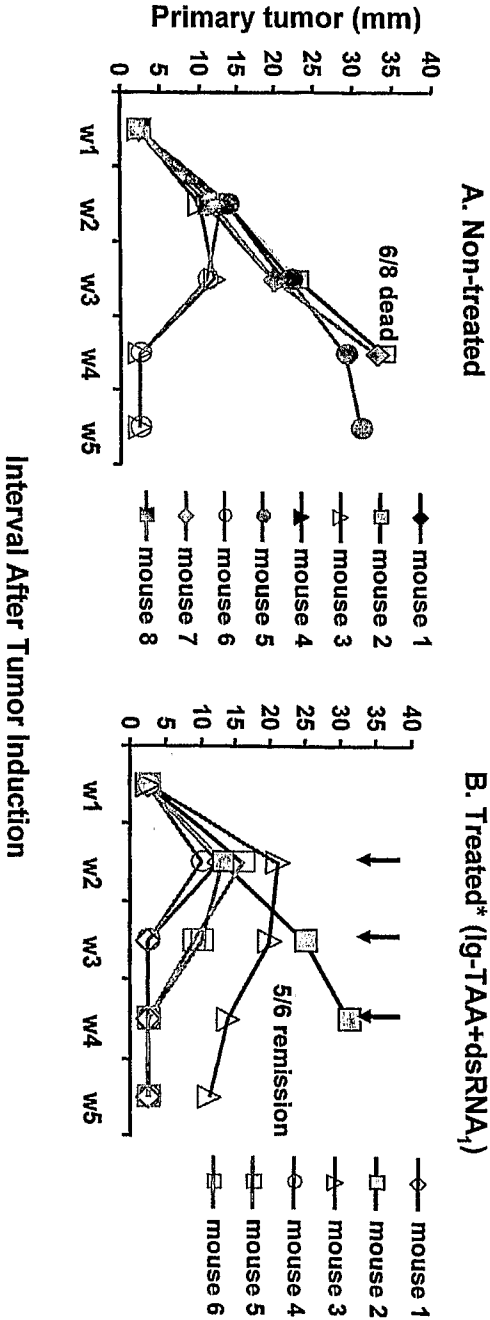


Figure 42:

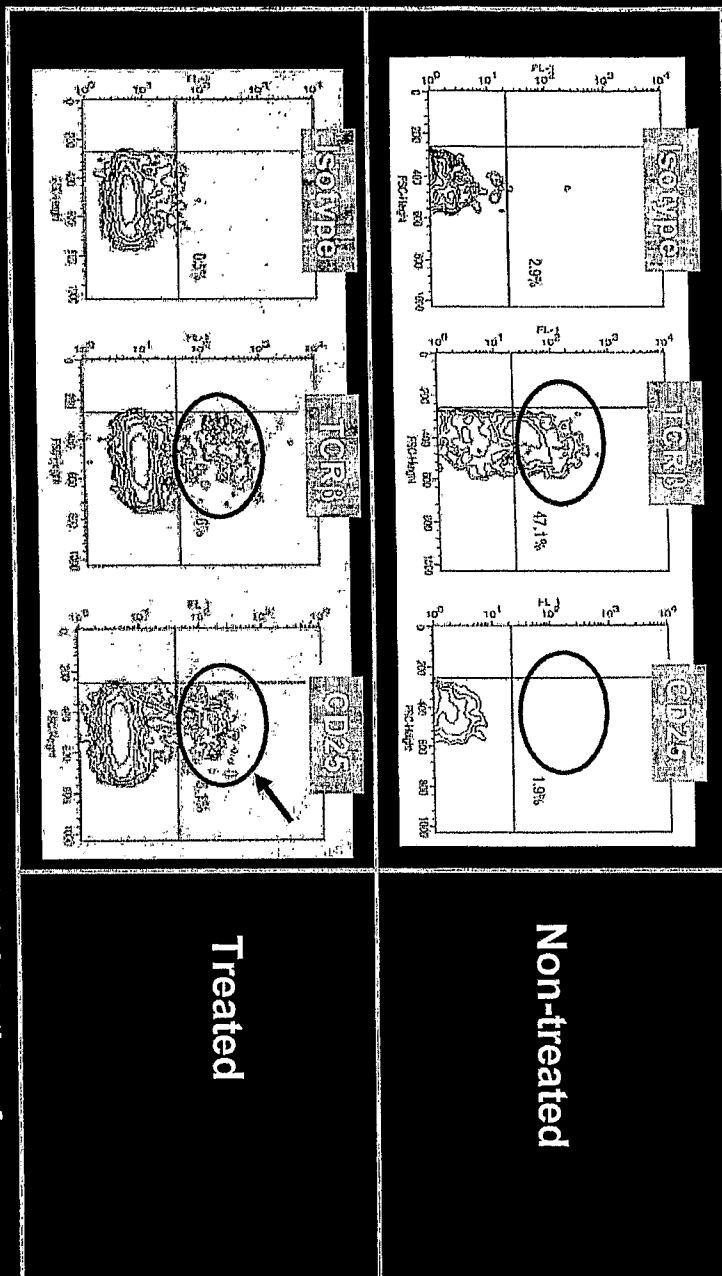


Figure 43:

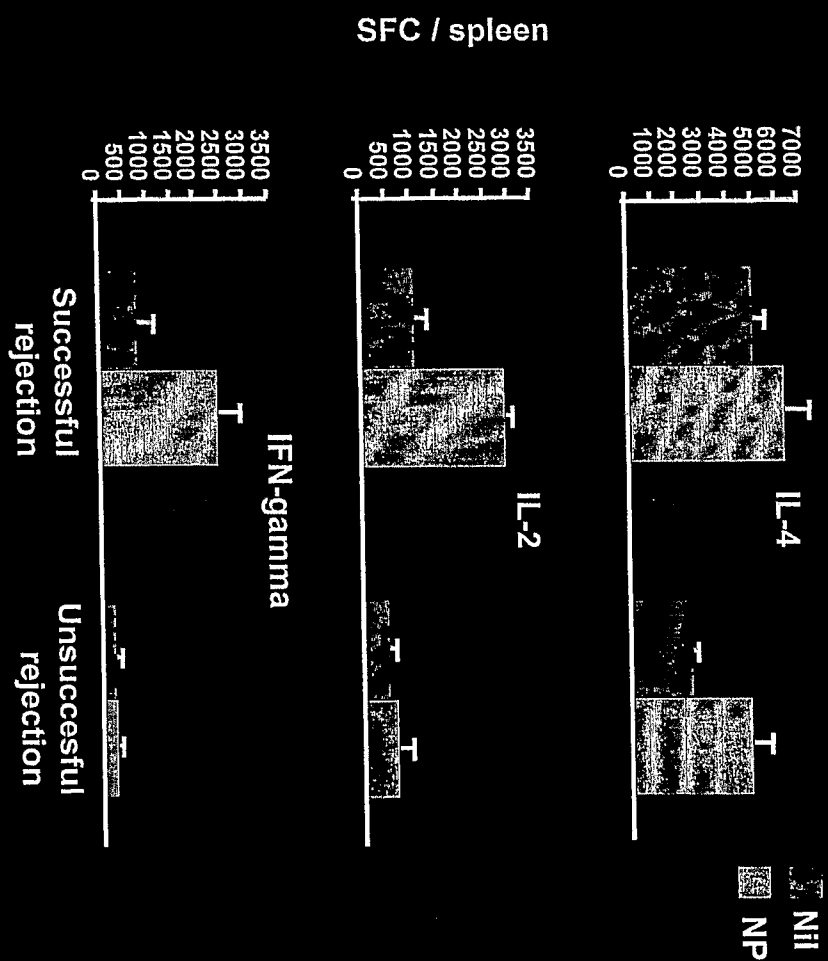


Figure 44:

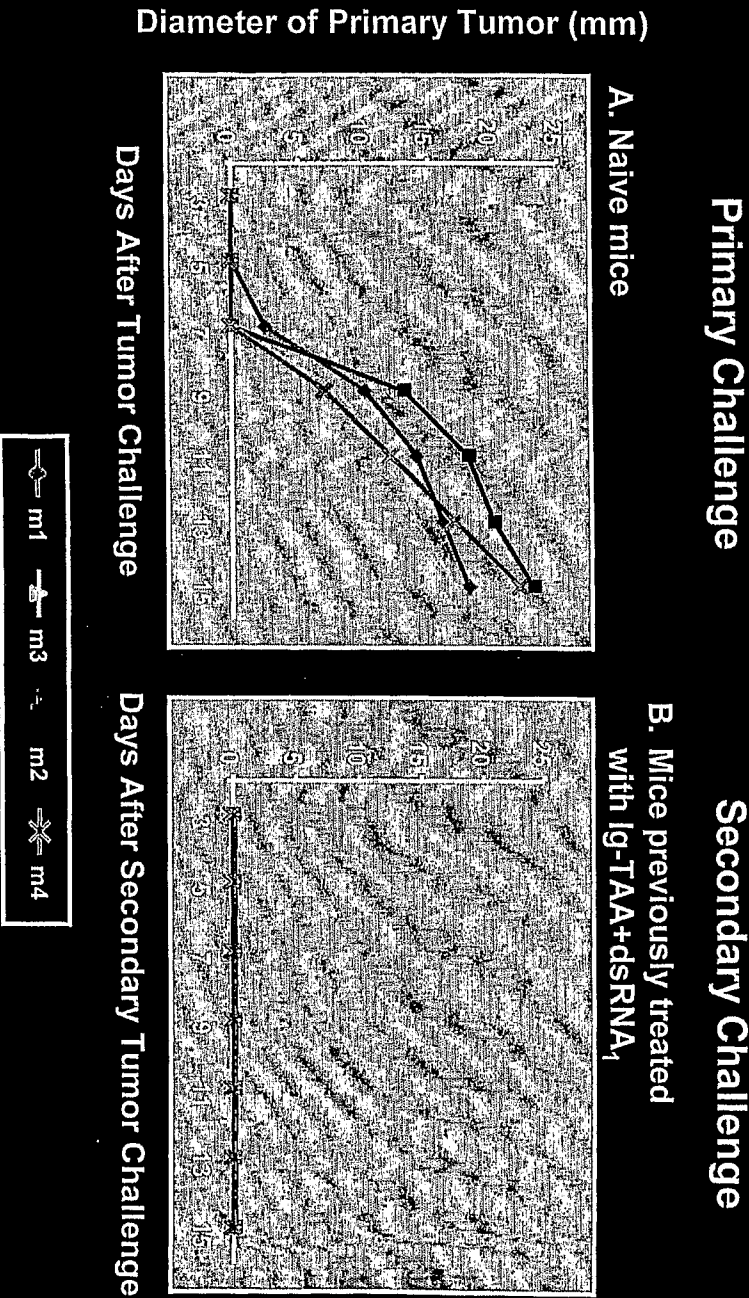


Figure 38:

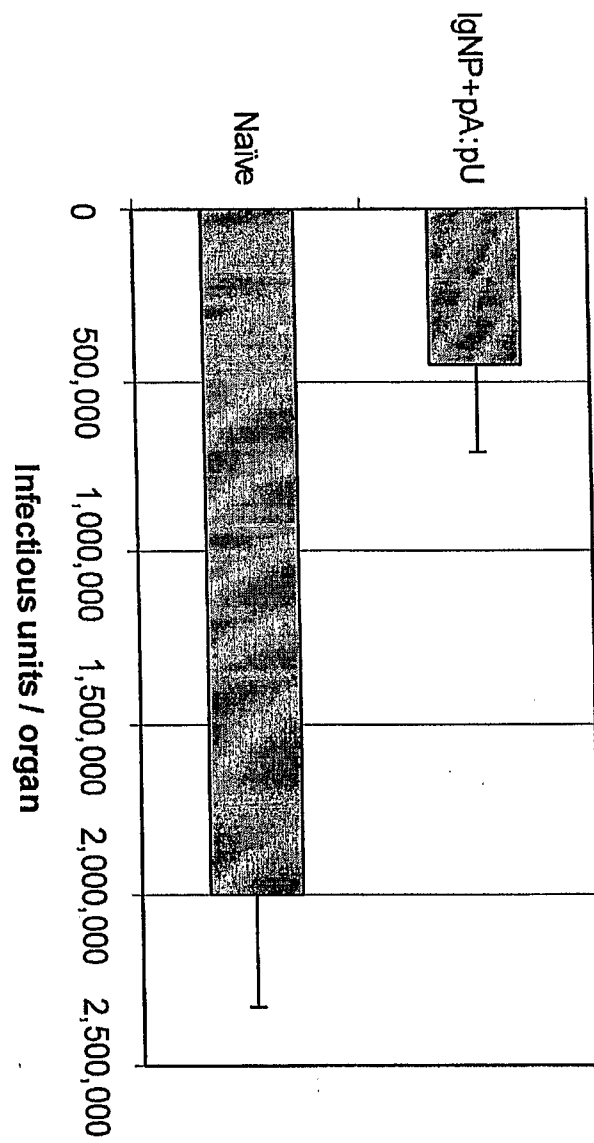
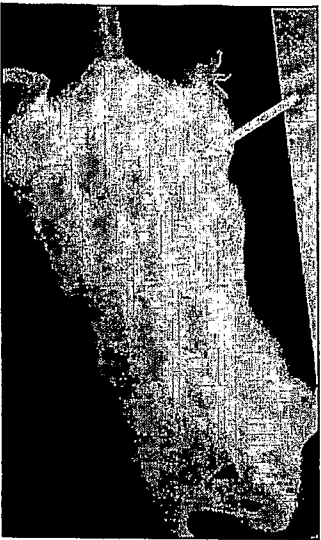


Figure 39:



Primary Solid Tumor



Metastasis:

- Peritoneal**
- Pericardial**
- Pleural**
- Hepatic**
- Splenic**

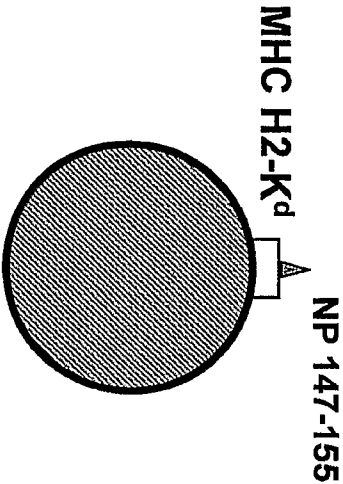


Figure 45A:

Tumor challenge	Status of animals prior to the challenge	Rate of tumoral disease upon subsequent challenge
SP2/0-IgNP (homologous)	Naïve Cured	4/4 0/10
SP2/0-IgHA (heterologous)	Naïve Cured	4/4 0/10
SP2/0 (heterologous)	Naïve Cured	4/4 0/8
SP2/0-IgW (heterologous)	Naïve Cured	4/4 0/2
4T-1 (breast cancer)	Naïve Cured	4/4 4/4

Figure 45B:

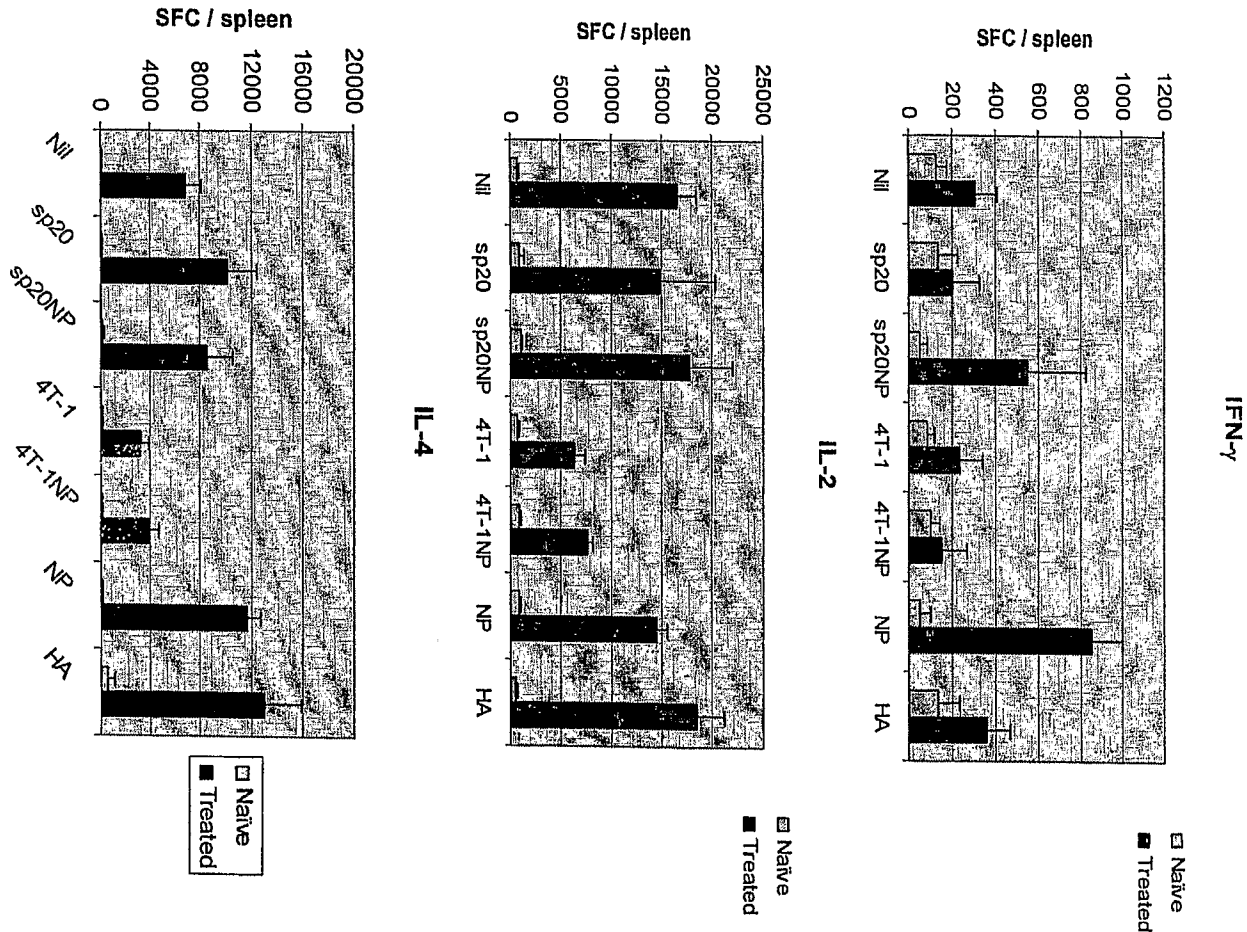


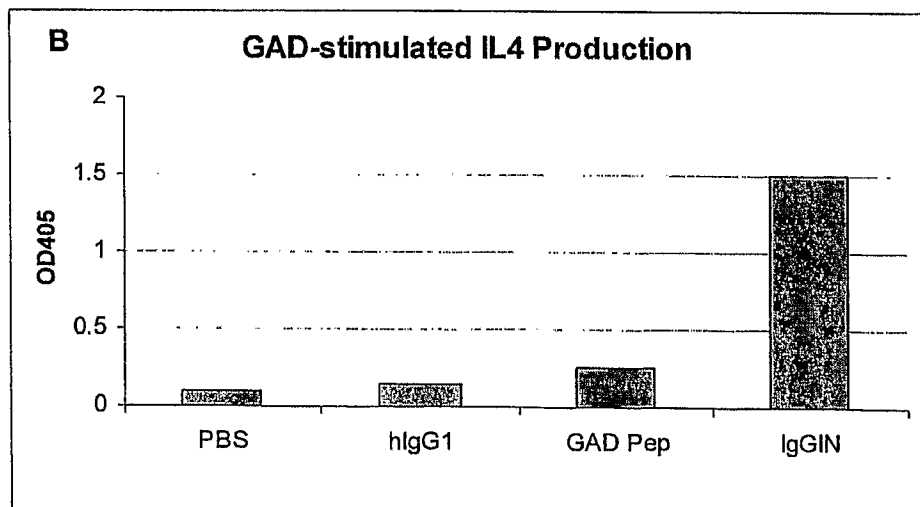
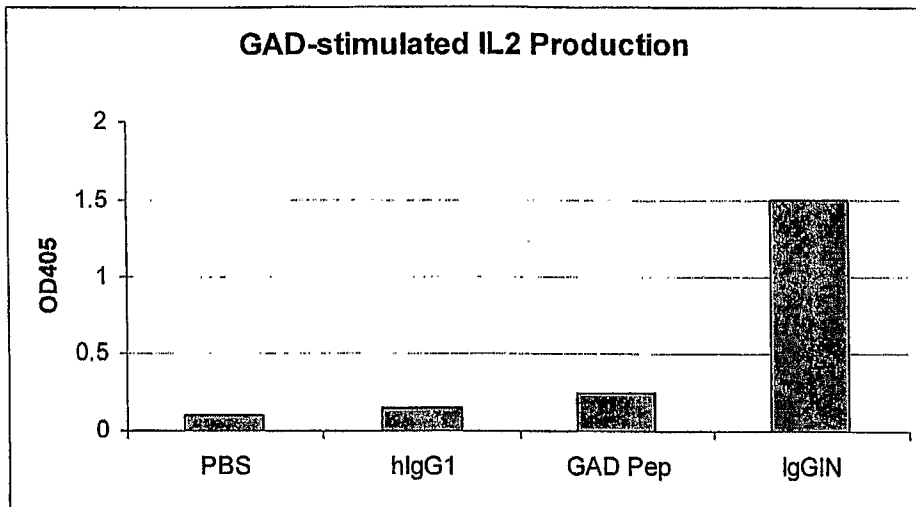
Fig. 46A

Fig. 46 B.

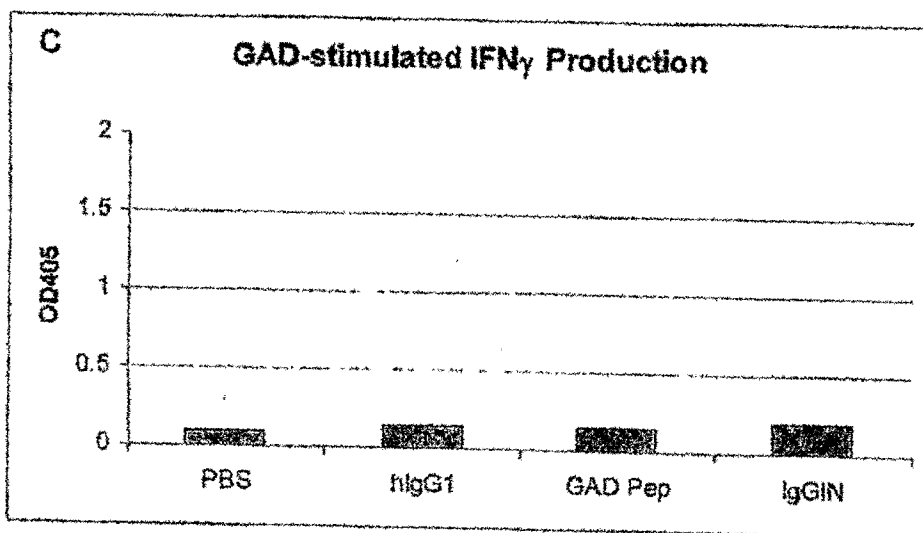


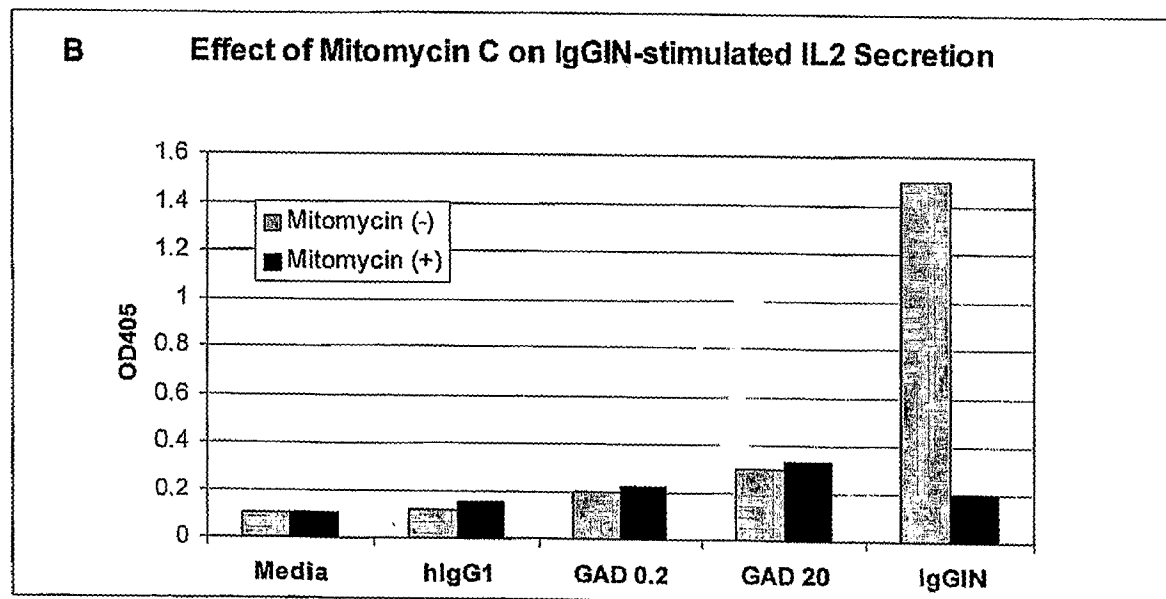
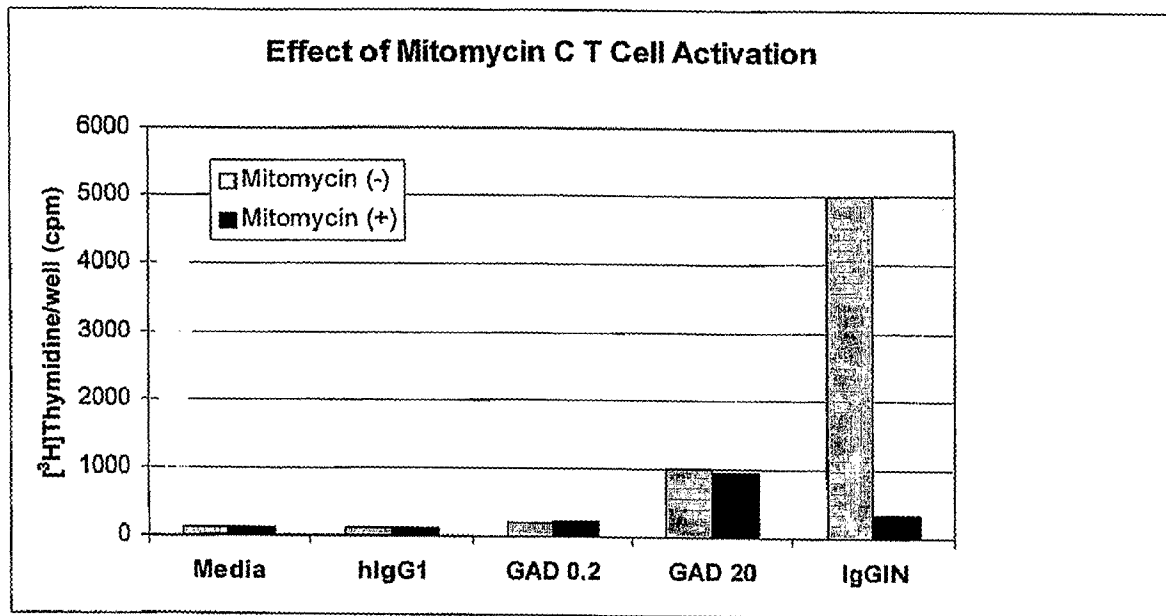
FIG. 47

Fig. 48

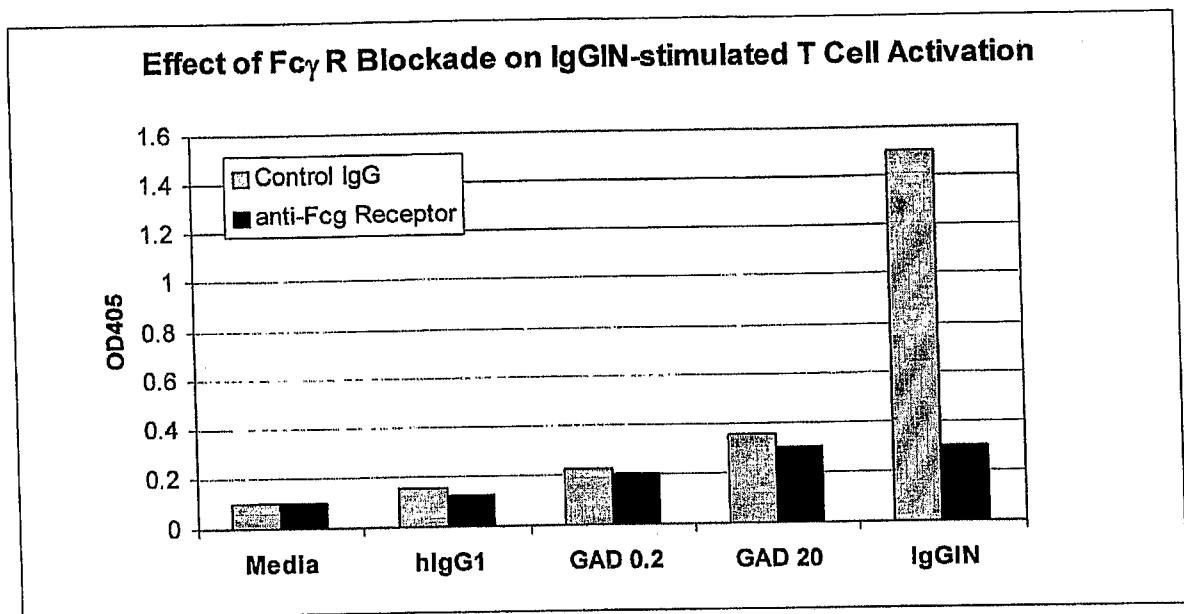


Fig. 49A

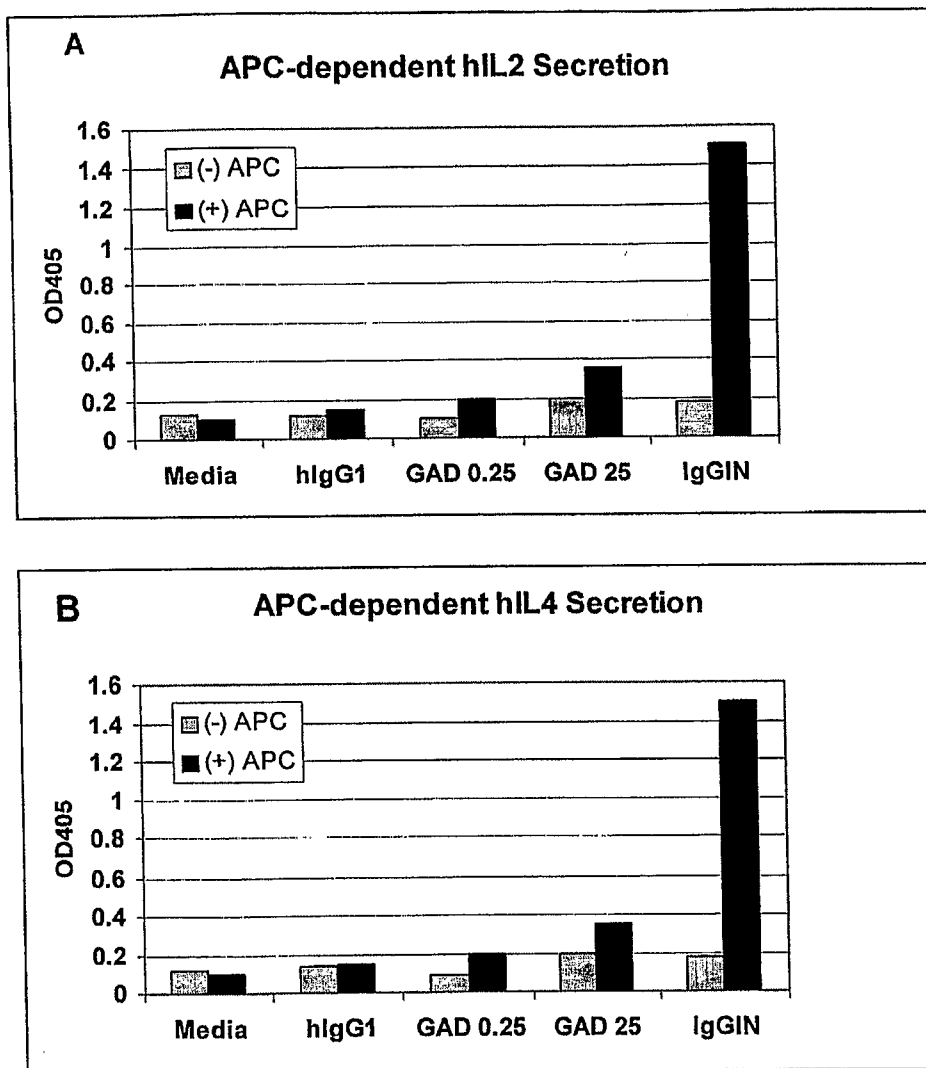


FIG. 49B

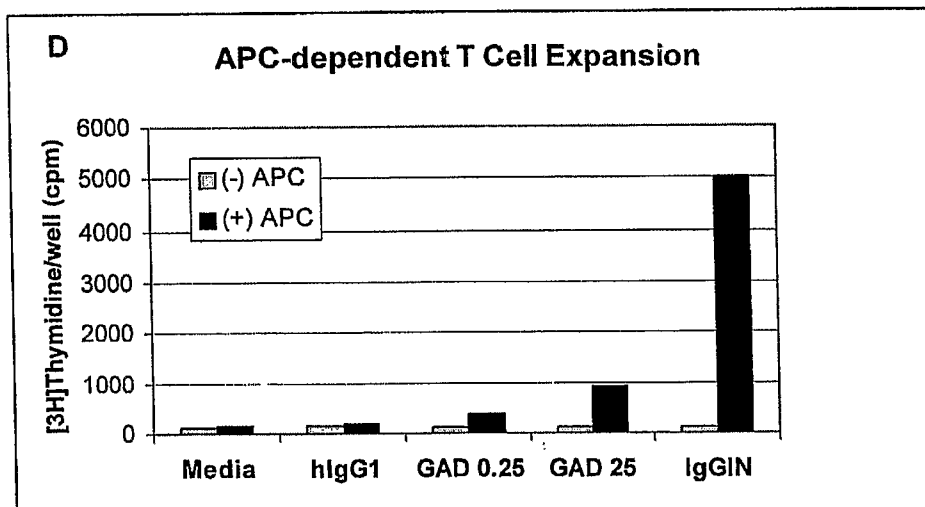
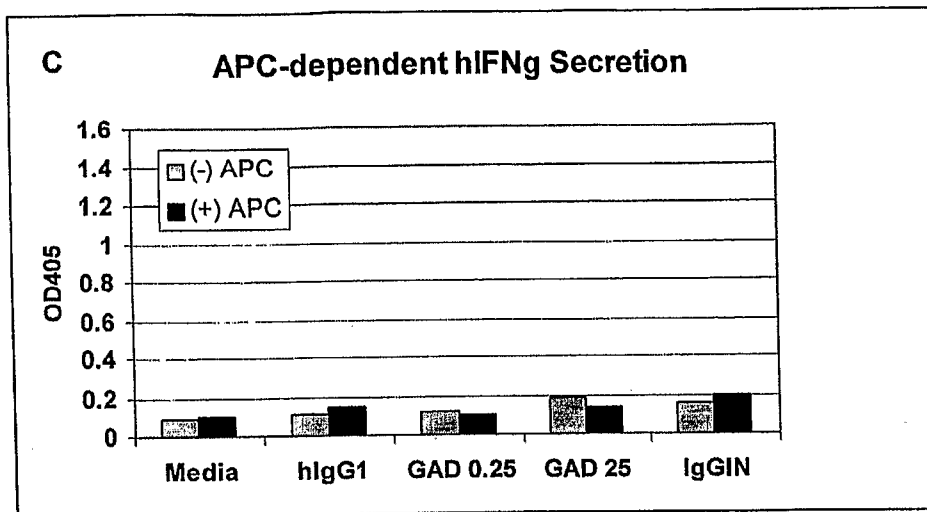


Fig. 50A

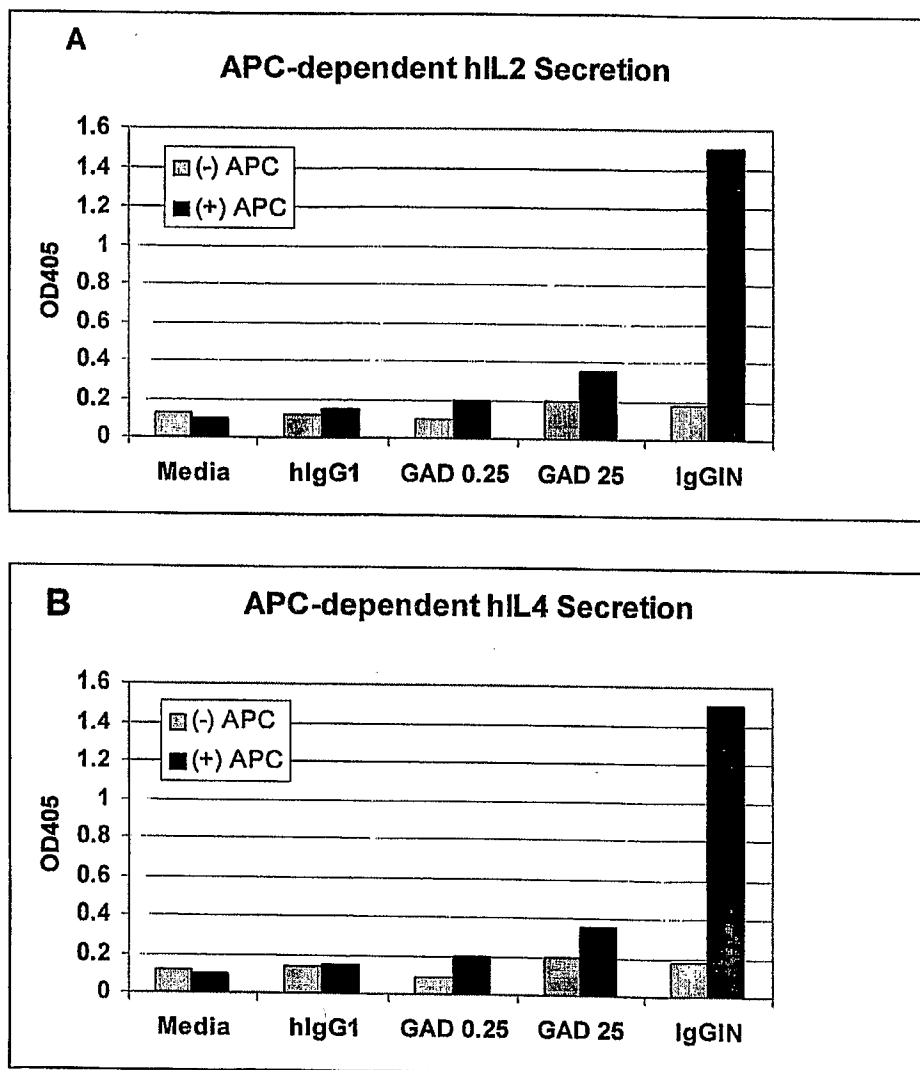


FIG. 50B

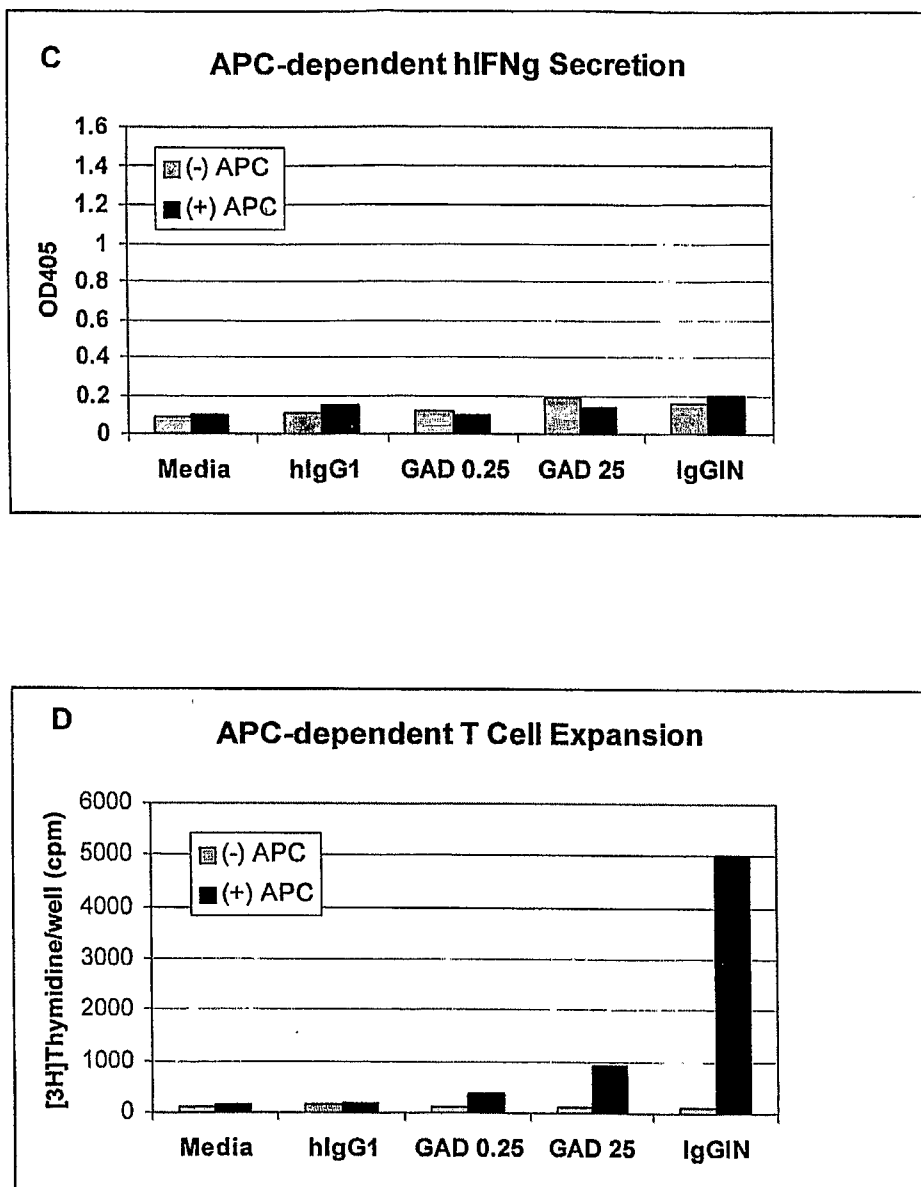


Fig. 51