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(54) Titre : ANTIGENES CIBLES PAR DES LYMPHOCYTES T PATHOGENES PREDOMINANT DANS LE DIABETE
TYPE 1 ET UTILISATIONS CORRESPONDANTES
(54) Title: ANTIGENS TARGETED BY PREVALENT PATHOGENIC T CELLS IN TYPE 1 DIABETES AND USES
THEREOF

(57) **Abrégé/Abstract:**

The present invention is based on the identification of a predominant ligand of CD8⁺ T cells that are responsible for type 1 diabetes. That ligand is islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). Several CD8⁺T cell-binding peptides from IGRP are identified, including the peptide comprising amino acids 206-214 of the IGRP sequence, which has high avidity to the most prevalent T cell receptor of pathogenic CD8⁺ T cells in autoimmune diabetes. The invention thus provides oligopeptide and polypeptide compositions comprising YLKTN/A/I/L/V)FL, FLWSVFWLI, (T/A)YY/G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP. Also provided are oligopeptide compositions 8-10 amino acids in length and completely homologous with a mammalian IGRP, where the oligopeptide is capable of binding a human MHC class I molecule. Additionally, various methods of treating a mammal using the above compositions are provided, where the mammal is at risk for or has type 1 diabetes. Also provided are methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a mammalian β -cell, where the methods also use the above compositions. Further provided are methods for determining whether a mammal is at risk for or has type 1 diabetes, where the methods use the above compositions.



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(54) Title: ANTIGENS TARGETED BY PREVALENT PATHOGENIC T CELLS IN TYPE 1 DIABETES AND USES THEREOF

(57) Abstract: The present invention is based on the identification of a predominant ligand of CD8⁺ T cells that are responsible for type 1 diabetes. That ligand is islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). Several CD8⁺ T cell-binding peptides from IGRP are identified, including the peptide comprising amino acids 206-214 of the IGRP sequence, which has high avidity to the most prevalent T cell receptor of pathogenic CD8⁺ T cells in autoimmune diabetes. The invention thus provides oligopeptide and polypeptide compositions comprising YLKTN/A/I/L/V)FL, FLWSVFWLI, (T/A)YY/G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP. Also provided are oligopeptide compositions 8-10 amino acids in length and completely homologous with a mammalian IGRP, where the oligopeptide is capable of binding a human MHC class I molecule. Additionally, various methods of treating a mammal using the above compositions are provided, where the mammal is at risk for or has type 1 diabetes. Also provided are methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a mammalian β -cell, where the methods also use the above compositions. Further provided are methods for determining whether a mammal is at risk for or has type 1 diabetes, where the methods use the above compositions.



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ANTIGENS TARGETED BY PREVALENT PATHOGENIC T CELLS IN TYPE 1 DIABETES
AND USES THEREOF

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Cross-Reference to Related Application

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10 Statement Regarding Federally Sponsored Research or Development

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. P01 DK52956 awarded by the National Institutes of Health.

15 Background

(1) Field of the Invention

The present invention generally relates to diagnosis and therapy of type 1 diabetes. More specifically, the invention provides methods of diagnosis, prevention and therapy of type 1 diabetes based on the identification of the islet β cell antigen targeted by pathogenic T cells.

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(2) Description of the Related Art

References cited

Aichele, P., D. Kyburz, P. Ohashi, B. Odermatt, R. Zinkernagel, H. Hengartner, and H. Pircher. 1994. Peptide-induced T-cell tolerance to prevent autoimmune diabetes in a transgenic mouse model. Proc. Natl. Acad. Sci. USA. 91:444-448.

25

Alexander-Miller, M., G. Leggatt, and J. Berzofsky. 1996. Selective expansion of high- or low-avidity cytotoxic T-lymphocytes and efficacy for adoptive immunotherapy. Proc. Natl. Acad. Sci. USA. 93:4102-4107.

J. D. Altman et al., Science 274, 94 (1996).

30

A. Amrani et al., Nature 406, 739 (2000).

A. Amrani et al., J. Immunol. 167, 655 (2001).

B. Anderson, B. J. Park, J. Verdager, A. Amrani, P. Santamaria, Proc. Natl. Acad. Sci. U.S.A. 96, 9311 (1999).

Anderton, S., and D. Wraith. 1998. Hierarchy in the ability of T cell epitopes to induce peripheral tolerance to antigens from myelin. Eur. J. Immunol. 28:1251-1261.

35

-2-

Arden, S., T. Zhan, S. Steegers, S. Webb, B. Bergman, R. O'Brien, and J. Hutton. 1999. Molecular cloning of a pancreatic islet-specific glucose-6-phosphatase catalytic subunit-related protein. *Diabetes*. 48:531-540.

Arif, S., T.I. Tree, T.P. Astill, J.M. Tremble, A.J. Bishop, C.M. Dayan, B.O. Roep, and M. Peakman. 2004. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest*. 113:451-463.

S. Baekkeskov et al., *Nature* 347, 151 (1990).

Bielekova, B., B. Goodwin, N. Richert, I. Cortese, T. Kondo, G. Afshar, B. Gran, J. Eaton, J. Antel, J. Frank, H. McFarland, and R. Martin. 2000. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat. Med*. 6:1167-1175.

Burke, D. H., L. Scates, K. Andrews, and L. Gold., *J. Mol. Biol*. 264, 650 (1996).

Cocca, B.A., A.M. Cline, and M.Z. Radic. 2002. Blebs and apoptotic bodies are B cell autoantigens. *J Immunol*. 169:159-166.

L. Cox, E. L. Huczko, V. H. Engelhard, J. Shabanowitz, D. F. Hunt, in *MHC: A Practical Approach*, N. Fernandez, G. Butcher, Eds. (Oxford Univ. Press, New York, 1997), vol. 1, pp. 141.

T. P. DiLorenzo et al., *Proc. Natl. Acad. Sci. U. S. A.* 95, 12538 (1998).

T. P. DiLorenzo et al., *Clin. Immunol*. 105, 332 (2002).

Ellington, A. D., and J. W. Szostak, *Nature* 346,818 (1990).

Gammon et al., *Nature (London)* 319, 413 (1986).

Gross, D.A., S. Graff-Dubois, P. Opolon, S. Cornet, P. Alves, A. Bennaceur- Griscelli, O. Faure, P. Guillaume, H. Firat, S. Chouaib, F.A. Lemonnier, J. Davoust, I. Miconnet, R.H. Vonderheide, and K. Kosmatopoulos. 2004. High vaccination efficiency of low-affinity epitopes in antitumor immunotherapy. *J Clin Invest*. 113:425-433.

Group, D.P.T.-T.D.S. 2002. Effects of insulin in relatives of patients with type 1 diabetes mellitus. *N. Engl. J. Med*. 346:1685-1691.

K. Hamaguchi, H. R. Gaskins, E. H. Leiter, *Diabetes* 40, 842 (1991).

A. H. Hartemann et al., *Clin, Exper. Immunol*. 116, 225 (1999).

Hirao, I., M. Spingola, D. Peabody, and A. D. Ellington, *Mol Divers*. 4, 75 (1998).

Jaeger, J., T. Restle, and T. A. Steitz, *The EMBO Journal* 17, 4535 (1998).

Kappos, L., G. Comi, H. Panitch, J. Oger, J. Antel, P. Conlon, L. Steinman, and T.A.P.L.i.R.M.S. Group. 2000. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. *Nat. Med*. 6:1176-1182.

Karin, N., D. Mitchell, S. Brocke, N. Ling, and L. Steinman. 1994. Reversal of experimental

-3-

autoimmune encephalomyelitis by a soluble peptide variant of a myelin basic protein epitope: T cell receptor antagonism and reduction of interferon γ and tumor necrosis factor α production. *J. Exp. Med.* 180:2227-2237.

J. Karttunen, S. Sanderson, N. Shastri, *Proc. Natl. Acad. Sci. U. S. A.* 89, 6020 (1992).

5 Kensch, O., B. A. Connolly, H. J. Steinhoff, A. McGregor, R. S. Goody, and T. Restle, *J Biol Chem.* 275, 18271 (2000).

R. S. Liblau, F. S. Wong, L. T. Mars, P. Santamaria, *Immunity* 17, 1 (2002).

Lieberman, S., and T. DiLorenzo. 2003. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. *Tissue Antigens.* 62:359.

10 Lieberman, S., A. Evans, B. Han, T. Takaki, Y. Vinnitskaya, J. Caldwell, D. Serreze, J. Shabanowitz, D. Hunt, S. Nathenson, P. Santamaria, and T. DiLorenzo. 2003. Identity of the beta cell antigen targeted by a prevalent population of pathogenic CD8+ T cells in autoimmune diabetes. *Proc. Natl. Acad. Sci. USA.* 100:8384-8388.

15 Liu, G., and D. Wraith. 1995. Affinity for class II MHC determines the extent to which soluble peptides tolerize autoreactive T cells in naive and primed adult mice -- implications for autoimmunity. *Int. Immunol.* 7:1255-1263.

S. E. Martin, J. Shabanowitz, D. F. Hunt, J. A. Marto, *Anal. Chem.* 72, 4266 (2000).

C. C. Martin et al., *J. Biol. Chem.* 276, 25197 (2001).

20 McKown, K. et al., 1999. Lack of efficacy of oral bovine type II collagen added to existing therapy in rheumatoid arthritis. *Arthritis Rheum.* 42:1204-1208.

Metzler, B., and D. Wraith. 1993. Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Int. Immunol.* 5:1159-1165.

25 Metzler, B., S. Anderton, S. Manickasingham, and D. Wraith. 2000. Kinetics of peptide uptake and tissue distribution following a single intranasal dose of peptide. *Immunol. Invest.* 29:61-70.

M. Nagata, P. Santamaria, T. Kawamura, T. Utsugi, J. W. Yoon, *J. Immunol.* 152, 2042 (1994).

30 Nicholson, L.B., J.M. Greer, R.A. Sobel, M.B. Lees, and V.K. Kuchroo. 1995. An altered peptide ligand mediates immune deviation and prevents autoimmune encephalomyelitis. *Immunity.* 3:397-405.

J. P. Palmer et al., *Science* 222, 1337 (1983).

K. C. Parker, M. A. Bednarek, J. E. Coligan, *J. Immunol.* 152, 163 (1994).

35 Perez-Diez, A., P. Spiess, N. Restifo, P. Matzinger, and F. Marincola. 2002. Intensity of the vaccine-elicited immune response determines tumor clearance. *J. Immunol.* 168:338-347.

-4-

R. A. Pierce et al., *J. Immunol.* 163, 6360 (1999).

M. J. Pittet et al., *Trends. Immunol.* 23, 325 (2002).

F. Pociot, M. F. McDermott, *Genes Immun.* 3, 235 (2002).

5 Pozzilli, P. et al., 2000. No effect of oral insulin on residual beta-cell function in recent-onset type 1 diabetes (the IMDIAB VII). IMDIAB Group. *Diabetologia.* 43:1000-1004.

Princiotta, M.F., D. Finzi, S.B. Qian, J. Gibbs, S. Schuchmann, F. Buttgerit, J.R. Bennink, and J.W. Yewdell. 2003. Quantitating protein synthesis, degradation, and endogenous antigen processing. *Immunity.* 18:343-354.

D. U. Rabin, S. M. Pleasic, R. Palmer-Crocker, J. A. Shapiro, *Diabetes* 41, 183 (1992).

10 H. Rammensee, J. Bachmann, N. P. Emmerich, O. A. Bachor, S. Stevanovic, *Immunogenetics* 50, 213 (1999).

P. A. Reche, J. P. Glutting, E. L. Reinherz, *Hum. Immunol.* 63, 701 (2002).

Santamaria, P. 2001. Effector lymphocytes in autoimmunity. *Curr. Opin Immunol.* 13:663-669.

15 P. Santamaria et al., *J. Immunol.* 154, 2494 (1995).

Sauter, B., M.L. Albert, L. Francisco, M. Larsson, S. Somersan, and N. Bhardwaj. 2000. Consequences of cell death. Exposure To necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. *J Exp Med.* 191:423-434.

20 Schneider, D. J., J. Feigon, Z. Hostomsky, and L. Gold, *Biochemistry* 34, 9599 (1995).

D. V. Serreze, E. H. Leiter, *Curr. Dir. Autoimmun.* 4, 31 (2001).

D. V. Serreze et al., *Diabetes* 50, 1992 (2001).

M. Terajima et al., *J. Exp. Med.* 197, 927 (2003).

R. Tisch, H. McDevitt, *Cell* 85, 291 (1996).

25 Toes, R., R. Offringa, R. Blom, C. Melief, and W. Kast. 1996. Peptide vaccination can lead to enhanced tumor growth through specific T-cell tolerance induction. *Proc. Natl. Acad. Sci. USA.* 93:7855-7860.

Trentham, D. et al., 1993. Effects of oral administration of type II collagen on rheumatoid arthritis. *Science.* 261:1727-1730.

30 J. D. Trudeau et al., *J. Clin. Invest.* 111, 217 (2003).

Tuerk, C., and L. Gold, *Proc. Natl. Acad. Sci. USA* 89:6988 (1992).

Tuerk, C., and L. Gold, *Science* 249:505 (1990).

F. S. Wong et al., *Nat. Med.* 9, 1026 (1999).

35 F. S. Wong, A. K. Moustakas, L. Wen, G. K. Papadopoulos, C. A. Janeway, Jr., *Proc. Natl. Acad. Sci. U. S. A.* 99, 5551 (2002).

-5-

Verdaguer, J., J.-W. Yoon, B. Anderson, N. Averill, T. Utsugi, B. Park, and P. Santamaria. 1996. Acceleration of spontaneous diabetes in TCR- β -transgenic nonobese diabetic mice by β -cell cytotoxic CD8+ T cells expressing identical endogenous TCR- α chains. *J. Immunol.* 157:4726-4735.

J. Verdager et al., *J. Exp. Med.* 186, 1663 (1997).

5 Weiner, H. 1993. Double-blind pilot trial of oral tolerization with myelin antigens in multiple sclerosis. *Science.* 259:1321-1324.

Wraith, D.C., D.E. Smilek, D.J. Mitchell, L. Steinman, and H.O. McDevitt. 1989. Antigen recognition in autoimmune encephalomyelitis and the potential for peptide-mediated immunotherapy. *Cell* 59:247-255.

10 Zeh, H., D. Perry-Lalley, M. Dudley, S. Rosenberg, and J. Yang. 1999. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo anti-tumor efficacy. *J. Immunol.* 162:989-994.

Zhang, Y., B. O'Brien, J. Trudeau, R. Tan, P. Santamaria, and J.P. Dutz. 2002. In situ β -cell death promotes priming of diabetogenic CD8 T lymphocytes. *J. Immunol.* 168:1466-1472.

15 U.S. Pat. No. 5,472,841.

U.S. Pat. No. 5,496,938.

U.S. Pat. No. 5,503,978.

U.S. Pat. No. 5,567,588.

U.S. Pat. No. 5,580,737.

20 U.S. Pat. No. 5,582,981.

U.S. Pat. No. 5,637,459.

U.S. Pat. No. 5,654,151.

U.S. Pat. No. 5,683,867.

U.S. Pat. No. 5,705,337.

25 U.S. Pat. No. 5,712,375.

U.S. Pat. No. 5,726,017.

U.S. Pat. No. 5,773,598.

U.S. Pat. No. 5,786,462.

U.S. Pat. No. 6,028,186.

30 U.S. Pat. No. 6,083,696.

U.S. Pat. No. 6,110,900.

U.S. Pat. No. 6,124,449.

U.S. Pat. No. 6,127,119.

U.S. Pat. No. 6,140,490.

35 U.S. Pat. No. 6,147,204.

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U.S. Pat. No. 6,168,778.

U.S. Pat. No. 6,171,795.

The NOD mouse is a widely studied model of human type 1 diabetes, an autoimmune disease characterized by inflammation of pancreatic islets (insulinitis) followed by T cell-mediated destruction of the insulin-producing β cells (Serreze & Leiter, 2001). Both $CD4^+$ and $CD8^+$ T cells are required for this pathogenic process (Serreze & Leiter, 2001); however, $CD8^+$ T cells appear to be responsible for the initial β cell insult (Serreze & Leiter, 2001; Tisch & McDevitt, 1996; DiLorenzo et al., 1998). While the pathogenicity of B cells and autoantibodies is less clear, the autoantigens currently believed to contribute to autoimmune diabetes pathogenesis in NOD mice and humans were all originally identified based on the presence of specific autoantibodies rather than by T cell recognition (Palmer et al., 1983; Baekkeskov et al., 1990; Rabin et al., 1992). Little is known of the β cell antigens targeted by the pathogenic $CD8^+$ T cells. While one study identified an insulin peptide as the antigenic target of the majority of islet-infiltrating $CD8^+$ T cells in NOD mice (Wong et al., 1999), the prevalence of these insulin-reactive $CD8^+$ T cells was not confirmed in subsequent studies (Amrani et al., 2000; Trudeau et al., 2003). A substantial proportion of β cell-autoreactive $CD8^+$ T cells isolated from NOD islets express a shared T cell receptor (TCR) α chain (V α 17-J α 42), suggesting recognition of a common β cell peptide (DiLorenzo et al., 1998; Santamaria et al., 1995). These T cells do not recognize the antigenic insulin peptide mentioned above (Anderson et al., 1999; Serreze et al., 2001). The pathogenicity of this prevalent T cell population has been well established through studies of the 8.3 T cell clone (a representative T cell clone of the V α 17-J α 42-expressing T cell population) (Amrani et al., 2000; Trudeau et al., 2003; Anderson et al., 1999; Nagata et al., 1994; Verdaguer et al., 1997). 8.3-like T cells are present in the earliest islet infiltrates of NOD mice (DiLorenzo et al., 1998) and undergo avidity maturation as islet inflammation progresses to overt disease (Amrani et al., 2000). At any given time, 8.3-like T cells can constitute up to 30-40% of the islet-associated $CD8^+$ T cells (Trudeau et al., 2003). Additionally, quantitation of 8.3-like T cells in peripheral blood predicts diabetes development in individual NOD mice (Trudeau et al., 2003), unlike any other single immune indicator identified to date. While the prevalence and pathogenicity of 8.3-like T cells has been clearly established, the identity of their ligand has remained elusive. The 8.3 T cell clone is restricted to the class I major histocompatibility complex (MHC) molecule H-2K^d (Nagata et al., 1994). The artificial oligopeptides NRP-V7 and NRP-A7 have previously been shown to bind to 8.3-like T cells in the context of H-2K^d molecules (Anderson et al., 1999; Amrani et al., 2001). However, those studies do not suggest the identity of the natural 8.3-like T cell ligand responsible for type 1 diabetes autoimmunity.

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The determination of the 8.3-like T cell ligand, as well as other T cell ligands associated with type 1 diabetes is thus desirable since it would open up new options for diagnosis, prevention and therapy of that disease. A characterization of the precise role of these ligands in type 1 diabetes pathogenesis is also needed. This invention satisfies these needs.

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Summary of the Invention

Accordingly, the inventors have succeeded in identifying the 8.3-like T cell ligand present on β cells that is responsible for type 1 diabetes. That ligand is islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and its CD8⁺ T cell-binding peptide comprising amino acids
10 206-214 of the IGRP sequence (SEQ ID NO:1 for mice; SEQ ID NO:2 for humans). The inventors have also identified several other CD8⁺ T cell ligands from IGRP that contribute to type 1 diabetes, particularly when T cells reacting to IGRP₂₀₆₋₂₁₄ are eliminated with a high affinity ligand, for example IGRP₂₀₆₋₂₁₄.

Thus, in some embodiments, the invention is directed to isolated and purified oligopeptides or
15 polypeptides less than 355 amino acids comprising a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP.

In other embodiments, the present invention is directed to isolated and purified oligopeptides or polypeptides comprising at least one of the above sequences in a sterile pharmaceutical preparation.

20 Additionally, the present invention is directed to isolated and purified oligopeptides 8-10 amino acids in length, completely homologous with a mammalian IGRP having at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2, wherein the oligopeptide is capable of binding a human MHC class I molecule.

The present invention is also directed to antisense molecules complementary to at least a
25 portion of an mRNA encoding a mammalian IGRP at least 90% homologous to SEQ ID NO:1 or SEQ ID NO:2. In these embodiments, the antisense molecules are capable of inhibiting translation of a mammalian IGRP.

In related embodiments, the invention is directed to ribozymes specific for a portion of an mRNA encoding a mammalian IGRP at least 90% homologous to SEQ ID NO:1 or SEQ ID NO:2.
30 The ribozymes of these embodiments inhibit translation of a mammalian IGRP.

In other related embodiments, the invention is directed to RNAi molecules homologous to a portion of an mRNA encoding a mammalian IGRP at least 90% homologous SEQ ID NO:1 or SEQ ID NO:2. In these embodiments, the RNAi molecule is capable of inhibiting translation of a mammalian IGRP.

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The invention is also directed to methods of treating a mammal that is at risk for or has type 1 diabetes. The methods comprise administering an oligopeptide to the mammal in a manner sufficient to reduce CD8⁺ T cells reactive to IGRP. In these methods, the oligopeptide is 8-10 amino acids in length and comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL,

5 FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL.

In further embodiments, the invention is directed to other methods of treating a mammal that is at risk for or has type 1 diabetes. These additional methods comprise administering an oligopeptide to the mammal in a manner sufficient to reduce CD8⁺ T cells reactive to IGRP. In these methods, the
10 oligopeptide is 8-10 amino acids in length, completely homologous with a mammalian IGRP, and capable of binding an MHC class I molecule of the mammal.

The invention is further directed to additional methods of treating a mammal that is at risk for or has type 1 diabetes. The methods comprise administering to the mammal an oligopeptide 8-10 amino acids in length, wherein the oligopeptide is a medium- or low-affinity ligand to an IGRP₂₀₆₋₂₁₄-
15 reactive CD8⁺ T cell.

Additionally, the invention is directed to methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a β -cell. The methods comprise treating the β -cell with a compound capable of specifically binding an oligopeptide 8-10 amino acids in length, and completely homologous with a mammalian IGRP having at least 90% homology to SEQ ID NO:1 or
20 SEQ ID NO:2. In these methods, the oligopeptide is capable of binding an MHC class I molecule of the mammal.

The invention is also directed to other methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a β -cell. The methods comprise treating the CD8⁺ T cell with an oligopeptide of 8-10 amino acids comprising a sequence selected from the group consisting of
25 YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL, and an MHC class I molecule that is capable of binding the oligopeptide.

In related embodiments, the invention is directed to additional methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a β -cell. The methods comprise
30 treating the CD8⁺ T cell with an oligopeptide in a manner sufficient to prevent binding of the CD8⁺ T cell to IGRP. In these methods, the oligopeptide is 8-10 amino acids in length and comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL.

In additional embodiments, the invention is directed to other methods of treating a mammal at
35 risk for type 1 diabetes. These methods comprise administering to the mammal an antisense

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molecule, a ribozyme, or an RNAi molecule. In these embodiments, the antisense molecule, the ribozyme, and the RNAi molecule are capable of specifically inhibiting translation of a mammalian IGRP.

The present invention is also directed to additional methods of treating a mammal having type 1 diabetes. The methods comprise transplanting pancreatic islet β -cells into the pancreas of the mammal. In these embodiments, the β -cells are transfected with a vector that expresses an antisense molecule, a ribozyme, or an RNAi molecule that is capable of specifically inhibiting translation of a mammalian IGRP.

The invention is also directed to methods for determining whether a mammal is at risk for or has type 1 diabetes. The methods comprise determining the presence of $CD8^+$ T cells reactive to IGRP in the mammal by obtaining a sample of lymphocytes comprising $CD8^+$ T cells from the mammal, combining the lymphocytes with an oligopeptide, and determining whether any $CD8^+$ T cells specifically bind to the oligopeptide. In these methods, the oligopeptide is 8-10 amino acids in length and comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL. Additionally, the oligopeptide or the MHC molecule further comprises a detectable label. In these methods, $CD8^+$ T cell binding to the oligopeptide indicates that the mammal is at risk for or has type 1 diabetes.

In related embodiments, the invention is directed to other methods for determining whether a mammal is at risk for or has type 1 diabetes. The methods comprise determining the presence of $CD8^+$ T cells reactive to IGRP in the mammal by obtaining a sample of lymphocytes comprising $CD8^+$ T cells from the mammal, combining the lymphocytes with an oligopeptide of 8-10 amino acids that is completely homologous with a mammalian IGRP, and an MHC class I molecule that is capable of binding the oligopeptide, where the oligopeptide or the MHC molecule further comprises a detectable label, and determining whether any $CD8^+$ T cells specifically bind to the oligopeptide. In these methods, $CD8^+$ T cell binding to the oligopeptide indicates that the mammal is at risk for or has type 1 diabetes.

Brief Description of the Drawings

FIG. 1 is graphs of experimental data establishing the identification of IGRP₂₀₆₋₂₁₄ as the natural β cell peptide recognized by the pathogenic T cell clone 8.3. Panels A to C. Epitope reconstitution activity of first- (A), second- (B), and third-dimension (C) HPLC fractions of H-2K^d-eluted NIT-1 peptides. RMA/S/K^d cells were pulsed with peptide fractions and used as targets in ⁵¹Cr-release assays with 8.3 CTL as effectors (15). Panel D. Determination of candidate peptides by correlation of ion abundance curves (plotted on the left and bottom axes) with epitope

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reconstitution activity of third-dimension HPLC fractions (right and top axes). Peptide m/z values are indicated in the key. Panel E. Collision-activated dissociation (CAD) mass spectrum of candidate peptide $(M+2H)^{+2}$ ion with monoisotopic m/z of 548.845. X represents I or L. Ions observed in the spectrum are underlined; the b ions originate from the N-terminus of the peptide and the y ions from the C-terminus. Panel F. Recognition of VYLKTNVFL (IGRP₂₀₆₋₂₁₄) by 8.3 CTL. RMA-S/K^d cells were pulsed with the indicated peptides and used as targets in ⁵¹Cr-release assays with 8.3 CTL as effectors. Panel G. Verification of IGRP as the source of the natural antigenic peptide recognized by 8.3 CTL. COS-7 cells were transfected with varying concentrations of an IGRP expression construct, or vector alone, together with 10 ng/ml of an H-2K^d expression construct (solid lines). Separate cultures were transfected with the H-2K^d construct alone and pulsed with varying concentrations of IGRP₂₀₆₋₂₁₄ peptide (broken line). Following co-culture with 8.3 CTL, T cell response was measured as IFN- γ -release by ELISA and is presented as absorbance at 405 nm (A405).

FIG. 2 is graphs of experimental results establishing that multiple early insulinitic T cell clones recognize IGRP₂₀₆₋₂₁₄. 58 $\alpha\beta$ transfectants expressing the indicated TCRs were cultured with RMA-S/K^d cells pulsed with the indicated peptides and IL-2 release (mean $\pm$ SD) was measured by ELISA. The partial TCR α and β chain sequences for 8.3 and for the early insulinitic T cell clones AI12.B1.3 and AI15.F5 were previously reported (DiLorenzo et al., 1998; Santamaria et al., 1995, Nagata et al., 1994). The AI4, AI12.B1.1, AI12.B1.2, and AI15.A10 lines, all of which express non-V α 17 TCR α chains, did not respond to any of the peptides tested, although they were capable of signaling through the transfected TCR as evidenced by their release of IL-2 in response to plate-bound anti-CD3 ϵ (S. M. Lieberman et al., data not shown). IGRP, IGRP₂₀₆₋₂₁₄; INS, INS B₁₅₋₂₃; FW, framework residues; CDR, complementarity-determining region.

FIG. 3 is a graph of experimental results establishing that IGRP₂₀₆₋₂₁₄ does not demonstrate poor peptide binding to H-2K^d. RMA-S/K^d cells were pulsed with the indicated peptides, stained with an anti-H-2K^d antibody, and analyzed by flow cytometry. IGRP, IGRP₂₀₆₋₂₁₄; INS, INS B₁₅₋₂₃; MFI, mean fluorescence intensity.

FIG. 4 is graphs of experimental results establishing that IGRP₂₀₆₋₂₁₄-reactive T cells are readily detected in islets and peripheral blood of NOD mice directly *ex vivo*. Cells from islets or peripheral blood of 9- or 20-week-old nondiabetic NOD mice were stained with anti-CD8 and the indicated peptide/H-2K^d tetramers. The left panel shows representative tetramer staining patterns of samples from 20-week-old mice gated on the CD8⁺ population. Numbers indicate percentage of tetramer-positive cells within the CD8⁺ population. The right panel shows the percentage (mean $\pm$ SD) of tetramer-positive cells within the CD8⁺ population for five individual mice per age group. TUM/H-2K^d tetramers stained 0% of cells in each group. IGRP, IGRP₂₀₆₋₂₁₄.

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FIG. 5 is graphs of experimental results showing the agonistic activity of APLs of 8.3-CD8+ T-cells. Naïve splenic 8.3-CD8+ cells (2×10^4) were incubated, in duplicate, with peptide-pulsed (0.01-1 μ M), γ -irradiated NOD splenocytes (10^5) for 2 or 3 days (cytokine secretion and proliferation assays, respectively) and pulsed with 1 μ Ci of (3 H)-thymidine, harvested and counted (for proliferation assays). The supernatants of the 2 day cultures (100 μ l) were assayed for IL-2, IL-4 and/or IFN- γ by ELISA. None of the libraries induced IL-4 secretion (not shown). Data are mean $\pm$ SE and are representative of two or three different experiments.

FIG. 6 is graphs of experimental results showing differences in the functional avidity of APLs correlate with differences in peptide/MHC-binding avidity and tolerogenic activity. Panel A shows the fluorescence intensity of tetramer staining. Naïve splenic 8.3-CD8+ T-cells were stained with different APL/K^d-PE tetramers and a CD8-FITC mAb. Panel B shows the tolerogenic activity of APLs against 8.3-CD8+ T-cells *in vivo*. The graph compares total numbers of total CD8+ splenocytes in untreated (10 ± 1 wk-old; n=9) or APL-treated 8.3-NOD mice. Mice received 1 i.p. injection of 100 μ g of peptide in PBS 7 days prior to analysis. The age and number of mice that were studied is as follows: TUM-treated (11 ± 1 wk-old; n=5); NRP-I4-treated (9 ± 1 wk-old; n=3); NRP-A7-treated (11 ± 1 wk-old; n=7); and NRP-V7-treated (14 ± 2 wk-old; n=3). Panel C shows tetramer-binding ability of islet-associated CD8+ T-cells from 20 wk-old non-transgenic NOD mice. Vertical panels correspond to three individual mice. Numbers in the upper right quadrants of each panel correspond to percentage of tetramer-positive CD8+ cells. Note that NRP-V7 stains these cells with higher fluorescence intensity than NRP-A7. Panel D shows CD8+ T-cells propagated from islets of 9 wk-old female NOD mice bind NRP-V7/K^d tetramers (n=6 mice) with higher avidity (lower K_d) than NRPA7/K^d (n=6 mice).

FIG. 7 is graphs of experimental results showing the anti-diabetogenic activity of APLs in wild-type NOD mice. Panel A (upper graphs) show the incidence of diabetes in TUM vs. APL-treated female NOD mice. N values were as follows: TUM: ≤ 25 μ g (n=29), 50 μ g (n=10), 100 μ g (n=69); NRP-I4: ≤ 25 μ g (n=30), 35 μ g (n=10), 50 μ g (n=10), 100 μ g (n=10); NRP-A7: ≤ 25 μ g (n=29), 35 μ g (n=10), 50 μ g (n=10), 75 μ g (n=10), 100 μ g (n=50); NRPV7: ≤ 25 μ g (n=30), 35 μ g (n=10), 50 μ g (n=10), 100 μ g (n=59). Panel A (lower graphs) show the effects of APL vs. TUM-treatment on the percentages and avidity of NRP-V7/K^d tetramer-binding cells within islet-derived CD8+ cells. Mice were sacrificed at diabetes onset or at the end of the study (32 wk) to isolate islet-associated CD8+ T-cells. Data are presented as differences in values obtained in APL- vs. TUM-treated mice ($x \pm$ SE). Values above or below zero indicate that APL treatment resulted in recruitment of low or high avidity T-cells, and/or in recruitment of higher or lower percentages of tetramer-reactive CD8+ T-cells, respectively. N values were as follows: NRP-I4: ≤ 25 μ g (n=15, 7 T1D), 35 μ g (n=4, 3 T1D), 50 μ g (n=3, 1 T1D), 100 μ g (n=7, 0 T1D); NRP-A7: < 25 μ g (n=9, 5

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T1D), 35 μ g (n=4, 4 T1D), 50 μ g (n=5, 1 T1D), 75 μ g (n=3, 0 T1D), 100 μ g (n=10); NRP-V7: <25 μ g (n=10, 7 T1D), 35 μ g (n=6, 3 T1D), 50 μ g (n=10, 5 T1D), 100 μ g (n=47, 31 T1D). Measurements of avidity were only possible in mice containing tetramer-positive cells in islets. The n values were as follows: NRP-I4: $\leq$ 25 μ g (n=15), 35 μ g (n=4), 50 μ g (n=2), 100 μ g (n=7); NRP-A7: $\leq$ 25 μ g (n=6), 35 μ g (n=4), 50 μ g (n=5), 75 μ g (n=2), 100 μ g (n=10); NRP-V7: $\leq$ 25 μ g (n=7), 50 μ g (n=2). Panel B shows the absolute number of NRP-V7/K^d tetramer-binding CD8⁺ T-cells in mice treated with different peptides at 100 μ g/injection.

FIG. 8 is graphs of experimental results demonstrating that NRP-V7 and IGRP₂₀₆₋₂₁₄ cannot blunt diabetes progression despite depleting the IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T-cell pool. Panel A shows the cumulative incidence of T1D in TUM (n=69), NRP-V7 (n=59) and IGRP₂₀₆₋₂₁₄-treated NOD mice (n=19). All mice received 100 μ g (TUM, NRP-V7 and IGRP₂₀₆₋₂₁₄) or 75 μ g of peptide/injection (IGRP₂₀₆₋₂₁₄; n=9). No differences were noted in mice receiving 75 or 100 μ g of IGRP₂₀₆₋₂₁₄, hence the data were pooled. Panel B shows the percentage of NRP-V7/K^d tetramer-reactive cells (left) and IFN- γ secretion (right) by islet-associated CD8⁺ T-cells from untreated, or TUM- and IGRP₂₀₆₋₂₁₄-treated NOD mice. No significant differences were noted between diabetic and non-diabetic mice within individual treatment groups. N values for tetramer staining were: Untreated group: n=11 (2 diabetic); TUM-treated group: n=20 (9 diabetic); NRP-V7-treated group: n=52 (32 diabetic); IGRP₂₀₆₋₂₁₄-treated group: n=17 (8 diabetic). N values for IFN- γ secretion were: Untreated group: n=28 (19 diabetic); TUM-treated group: n=11 (7 diabetic); and IGRP₂₀₆₋₂₁₄-treated group: n=12 (6 diabetic). Panel C is the same as Panel B, but employing IGRP₂₀₆₋₂₁₄/K^d tetramers or IGRP₂₀₆₋₂₁₄ peptide. N values for tetramer staining were: Untreated group: n=28 (19 diabetic); TUM-treated group: n=3 (0 diabetic); IGRP₂₀₆₋₂₁₄-treated group: n=14 (6 diabetic). N values for IFN- γ secretion were: Untreated group: n=29 (9 diabetic); TUM-treated group: n=7 (4 diabetic); and IGRP₂₀₆₋₂₁₄-treated group: n=11 (5 diabetic).

FIG. 9 is graphs of experimental results showing that NOD mice spontaneously mount intra-islet CD8⁺ T-cell responses against multiple IGRP epitopes. Panel A (left) shows the percentage of diabetic and non-diabetic NOD mice that contain intra-islet CD8⁺ T-cells recognizing epitopes of IGRP. Panel A (right) shows the average amounts of IFN- γ secreted by islet-derived CD8⁺ T-cells from mice that responded to the corresponding peptide (excluding mice that did not respond). Data are from Table II. Panel B shows examples of mice containing IGRP₃₂₄₋₃₃₂- or IGRP₂₁₋₂₉-reactive CD8⁺ T-cells within islets, as determined with tetramers. Panel C shows the cumulative amounts of IFN- γ secreted by islet-derived CD8⁺ T-cells in response to IGRP peptides.

FIG. 10 is graphs of experimental results showing that IGRP₂₀₆₋₂₁₄-treatment reduces IGRP₂₀₆₋₂₁₄-reactivity but increases responses against other IGRP epitopes, particularly in mice progressing to overt diabetes. Panel A (top graphs) shows the percentage of untreated, and TUM- or

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IGRP₂₀₆₋₂₁₄-treated NOD mice that contain intra-islet CD8⁺ T-cells recognizing epitopes of IGRP. Panel A (bottom graphs) shows the average amounts of IFN- γ secreted by islet-derived CD8⁺ T-cells from mice responding to the corresponding peptides. Data are from Table II. Panel B shows the cumulative amounts of IFN- γ secreted by islet-derived CD8⁺ T-cells in response to IGRP peptides. Panel C is the same as in Panel B except that Panel C compares IGRP₂₀₆₋₂₁₄-treated NOD mice that developed diabetes vs. those that did not. Panel D shows the cumulative amounts of IFN- γ secreted by islet-derived CD8⁺ T-cells of diabetic and non-diabetic IGRP₂₀₆₋₂₁₄-treated NOD mice in response to IGRP peptides.

10 Detailed Description of the Invention

The present invention is based on the identification of the 8.3-like T cell ligand present on β cells that is responsible for type 1 diabetes. As established by the research described in Example 1, the ligand is islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), and its predominant CD8⁺ T cell-binding peptide comprising amino acids 206-214 of the IGRP sequence, having the sequence VYLKTNVFL in the mouse, using the commonly accepted single letter amino acid abbreviations. Other mouse IGRP peptides capable of binding CD8⁺ T cells that are involved in the pathology of type 1 diabetes are FLWSVFWLI (amino acids 152-160); TYYGFLNFM (21-29); LRLFGIDLL (225-233); KWCANPDWI (241-249) and SFCKSASIP (324-332). See Examples 3 and 4.

As used herein, an IGRP amino acid sequence includes any naturally occurring mammalian amino acid sequence that is islet specific and is at least 90% identical to SEQ ID NO:1 (Mouse IGRP, from GenBank NP 067306) or SEQ ID NO:2 (Human IGRP, from GenBank NP 066999). The IGRP mouse and human amino acid sequences are not limited to SEQ ID NO:1 and SEQ ID NO:2, respectively, but includes any variants naturally present in mice or humans. The identification of any mammalian IGRP amino acid sequence can be readily made without undue experimentation, e.g., by identifying mRNA sequences limited to islet cells that are highly (i.e., >90%) homologous to already-identified mRNA sequences of mouse or human IGRP (e.g., as found in GenBank accessions NP 021331 and NP 021176, respectively), and determining the amino acid sequence of the expressed protein.

As further discussed in Example 1, the mouse IGRP₂₀₆₋₂₁₄ peptide has the sequence VYLKTNVFL. The analogous human sequence, from GenBank NP 066999, is TYLKTNLFL. Thus, the mouse and human sequences differ at the first (V vs. T) and seventh position (V vs. L). It is also known that the oligopeptide sequences KYNKANVFL (NRP-V7) and KYNKANAFLL (NRP-A7) are additional 8.3-like T cell ligands (Anderson et al., 1999; Amrani et al., 2001). These differ from the naturally occurring mouse and human sequences at the first, third, fifth, and seventh positions. The

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differences among these sequences at the first position is not conservative, since V (Val) is nonpolar, T (Thr) is polar, and K (Lys) is positively charged. This indicates that the first position of these peptides do not contribute to 8.3 T cell binding. However, the three different amino acids at the seventh position, V (Val), L (Leu) and A (Ala), are all nonpolar, indicating that a nonpolar residue is required for that position in order to retain 8.3 T cell binding. Therefore, one can conclude that an oligopeptide having the naturally-conserved sequence YLKTN(A/I/L/V)FL are 8.3 T cell ligands. As used herein, (A/I/L/V) signifies that the amino acid at that position can be any of the non-polar amino acids A, I, L or V. Since the mouse H-2K^d class I molecule is known to require at least a 9-mer, at least nine amino acids is preferred where mouse MHC molecules and T cells are utilized. A Blast search revealed that none of the 8-mers are present in any known amino acid sequence, except for IGRP.

With regard to the other IGRP peptides identified as capable of binding CD8⁺ T cells, peptides 152-160 (FLWSVFWLI), 241-249 (KWCANPDWI), and 324-332 (SFCKSASIP) are identical in humans and mice. However, peptides 21-29 and 225-233 are different in mice and humans. Peptide 21-29 is TYYGFKNFM in mice and AYYTFLNFM in humans, and peptide 225-231 is LRLFGIDLL in mice and LRVLNIDLL in humans. The human peptides would also be expected to be capable of binding CD8⁺ T cells.

Thus, in some embodiments, the present invention is directed to isolated and purified oligopeptides or polypeptides less than 355 amino acids comprising a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP. These oligopeptides and polypeptides are also useful in that they are CD8⁺ T cell ligands comprising peptide sequences involved in type 1 diabetes and are thus useful for diagnostic or therapeutic applications.

As used herein, "isolated and purified" means present in a greater concentration than would be found in nature. Preferably, an isolated and purified oligopeptide or polypeptide is at least about 10% of the peptide component of the preparation; more preferably at least about 25%; even more preferably at least about 50%; still more preferably at least about 75%; and most preferably at least about 90% of the peptide component of a preparation.

In preferred embodiments, these oligopeptides or polypeptides are completely homologous to a mammalian IGRP (e.g., mouse and human) having at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2. In other preferred embodiments, the oligopeptides or polypeptides comprise 100 amino acids or less, more preferably 25 amino acids or less, even more preferably 13-25 amino acids, and most preferably 8-10 amino acids, since CD8⁺ T cells generally only bind oligopeptides of 8-10 amino acids.

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In these embodiments, preferred polypeptides or oligopeptides comprise the above peptides having the sequences that VYLKTNVFL or TYLKTNLFL, since those sequences are present in the naturally occurring IGRP from mice and humans, respectively.

5 In further aspects of these embodiments, the oligopeptide or polypeptide also comprises an antigenic carrier, in order to more effectively use the peptides in immunization protocols, to tolerize a mammal to IGRP, preventing development of type 1 diabetes, as was achieved in the experiment described in Example 2. A nonlimiting example of an antigenic carrier is incomplete Freund's adjuvant. See also Gammon et al., 1986.

10 In other aspects, the oligopeptide or polypeptide further comprises a detectable label. Such labeled peptides are useful in diagnostic protocols, e.g., to determine the presence of 8.3-like CD8⁺ T cells, to identify a mammal that has, or is at risk for, type 1 diabetes. The invention is not limited to any particular detectable label, and the skilled artisan can select a label most appropriate for any particular application without undue experimentation. Examples include a fluorescent moiety, a radioactive molecule, and an assayable enzyme (e.g., β -galactosidase or streptavidin). Methods for
15 labeling peptides with any of these detectable moieties are well known.

In further aspects, the above-identified oligopeptides of 8-10 amino acids can be usefully combined with an MHC class I molecule that is capable of binding the oligopeptide, for example a mouse H-2K^d molecule, which binds the IGRP₂₀₆₋₂₁₄. An MHC class I molecule for any of the other peptides can be identified using methods known in the art. Since CD8⁺ T cells only bind to an antigen
20 in the context of an MHC class I molecule, the oligopeptide-MHC class I mixtures are useful for creating a T cell ligand. The MHC class I molecules are preferably employed in the form of tetramers. See, e.g., Altman et al., 1996; Trudeau et al., 2003. In some applications, e.g., diagnostics, the oligopeptide of the oligopeptide-MHC class I mixtures further comprises a detectable label, such as those previously discussed, i.e., conjugated to the oligopeptide. Alternatively or additionally, the
25 MHC class I molecule could employ a detectable label.

In some methods of treatment, directed toward eliminating CD8⁺ T cells, the oligopeptide or the MHC class I molecule can also include a cytotoxic molecule. In these methods, the cytotoxic oligopeptide-MHC class I mixture binds to the 8.3-like T cell, where the cytotoxic molecule kills the T cell.

30 The invention is not narrowly limited to any particular cytotoxic molecule that is bound to the oligopeptide or MHC class I molecule. The skilled artisan could identify various cytotoxic molecules useful in these aspects, and could select the appropriate cytotoxic molecule for any particular application without undue experimentation. Examples of potentially useful cytotoxic molecules include radioactive molecules (e.g., ¹³¹I, ⁹⁰Y), and toxic chemicals or proteins (e.g., 5-fluorouridine or
35 ricin).

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Since CD4⁺ T cells are also involved in the pathogenic process of type 1 diabetes, and since CD4⁺ T cells bind oligopeptides that are 13-25 amino acids, and only when presented on MHC class II molecules, mixtures of oligopeptides with MHC class II molecules are also within the scope of the invention. In these embodiments, the oligopeptides are 13-25 amino acids and comprise a sequence
5 selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP. Analogous to previously described oligopeptide-MHC class I mixtures, the oligopeptides or MHC class II molecules of the oligopeptide-MHC class II mixtures can also usefully comprise a detectable label or a cytotoxic molecule.

It is also envisioned that any of the above-described oligopeptides or polypeptides are
10 usefully provided in a sterile pharmaceutical preparation, particularly when the oligopeptide or polypeptide is to be utilized for therapeutic treatments. Thus, in some embodiments, the oligopeptide or polypeptide in a sterile pharmaceutical preparation is capable of tolerizing a mammal to reduce CD8⁺ T cells reactive to IGRP. Also included as useful in a sterile pharmaceutical preparation is a mammalian IGRP protein itself, i.e., having at least 90% homology to SEQ ID NO:1 or SEQ ID
15 NO:2. Preferably, this mammalian IGRP is a mouse IGRP or a human IGRP.

As indicated in Examples 2 and 3, it appears that a general characteristic of IGRP itself makes it unusually capable of contributing peptides that are ligands for T cells involved in type 1 diabetes.

Thus, the invention is also directed to isolated and purified oligopeptides 8-10 amino acids in length and completely homologous with a mammalian IGRP (e.g., mouse and human), where the
20 oligopeptide is capable of binding a MHC class I molecule of the mammal. Preferably, the oligopeptide has at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2. In preferred embodiments, the isolated and purified oligopeptide comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP. In some preferred embodiments,
25 particularly where the peptide is to be used diagnostically, the oligopeptide further comprises a detectable label as previously described, e.g., a fluorescent molecule, a radioactive molecule, or an enzyme. In other preferred embodiments, the peptide further comprises an MHC class I molecule that is capable of binding the oligopeptide. These oligopeptides, either with or without the detectable label, can also comprise an MHC class I molecule that is capable of binding the oligopeptide. As with
30 previously discussed embodiments, the oligopeptide-MHC class I mixture can also comprise a cytotoxic molecule, conjugated to the oligopeptide or to the MHC class I molecule.

Despite efforts to identify a function for IGRP, none has been found. It is therefore likely that therapy to reduce or eliminate expression of IGRP on islet cells would be beneficial in reducing or
35 eliminating pathogenic islet cell destruction in type 1 diabetes, since such a therapy would eliminate the target ligand for the autoimmune reactions leading to type 1 diabetes. The reduction or

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elimination of IGRP expression can be achieved by treatment with antisense molecules, ribozymes, or RNAi molecules that target IGRP mRNA. In these embodiments, the antisense molecule, ribozyme, or RNAi molecules can be comprised of nucleic acid (e.g., DNA or RNA) or nucleic acid mimetics (e.g., phosphorothionate mimetics) as are known in the art.

5 Thus, the present invention is additionally directed to antisense molecules complementary to at least a portion of an mRNA encoding a mammalian (e.g., mouse and human) IGRP. In these embodiments, the antisense molecule is capable of inhibiting translation of a mammalian IGRP. Preferably, the mammalian IGRP is at least 90% homologous to SEQ ID NO:1 or SEQ ID NO:2. The antisense molecule for any mammalian IGRP can be designed without undue experimentation using
10 known IGRP sequence information, e.g., as provided in GenBank accessions NM 021331 (mouse) and NM 021176 (human).

 In related embodiments, the invention is directed to ribozymes that specifically cleave an mRNA encoding a mammalian (e.g., mouse or human) IGRP, thus inhibiting translation of the mammalian IGRP. Preferably, the mammalian IGRP is at least 90% homologous to SEQ ID NO:1 or
15 SEQ ID NO:2. Since ribozyme technology is well established, the skilled artisan could design a ribozyme as described above without undue experimentation.

 In additional related embodiments, the invention is directed to RNAi molecules that are homologous to a portion of an mRNA encoding a mammalian IGRP, and that are capable of inhibiting translation of a mammalian (e.g., mouse or human) IGRP. As is well known, an RNAi
20 (including siRNA) molecule is a short double stranded nucleic acid that interferes with transcription or translation of a homologous gene. As with previous embodiments, the mammalian IGRP is at least 90% homologous SEQ ID NO:1 or SEQ ID NO:2. Since RNAi technology is well established, the skilled artisan could design an RNAi molecule as described above without undue experimentation.

 Many of the above-described compositions are useful in methods of treating mammals
25 (including but not limited to humans and rodents such as mice) that are at risk for, or have type 1 diabetes. As such, the above-described compositions can be formulated without undue experimentation for administration to a mammal, including humans, as appropriate for the particular application. Additionally, proper dosages of the compositions can be determined without undue experimentation using standard dose-response protocols.

30 Accordingly, the compositions designed for oral, lingual, sublingual, buccal and intrabuccal administration can be made without undue experimentation by means well known in the art, for example with an inert diluent or with an edible carrier. The compositions may be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the pharmaceutical compositions of the present invention may be incorporated with excipients and used in
35 the form of tablets, troches, capsules, elixirs, suspensions, syrups, wafers, chewing gums and the like.

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Tablets, pills, capsules, troches and the like may also contain binders, recipients, disintegrating agent, lubricants, sweetening agents, and flavoring agents. Some examples of binders include microcrystalline cellulose, gum tragacanth or gelatin. Examples of excipients include starch or lactose. Some examples of disintegrating agents include alginic acid, corn starch and the like.

5 Examples of lubricants include magnesium stearate or potassium stearate. An example of a glidant is colloidal silicon dioxide. Some examples of sweetening agents include sucrose, saccharin and the like. Examples of flavoring agents include peppermint, methyl salicylate, orange flavoring and the like. Materials used in preparing these various compositions should be pharmaceutically pure and nontoxic in the amounts used.

10 The compositions of the present invention can easily be administered parenterally such as for example, by intravenous, intramuscular, intrathecal or subcutaneous injection. Parenteral administration can be accomplished by incorporating the compositions of the present invention into a solution or suspension. Such solutions or suspensions may also include sterile diluents such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other
15 synthetic solvents. Parenteral formulations may also include antibacterial agents such as for example, benzyl alcohol or methyl parabens, antioxidants such as for example, ascorbic acid or sodium bisulfite and chelating agents such as EDTA. Buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose may also be added. The parenteral preparation can be enclosed in ampules, disposable syringes or multiple dose vials made of glass or
20 plastic.

Rectal administration includes administering the pharmaceutical compositions into the rectum or large intestine. This can be accomplished using suppositories or enemas. Suppository formulations can easily be made by methods known in the art. For example, suppository formulations can be prepared by heating glycerin to about 120° C., dissolving the composition in the glycerin,
25 mixing the heated glycerin after which purified water may be added, and pouring the hot mixture into a suppository mold.

Transdermal administration includes percutaneous absorption of the composition through the skin. Transdermal formulations include patches (such as the well-known nicotine patch), ointments, creams, gels, salves and the like.

30 The present invention includes nasally administering to the mammal a therapeutically effective amount of the composition. As used herein, nasally administering or nasal administration includes administering the composition to the mucous membranes of the nasal passage or nasal cavity of the patient. As used herein, pharmaceutical compositions for nasal administration of a composition include therapeutically effective amounts of the composition prepared by well-known methods to be

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administered, for example, as a nasal spray, nasal drop, suspension, gel, ointment, cream or powder. Administration of the composition may also take place using a nasal tampon or nasal sponge.

Accordingly, in some embodiments, the invention is directed to methods of treating a mammal, where the mammal is at risk for or has type 1 diabetes. The methods comprise

5 administering an oligopeptide in a sterile pharmaceutical preparation to the mammal in a manner sufficient to reduce T cells reactive to IGRP. In these embodiments, the oligopeptide comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL. The last sequence is the sequence of the peptide NRP-I4, which is a peptide known to react to 8.3-like
10 T cells, and which is shown in Example 3 to be an effective treatment for type 1 diabetes. The T cells reduced by these methods can be CD4⁺, or preferably, CD8⁺. In these methods, the oligopeptide or polypeptide treatment tolerizes the mammal to eliminate the T cells. Where the T cells are CD8⁺, these methods would reduce or eliminate T cells reactive to IGRP in β -cells. See Examples 2-4. The oligopeptides or polypeptides of these embodiments are described above. They can be any size
15 considered appropriate by the skilled artisan as appropriate for tolerization protocols, including 8-10mers, 13-25mers, or less than 25, or less than 50, or less than 100, or less than 300, or even larger, including an entire IGRP protein, or several of the peptides conjugated to an inert or immunogenic carrier. Protocols for tolerizing a mammal to T cell reactivity are well known. See, e.g., Gammon et al., 1986.

20 These methods include embodiments where more than one of the above-identified oligopeptides is used in the treatment, in order to eliminate other reactive CD8⁺ T cells.

In other embodiments, the invention is directed to additional methods of treating a mammal, where the mammal is at risk for or has type 1 diabetes. These methods comprise administering, to the mammal, an oligopeptide of 8-10 amino acids comprising a sequence selected from the group
25 consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL. In these methods, a class I MHC molecule capable of binding the oligopeptide is also included with the oligopeptide, in such a manner as to cause oligopeptide-MHC binding. The oligopeptide and/or the class I MHC molecule in these embodiments further comprises a cytotoxic molecule, as described above. The
30 oligopeptide-cytotoxic molecule is administered to the mammal in a manner sufficient to reduce CD8⁺ T cells reactive to IGRP. The reduction in CD8⁺ T cells is achieved when the peptide-MHC molecule-cytotoxic molecule binds to the 8.3-like T cell, which is killed by the cytotoxic molecule. As with the embodiments described above, more than one oligopeptide can be administered.

In these embodiments, the MHC class I molecules are preferably employed in the form of
35 tetramers. See, e.g., Altman et al., 1996; Trudeau et al., 2003. However, the MHC class I molecule

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can also be present as part of an antigen-presenting cell. Such compositions are particularly useful for measuring cytokine (particularly interferon- γ) production from CD8⁺ T cells. See, e.g., Example 2 and Terajima et al., 2003.

In related embodiments, the invention is directed to additional methods of treating a mammal, where the mammal is at risk for or has type 1 diabetes. These methods comprise administering to the mammal an oligopeptide 8-10 amino acids in length that is completely homologous with a mammalian IGRP, and is capable of binding a mammalian MHC class I molecule, as described above. The oligopeptide is preferably completely homologous with a mammalian IGRP having at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2. In these methods, a class I MHC molecule capable of binding the oligopeptide is also included with the oligopeptide, in such a manner as to cause oligopeptide-MHC binding. In these embodiments, the oligopeptide and/or the class I MHC molecule further comprise a cytotoxic molecule, as described above. The oligopeptide-cytotoxic molecule is administered to the mammal in a manner sufficient to reduce CD8⁺ T cells reactive to IGRP. The reduction in CD8⁺ T cells is achieved when the peptide-MHC molecule-cytotoxic molecule binds to the IGRP-reactive T cell, which is then killed by the cytotoxic molecule. More than one oligopeptide can be administered in these embodiments.

As shown in Example 3, a particularly effective type 1 diabetes treatment is with an oligopeptide that has medium- or low-affinity binding to an IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cell. Thus, the invention is also directed to additional methods of treating a mammal, wherein the mammal is at risk for or has type 1 diabetes. The methods comprising administering an oligopeptide 8-10 amino acids in length to the mammal, wherein the oligopeptide is a medium- or low-affinity ligand to an IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cell. Preferred examples of useful oligonucleotides for these methods are KYNIANWFL and KYNKANAFLL, as utilized in Example 3.

In further embodiments, the invention is directed to methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a β -cell. These methods comprise treating the β -cell with a compound capable of binding an oligopeptide, where the oligopeptide is 8-10 amino acids in length and is completely homologous with a mammalian IGRP, as described above. By binding domains on β -cell IGRP, reaction of CD8⁺ T cells with IGRP, which can lead to the destruction of the β -cell, can be prevented. The oligopeptide is preferably completely homologous with a mammalian IGRP having at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2. In these embodiments, the oligopeptide-binding compound prevents binding of the oligopeptide to the MHC, or prevents binding of the oligopeptide-MHC complex to a CD8⁺ T cell.

In preferred embodiments, the compound can bind an oligopeptide or polypeptide comprising a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP.

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In these embodiments, the compound capable of binding the oligopeptide can be any compound capable of interfering with the CD8⁺ binding to the β cell, either by reducing the numbers of oligopeptide-MHC binding, or by causing a physical interference to the T cell binding to the oligopeptide-MHC complex. In preferred embodiments, the compound is an antibody or an aptamer.

5 Methods of making antibodies to an oligopeptide are routine, and the skilled artisan would expect that such an antibody could be made to any IGRP oligopeptide without undue experimentation. The antibodies can be from a polyclonal, monoclonal, or recombinant source. As used herein, "antibodies" also include a fragment of a whole antibody that comprises a typical immunoglobulin antigen binding site (e.g., Fab or Fab2). The antibodies can also be of any vertebrate (e.g., mouse,
10 chicken, rabbit, goat or human), or of a mixture of vertebrates (e.g., humanized mouse).

Aptamers are single stranded oligonucleotides or oligonucleotide analogs that bind to a particular target molecule, such as a protein or a small molecule (e.g., a steroid or a drug, etc.). Thus, aptamers are the oligonucleotide analogy to antibodies. However, aptamers are smaller than antibodies, generally in the range of 50-100 nt. Their binding is highly dependent on the secondary
15 structure formed by the aptamer oligonucleotide. Both RNA and single stranded DNA (or analog), aptamers are known. See, e.g., Burke et al., 1996; Ellington and Szostak 1990; Hirao et al., 1998; Jaeger et al., 1998; Kensch et al, 2000; Schneider et al., 1995; and U.S. Pats. No. 5,773,598; 5,496,938; 5,580,737; 5,654,151; 5,726,017; 5,786,462; 5,503,978; 6,028,186; 6,110,900; 6,124,449; 6,127,119; 6,140,490; 6,147,204; 6,168,778; and 6,171,795.

20 Aptamers that bind to virtually any particular target can be selected by using an iterative process called SELEX, which stands for Systematic Evolution of Ligands by EXponential enrichment (Burke et al., 1996; Ellington and Szostak, 1990; Schneider et al., 1995; Tuerk and Gold, 1992; Tuerk and Gold, 1990). Several variations of SELEX have been developed which improve the process and allow its use under particular circumstances. See, e.g., U.S. Pats. No. 5,472,841; 5,503,978;
25 5,567,588; 5,582,981; 5,637,459; 5,683,867; 5,705,337; 5,712,375; and 6,083,696. Thus, the production of aptamers to any particular oligopeptide, including IGRP oligopeptides, requires no undue experimentation.

Additionally, the pancreatic islet β -cell in these methods can be treated *ex vivo* or *in vitro* (e.g., on islet β cells that are for transplanting into a patient having type 1 diabetes). In preferred
30 embodiments, the islet β -cell is part of a pancreas of a mammal at risk for or having type 1 diabetes. While the methods are not limited to the use with any particular mammal, a mouse or a human is preferred.

The present invention is also directed to methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a β -cell. The methods comprise treating the CD8⁺ T cell
35 with an oligopeptide of 8-10 amino acids comprising a sequence selected from the group consisting of

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YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP, as described above, along with an MHC class I molecule that is capable of binding the oligopeptide, as described above. The MHC class I molecules in these embodiments is preferably in the form of a tetramer, as described above. In these embodiments, the CD8⁺ T cell binds the oligopeptide-MHC combination, preventing the T cell from binding to the oligopeptide-MHC on the β -cell.

In some embodiments of these methods, the oligopeptide or MHC molecule further comprises a cytotoxic molecule, as described above, such that the T cell is killed when it binds to the oligopeptide-MHC-cytotoxic molecule combination.

The pancreatic islet β -cell in these methods can also be treated *ex vivo* or *in vitro* (e.g., on islet β cells that are for transplanting into a patient having type 1 diabetes). In preferred embodiments, the islet β -cell is part of a pancreas of a mammal at risk for or having type 1 diabetes. While the methods are not limited to the use with any particular mammal, a mouse or a human is preferred.

In related embodiments, the invention is directed to other methods of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a β -cell. The methods comprise treating the CD8⁺ T cell with an oligopeptide of 8-10 amino acids completely homologous with a mammalian IGRP, as described above, along with an MHC class I molecule that is capable of binding the oligopeptide, as described above. The oligopeptide or these embodiments is capable of binding a human MHC class I molecule. Preferably, the oligopeptide has at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2.

The MHC class I molecules in these embodiments is preferably in the form of a tetramer, as described above. In these embodiments, the CD8⁺ T cell binds the oligopeptide-MHC combination, preventing the T cell from binding to the oligopeptide-MHC on the β -cell.

In some embodiments of these methods, the oligopeptide or MHC molecule further comprises a cytotoxic molecule, as described above, such that the T cell is killed when it binds to the oligopeptide-MHC-cytotoxic molecule combination.

The pancreatic islet β -cell in these methods can also be treated *ex vivo* or *in vitro* (e.g., on islet β cells that are for transplanting into a patient having type 1 diabetes). In preferred embodiments, the islet β -cell is part of a pancreas of a mammal at risk for or having type 1 diabetes. While the methods are not limited to the use with any particular mammal, a mouse or a human is preferred.

Additionally, the present invention is directed to methods of treating a mammal at risk for type 1 diabetes. The methods of these embodiments comprise administering to the mammal a compound capable of specifically decreasing expression of IGRP in the mammal. In these embodiments, the compound is selected from the group consisting of the antisense molecule, the ribozyme, and the RNAi molecule previously described, where the compound is administered in a

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manner sufficient to decrease expression of IGRP in the mammal. While the methods are not limited to the use with any particular mammal, a mouse or a human is preferred.

A known method of treatment of mammals (including humans) having type 1 diabetes is transplanting pancreatic islet β -cells into the pancreas of the mammal. However, since β -cell-autoreactive $CD8^+$ T cells are generally present after transplant, the transplanted β -cells are often also destroyed. The present invention provides methods for reducing or eliminating this destruction of the transplanted β -cells. The methods involve transfecting the β -cells with a vector expressing a nucleic acid that reduces or eliminates expression of the IGRP in the cells. The transfected cells, which are expressing little or no IGRP, are then transplanted into the mammal. Since those cells express little or no IGRP, autoreactive $CD8^+$ T cells are not able to bind to and destroy the β -cells.

Thus, in these embodiments, the invention is directed to additional methods of treating a mammal having type 1 diabetes. The methods comprise transplanting pancreatic islet β -cells into the pancreas of the mammal, where the β -cells are transfected with a vector that expresses a nucleic acid. The nucleic acid is selected from the group consisting of the antisense molecule described above, the ribozyme described above, and the RNAi molecule described above. In these embodiments, the nucleic acid is expressed in a manner sufficient to decrease expression of IGRP in the β -cells.

These embodiments are not limited to any particular type of vector. As is well known in the art, examples of suitable vectors include a naked DNA vector and a viral vector (e.g., adenoviral or lentiviral). The skilled artisan can select and synthesize an appropriate vector without undue experimentation.

As with other embodiments, the methods are not limited to use with any particular mammal. In preferred embodiments, the mammal is a mouse or a human.

The discovery of the role of IGRP in type 1 diabetes suggests several diagnostic methods.

Accordingly, in some embodiments, the invention is directed to methods for determining whether a mammal is at risk for or has type 1 diabetes. The methods comprise determining the presence of $CD8^+$ T cells reactive to IGRP in the mammal by

a. obtaining a sample of lymphocytes comprising $CD8^+$ T cells from the mammal by standard methods (e.g., venipuncture);

b. combining the lymphocytes with an oligopeptide of 8-10 amino acids comprising a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP, as described above, along with an MHC class I molecule that is capable of binding the oligopeptide, as described above, and where the oligopeptide or the MHC class I molecule further comprises a detectable label, as described above; and

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c. determining whether any CD8⁺ T cells specifically bind to the oligopeptide. In these methods, CD8⁺ T cell binding to the oligopeptide indicates that the mammal is at risk for or has type 1 diabetes.

These methods can be used with any mammal, although the mammal is preferably a mouse or a human.

The determination step (c.) can be by any known means. In some preferred embodiments, the determination step is performed by counting labeled CD8⁺ T cells using a cell sorter (e.g., a fluorescence activated cell sorter) or labeled cell counter (e.g., Coulter counter). In other preferred embodiments, the determination step is performed by microscopic observation of the lymphocytes under conditions where the label can be observed, e.g., with a fluorescence microscope if a fluorescent label is used, or light microscope if an enzyme label and colored enzyme substrate is used to visualize the bound T cells. In additional preferred embodiments, the determination step is performed by measuring activation of the CD8⁺ T cells, preferably by measuring interferon- γ production by known methods, for example using an ELISpot assay (see, e.g., Hartemann et al., 1999).

The invention is also directed to other methods for determining whether a mammal is at risk for or has type 1 diabetes. The methods comprise determining the presence of CD8⁺ T cells reactive to IGRP in the mammal by

a. obtaining a sample of lymphocytes comprising CD8⁺ T cells from the mammal by standard methods (e.g., venipuncture);

b. combining the lymphocytes with an oligopeptide 8-10 amino acids in length that is completely homologous with a mammalian IGRP, where the oligopeptide is capable of binding a human MHC class I molecule, as described above, along with an MHC class I molecule that is capable of binding the oligopeptide, as described above, and where the oligopeptide or the MHC class I molecule further comprises a detectable label, as described above; and

c. determining whether any CD8⁺ T cells specifically bind to the oligopeptide. In these methods, CD8⁺ T cell binding to the oligopeptide indicates that the mammal is at risk for or has type 1 diabetes.

In preferred embodiments, the oligopeptide used in these methods has at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2. As with the other diagnostic method described above, the determination step is preferably performed by counting labeled CD8⁺ T cells using a cell sorter or labeled cell counter, or by microscopic observation of the lymphocytes under conditions where the label can be observed, or by measuring activation of the CD8⁺ T cells, e.g., by measuring interferon- γ production, for example using an ELISpot assay.

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All of the above treatment or diagnostic methods suggest second medical use embodiments (e.g., the use of an oligopeptide or polypeptide comprising a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP, in a sterile pharmaceutical preparation, for the manufacture of a medicament for treating a mammal, where the mammal is at risk for or has type 1 diabetes, by administering the oligopeptide or polypeptide to the mammal in a manner sufficient to reduce T cells reactive to IGRP); all such second medical use embodiments suggested by the above claims are specifically envisioned herein.

Preferred embodiments of the invention are described in the following examples. Other embodiments within the scope of the claims herein will be apparent to one skilled in the art from consideration of the specification or practice of the invention as disclosed herein. It is intended that the specification, together with the examples, be considered exemplary only, with the scope and spirit of the invention being indicated by the claims which follow the examples.

Example 1. Identity of the Antigen Targeted by Prevalent Pathogenic T Cells in Diabetes

Example summary

CD8⁺ T cells are essential for destruction of the insulin-producing pancreatic β -cells in autoimmune (type 1) diabetes. Yet their antigenic targets are largely unknown. Here we reveal that the β cell target of a prevalent population of pathogenic CD8⁺ T cells in NOD mice is islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP). The human IGRP gene maps to a diabetes susceptibility locus, indicating that IGRP is also an antigen for pathogenic T cells in human type 1 diabetes and, thus, a new target for diagnostic and therapeutic approaches.

Materials and Methods

Mice. NOD/Lt mice were maintained by brother-sister mating. 8.3-TCR $\alpha\beta$ -transgenic NOD mice, designated 8.3-NOD, have been described (Verdaguer et al., 1997). All mice were maintained under specific pathogen-free conditions and used in accordance with institutional guidelines for animal welfare.

Class I MHC-associated peptides. H-2K^d molecules were immunoaffinity purified from 1.4 X 10¹⁰ interferon- γ -treated NIT-1 pancreatic β cells (Hamaguchi et al., 1991) using monoclonal antibody SF1-1.1, and their associated peptides extracted as previously described (Cox et al., 1997). Peptide extracts were fractionated by two rounds of reverse phase HPLC as described (Pierce et al., 1999).

Epitope reconstitution assays. 8.3 CTL were generated by culturing splenocytes from 8.3-NOD mice with mitomycin C-treated NOD splenocytes pulsed with NRP-A7 peptide as described

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(DiLorenzo et al, 2002). CTL were used in 16 h ^{51}Cr -release cytotoxicity assays to test for recognition of peptide-pulsed RMA-S/ K^{d} target cells (provided by M. Bevan, University of Washington, Seattle, WA, USA) at an effector to target ratio of 40:1 as described (DiLorenzo et al., 2002). Synthetic peptides were used at concentrations as indicated in the figures, and 7×10^8 , 6×10^8 , and 2×10^9 NIT-1-cell-equivalents of peptide were used for assays of first-, second-, and third-dimension HPLC fractions, respectively.

Peptide analysis. Active second-dimension fraction 66 was loaded on a reverse-phase microcapillary column and analyzed by micro-ESI on a home-assembled FT-ICR MS, equipped with nanoflow liquid chromatography and an online effluent splitter (Cox et al., 1997; Pierce et al., 1999). Briefly, effluent from the microcapillary HPLC column was split such that one-nineteenth was directed into the FT-ICR MS for ESI MS analysis and the remaining eighteen-nineteenths were deposited directly into the wells of a 96-well plate for epitope reconstitution assays. In this way, each well could be correlated to a set of scans in the mass spectral data. Peptide masses eluting in the area of CTL activity and having an elution profile similar to the activity lysis profile were marked as antigen candidates. Good candidates had a deconvoluted $(\text{M}+\text{H})^+$ mass between 800 and 1600 Da, the mass range characteristic of peptides eluted from class I MHC molecules.

Sequence analysis and synthesis of candidate antigens. CAD mass spectra were recorded for selected peptide candidates using a ThermoFinnigan ion trap mass spectrometer (LCQ). Candidate masses were targeted throughout the chromatographic run. Candidate peptides (VYLKTNVFL and IYQKAFDLI) were synthesized by standard Fmoc chemistry using a Gilson peptide synthesizer (model AMS422) and purified to >95% by reverse phase HPLC. Candidate antigen sequences were confirmed by comparing CAD spectra to those of synthetic peptides. Further sequence confirmation and an estimation of copy number per cell was determined by running an aliquot of the active fraction and comparing antigen ion abundance to an identical run with synthetic antigen spiked in at a known level. Synthetic and naturally processed peptide co-elution further confirmed the identity of the antigen.

Synthetic peptides. NRP-A7 (KYNKANAF L), NRP-V7 (KYNKANVFL), INS B $_{15-23}$ (LYLVCGERG), INS-I9 (LYLVCGERI), TUM (KYQAVTTTL), and G6Pase (KYCLITIFL) peptides were synthesized by standard solid-phase methods using Fmoc chemistry in an automated peptide synthesizer (Applied Biosystems model 433A), and their identities were confirmed by mass spectrometry.

Transient transfection. COS-7 cells were transfected using a DEAE-dextran protocol as described (Karttunen et al., 1992). 10 ng/ml of DNA for the class I MHC molecule (H-2K $^{\text{d}}$ or H-2D $^{\text{b}}$) expression constructs was used along with concentrations of IGRP expression construct or vector alone as indicated in the figure. Separate cultures were transfected with the H-2K $^{\text{d}}$ construct alone

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and pulsed with varying concentrations of IGRP₂₀₆₋₂₁₄ peptide. Following co-culture with 8.3 CTL, T cell response was measured as IFN- γ release by ELISA.

T cell receptor transfectants. T cell receptors from the previously isolated NOD-derived β cell-autoreactive class I MHC-restricted T cell clones AI4, AI12.B1.1, AI12.B1.2, AI12.B1.3, AI15.A10, AI15.F5 (DiLorenzo et al., 1998), and 8.3 (Verdaguer et al., 1997) were expressed in the TCR⁻ T cell hybridoma 58 $\alpha\beta$ ⁻ (engineered to express the TCR ζ chain and CD8 $\alpha\beta$ and provided by H.-C. Chang, Dana Farber, Boston, MA, USA) as described (Serreze et al., 2001). TCR expression was verified by flow cytometric analysis of growing clones using an antibody to CD3 ϵ (145-2C11). RMA-S/K^d cells incubated overnight at 28 °C were used to present exogenously added synthetic peptides at concentrations as indicated in the figures. Peptide recognition by the TCR transfectants was measured by IL-2 production as previously described (Serreze et al., 2001).

H-2K^d stabilization assay. RMA-S/K^d cells, cultured overnight at 28 °C, were pulsed with peptides in complete DMEM for 1 h at 28 °C, incubated at 37 °C for 3 h, washed, stained with anti-H-2K^d monoclonal antibody SF1-1.1, counter-stained with FITC-conjugated polyclonal goat anti-mouse antibody, and analyzed by flow cytometry. Data were calculated by subtracting mean fluorescence intensity of H-2K^d on non-peptide-pulsed cells from that on peptide-pulsed cells.

Tetramer staining and flow cytometry. Islet-associated and peripheral blood-derived CD8⁺ T cells were isolated from NOD mice as previously described (Amrani et al., 2000; Trudeau et al., 2003). Tetramers were prepared and used as previously described (Amrani et al., 2000).

Results and Discussion

To identify the pancreatic β cell antigen naturally recognized by the diabetogenic 8.3-like CD8⁺ T cell population, we purified H-2K^d class I MHC molecules from the NOD-derived pancreatic β cell line NIT-1 by immunoaffinity chromatography. Peptides were eluted from the H-2K^d molecules and fractionated by reverse phase high-performance liquid chromatography (HPLC). Fractions were tested for their ability to elicit 8.3 cytotoxic T lymphocyte (CTL)-mediated lysis of peptide-pulsed target cells (FIG. 1A). Fractions composing the active peak were pooled and subjected to another round of HPLC under different conditions. A single peak of activity was observed (FIG. 1B). Second-dimension fraction 66 was rechromatographed and a portion of the effluent was analyzed by electrospray ionization (ESI) on a Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) equipped with nanoflow liquid chromatography and an online effluent splitter. For this splitter experiment, a portion of the effluent was deposited in 96-well plates for determination of epitope reconstitution activity, and the remainder was directed to the mass spectrometer. Testing of peptide fractions for recognition by 8.3 CTL yielded a single peak of activity (FIG. 1C). Candidate peptides were identified by comparing the abundances of ions observed in the active and adjoining fractions with the lysis profile from the epitope reconstitution assay (FIG.

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1D). Over 100 peptide candidates were present in the scan window; of these, approximately 35 eluted entirely within the window. Candidates were ranked based on the extent of alignment between their ion abundance curves and the lysis profile. Only the abundance curves for the best candidates and one very abundant later-eluting peptide are shown in FIG. 1D. The millimass accuracy capability of the FT-ICR MS allowed co-eluting peptides differing by <0.1 mass unit to be easily distinguished, and the detection limit of the instrument (2-10 attomoles) made it possible to detect peptide candidates present at only a few copies per cell (Martin et al., 2000).

Based on alignment between abundance curves and the lysis profile, the best peptide candidate, while not the most abundant, was the one having a doubly charged monoisotopic m/z of 548.845 (FIG. 1D). Sequence analysis yielded VYXKTNVFX (FIG. 1E), where X represents either I or L, amino acids of identical mass which cannot be differentiated by the instrument. Similarly, the sequence of the abundant later-eluting peptide (m/z of 555.835⁺²) was determined to be XYQKAFDXX (S. M. Lieberman et al., data not shown). Protein database searches yielded one perfect match each for VYXKTNVFX and XYQKAFDXX (VYLKTNVFL and IYQKAFDLI, respectively). These peptides were synthesized and tested in epitope reconstitution assays. FIG. 1F reveals that VYLKTNVFL is the natural β cell peptide recognized by 8.3 and that activated 8.3 CTL respond to it in an equivalent dose-dependent manner as to the previously described synthetic agonist NRP-A7 and superagonist NRP-V7 (Anderson et al., 1999; Amrani et al., 2001), with half-maximal activity observed at a peptide concentration of ~ 50 pM. Approximately 100 copies of VYLKTNVFL/H-2K^d are present per interferon (IFN)- γ -treated NIT-1 cell.

A BLAST search of the entire NCBI non-redundant protein database resulted in only one exact hit for VYLKTNVFL corresponding to residues 206-214 of murine islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) (Arden et al., 1999). To confirm that the source of the antigenic peptide was indeed IGRP, we transfected COS-7 cells with varying concentrations of an expression construct for IGRP, or vector alone, together with an H-2K^d expression construct and tested for recognition by 8.3 CTL. Cells transfected with IGRP, but not vector alone, stimulated 8.3 CTL to release IFN- γ in a dose-dependent manner upon co-culture (FIG. 1G). This response required the expression of H-2K^d; transfection of the IGRP construct along with an H-2D^b expression construct resulted in a T cell response profile similar to that of vector alone (S. M. Lieberman et al., data not shown). To our knowledge, IGRP has not previously been implicated as either a T cell or B cell antigen in NOD mice or in type 1 diabetes patients. Thus, IGRP is the natural target of the prevalent and pathogenic 8.3-like T cell population in NOD mice and represents a new β cell autoantigen.

We previously isolated a set of six β cell-autoreactive CD8⁺ T cell clones of unknown antigenic specificity from early insulitic lesions of young NOD mice (designated AI4, AI12.B1.1,

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AI12.B1.2, AI12.B1.3, AI15.A10, and AI15.F5) (DiLorenzo et al., 1998). We had earlier reported that AI12.B1.3, which expresses a V α 17-J α 42 TCR α chain nearly identical to that of 8.3, recognizes NRP-A7 (Serreze et al., 2001). Thus, we hypothesized it would also recognize IGRP₂₀₆₋₂₁₄. To test whether this and any other clones in our panel were IGRP₂₀₆₋₂₁₄-reactive, we assayed their ability to recognize IGRP₂₀₆₋₂₁₄. AI12.B1.3 recognized IGRP₂₀₆₋₂₁₄, as well as NRP-V7 and NRP-A7, but not the previously identified antigenic insulin peptide (INS B₁₅₋₂₃) or its I9 variant (INS-I9) (Wong et al., 1999; Wong et al., 2002) (FIG. 2). Hence, the 8.3 clonotype is not unique; other β cell-autoreactive CD8⁺ T cells sharing the prevalent V α 17-J α 42 TCR α chain also recognize IGRP₂₀₆₋₂₁₄. Importantly, AI15.F5, which expresses a V α 17-J α 5 TCR α chain and a similar TCR β chain to 8.3, also responded to IGRP₂₀₆₋₂₁₄ and NRP-V7, although it did not recognize NRP-A7 (FIG. 2). This demonstrates that reactivity to IGRP₂₀₆₋₂₁₄ is not limited strictly to T cells expressing a V α 17-J α 42 TCR α chain. Further, the ability of AI15.F5 to recognize NRP-V7, but not NRP-A7, is consistent with the recent observation that NRP-V7/H-2K^d tetramers stain a larger population of islet T cells than do NRP-A7 tetramers (Trudeau et al., 2003). Thus, previous measurements of NRP-A7 reactivity (Amrani et al., 2000) have underestimated the prevalence of the IGRP₂₀₆₋₂₁₄-reactive T cell population.

The only other known natural ligand for an NOD-derived diabetogenic CD8⁺ T cell clone is INS B₁₅₋₂₃ presented by H-2K^d (Wong et al., 1999). The insulin peptide exhibits very poor binding to H-2K^d, and it has been suggested that this results in insufficient peptide presentation for T cell negative selection in the thymus (Wong et al., 2002). To determine whether poor MHC binding is a characteristic of autoantigenic peptides in general, we tested the ability of IGRP₂₀₆₋₂₁₄ to bind H-2K^d. In H-2K^d stabilization assays, IGRP₂₀₆₋₂₁₄ demonstrated good MHC binding, comparable to that of the synthetic ligands NRP-V7 and NRP-A7 and considerably better than that of INS B₁₅₋₂₃, which was barely detectable even at the highest peptide concentration tested (FIG. 3). Like NRP-V7 and NRP-A7, IGRP₂₀₆₋₂₁₄ contains the expected H-2K^d anchor residues, i.e., Y at position 2 and L at 9, while INS B₁₅₋₂₃ has G at position 9, which makes its binding to H-2K^d unfavorable. When G at position 9 is replaced with I, the resulting INS-I9 peptide shows improved binding to H-2K^d (FIG. 3) (Wong et al., 2002). Furthermore, when the protein sequence of murine IGRP is analyzed by several different algorithms designed to identify good MHC-binding peptides (SYFPEITHI (Rammensee et al., 1999), BIMAS (Parker et al., 1994), or RANKPEP (Reche et al., 2002)), IGRP₂₀₆₋₂₁₄ consistently ranks among the best H-2K^d binders (third, first, or second, respectively). Taken together, these observations indicate that poor MHC class I binding is not a requirement for self-peptides recognized by autoreactive CD8⁺ T cells.

A recent study demonstrated that NRP-V7/H-2K^d tetramers could be used to detect and to quantify islet- and peripheral blood-derived NRP-V7-reactive CTL directly *ex vivo* (Trudeau et al., 2003). However, the use of natural self-peptides in tetramer studies to identify autoreactive T cells

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directly *ex vivo* has only been reported for melanocyte-antigen-specific T cells (Pettet et al., 2002). To evaluate whether the natural IGRP peptide, which differs from the 8.3 superagonist NRP-V7 (KYNKANVFL) by only three residues, could similarly be used to quantify the prevalent β cell-autoreactive T cell population, we isolated T cells from both islets and peripheral blood of 9- and 20-week-old nondiabetic NOD mice and stained them with IGRP₂₀₆₋₂₁₄, NRP-V7, or negative control peptide (TUM) tetramers. A sizeable proportion of islet T cells from 9- or 20-week-old mice was IGRP-reactive, and a population of IGRP-reactive cells was clearly measurable in peripheral blood (FIG. 4). Importantly, for both islet and peripheral blood samples of 9- or 20-week-old mice, the size of the CD8⁺ T cell population that stained with tetramer was similar whether IGRP₂₀₆₋₂₁₄ or NRP-V7 tetramers were used for detection. Thus, the natural peptide can be used to detect and to quantify IGRP₂₀₆₋₂₁₄-reactive islet and peripheral blood T cells directly *ex vivo*.

The role of CD8⁺ T cells in autoimmune diseases such as type 1 diabetes is becoming more widely recognized (Liblau et al., 2002); however, knowledge of the natural ligands of these pathogenic T cells in spontaneous autoimmune diseases is extremely limited. Here we have identified IGRP as the source of the natural peptide recognized by a prevalent population of pathogenic CD8⁺ T cells in NOD mice. IGRP is an islet-specific protein expressed in pancreatic β cells, and to a lesser extent in α cells, and shares approximately 50% identity with the catalytic subunit of the liver enzyme glucose-6-phosphatase (G6Pase) (Martin et al., 2001). Importantly, IGRP₂₀₆₋₂₁₄ differs from the homologous residues of murine G6Pase (KYCLITIFL) at six of nine positions. Accordingly, this liver G6Pase peptide is not recognized by 8.3 CTL (S. M. Lieberman et al., data not shown). Despite its homology to G6Pase, no catalytic activity has been demonstrated for IGRP, and its function is unknown (Ardin et al., 1999; Martin et al., 2001). From its sequence, it is predicted to be an ER-resident protein that spans the membrane nine times (Arden et al., 1999). The abundance of its RNA places IGRP among β cell genes expressed at moderate to high levels (Id.), and the protein can be readily detected in islets by immunohistochemistry (Martin et al., 2001). Intriguingly, the human IGRP gene, which maps to chromosome 2q28-32 (26), overlaps a diabetes susceptibility locus, IDDM7 (2q31) (Pociot & McDermott, 2002), for which the responsible gene is unknown.

The IGRP-reactive T cell population constitutes a substantial component of even the earliest NOD islet infiltrates (FIG. 2) (DiLorenzo et al., 1998), and its pathogenicity has been clearly established (Amrani et al., 2000; Trudeau et al., 2003; Anderson et al., 1999; Nagata et al., 1994; Verdaguer et al., 1997). Thus, the response to IGRP appears to be one of the first events leading to β cell destruction by CD8⁺ T cells in autoimmune diabetes. Now, with the identification of IGRP as the natural β cell antigen targeted by 8.3-like T cells, critical aspects of their development, activation, and expansion can be further investigated. Already this knowledge has made possible the elucidation of the mechanisms responsible for the avidity maturation of this pathogenic T cell population in NOD

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mice (Han et al, in preparation). In addition, it will now be possible to confirm that a similarly prevalent IGRP-reactive T cell population participates in the pathogenesis of human type 1 diabetes. As several β cell autoantigens in NOD mice overlap those that have been identified in patients (Tisch & McDevitt, 1996), and the human IGRP gene maps to a diabetes susceptibility locus, it is likely that IGRP will demonstrate considerable importance in the development of the human disease.

Example 2. Protection of NOD Mice from Type 1 Diabetes Using IGRP₂₀₆₋₂₁₄

Ten NOD female mice were treated with 100 micrograms of IGRP₂₀₆₋₂₁₄ peptide (in phosphate-buffered saline) intraperitoneally, starting at 3.5 weeks of age. Forty mice were used as controls. Each control mouse was treated with the TUM peptide (KYQAVTTTL). The mice received one injection of peptide every two weeks for the first three injections, and then one injection every three weeks thereafter. At ~25 weeks-old, two (20%) of the IGRP₂₀₆₋₂₁₄ mice developed diabetes, vs. 26 control mice (~65%). The two mice that have developed diabetes did not have IGRP₂₀₆₋₂₁₄-reactive T-cells in islets, as determined by T-cell staining with NRP-V7/K^d tetramers. This was also confirmed by determining whether islet-derived T-cells from these mice produced interferon- γ upon stimulation with IGRP₂₀₆₋₂₁₄ and NRP-V7 *in vitro*. There were no significant differences in interferon- γ production vs. cells stimulated with the negative control peptide TUM. In one of these two mice, the islets contained T-cells that responded to two IGRP peptides other than IGRP₂₀₆₋₂₁₄. These results indicate that tolerization to IGRP₂₀₆₋₂₁₄ protects against the development of type 1 diabetes, and that other IGRP peptides can contribute to the development of type 1 diabetes.

Further supporting the assertion that other IGRP peptides can contribute to the development of type 1 diabetes, the inventors have also discovered that the peptide CALTSLTTM, present in mouse IGRP at amino acid residues 296-304, is presented by the MHC class I molecule H-2Db.

Example 3. Prevention of Diabetes by Manipulation of anti-IGRP Autoimmunity: High Efficiency of a Low Affinity Altered Peptide Ligand

Example Summary

Antigen therapy holds great promise for the prevention of organ-specific autoimmunity. However, limited clinical trials in humans have almost invariably met with failure, possibly because the principles guiding the choice of treatment remain poorly defined. Here, we have examined the antidiabetogenic properties of wild type or altered peptide ligands of CD8⁺ T-cells recognizing an epitope from islet-specific glucose 6 phosphatase catalytic subunit-related protein (IGRP₂₀₆₋₂₁₄), a prevalent population of autoreactive T-cells in murine autoimmune diabetes. We show that pancreatic islet-associated CD8⁺ T-cells in nonobese diabetic mice recognize several additional epitopes of IGRP, and that these cells play a role in the outcome of peptide-treatment protocols designed to

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induce IGRP₂₀₆₋₂₁₄-specific tolerance. Altered peptide ligands targeting IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells could effectively curb the progression of diabetes, but only at doses that spared low avidity IGRP₂₀₆₋₂₁₄-specific clonotypes. In contrast, near complete depletion of the IGRP₂₀₆₋₂₁₄-reactive T-cell pool by relatively high doses of these ligands resulted in enhanced recruitment of sub-dominant IGRP epitope-reactive T-cell specificities and did not significantly inhibit diabetes progression. These results suggest that peptide therapy in organ-specific autoimmunity is most effective under conditions that foster the occupation of the target organ lymphocyte niche by non-pathogenic, low avidity clonotypes.

Introduction

Administration of autoantigenic proteins or peptides in solution can blunt the initiation and/or progression of autoimmunity in experimental models of autoimmune disease (Wraith et al, 1989; Metzler and Wraith, 1993; Liu and Wraith, 1995; Anderton and Wraith, 1998; Karin et al., 1994). However, limited clinical trials in humans employing similar strategies have almost invariably met with failure (Weiner, 1993; Trentham, et al., 1993; McKown et al., 1999; Pozzalli et al., 2000; Group, D.P.T.-T.D.S., 2002; Kappos et al., 2000; Bielekova et al., 2000). This suggests that the principles guiding the choice and conditions of treatment are poorly defined and, as a result, inadequate for human application.

Unlike their experimental counterparts, spontaneous organ-specific autoimmune disorders result from complex responses against numerous epitopes in multiple antigens that arise spontaneously in a stochastic and often unpredictable sequence. This complexity is compounded by the fact that lymphocyte clones recognizing identical epitopes engage antigen/MHC within a broad range of avidities, the strength of which correlates with pathogenic potential (Amrani et al., 2000; Santamaria, 2001; Liblau et al., 2002). Consequently, the outcome of any immunization strategy for the prevention of autoimmunity is likely to be influenced by the choice of autoantigen(s), dose, periodicity of treatment, and route and form of administration. Unfortunately, our current understanding of the independent contribution of these variables to treatment outcome is extremely limited.

Type 1 diabetes (T1D) in both humans and nonobese diabetic (NOD) mice is an autoimmune disease that results from selective destruction of pancreatic beta cells by T-lymphocytes. Human and murine T1D involve complex B- and CD4+ T-cell responses against a growing list of antigens (Lieberman and DiLorenzo, 2003). Although initiation of T1D clearly requires the recruitment of autoreactive CD4+ T-cells, there is compelling evidence that initiation and progression of T1D is CD8+ T-cell-dependent (Santamaria, 2001; Liblau et al., 2002). Others and we have shown that a large fraction of all islet-associated CD8+ cells in NOD mice use highly homologous TCR α chains (V α 17-J α 42) (Santamaria et al., 1995; Verdaguer et al., 1996; Verdaguer et al., 1997; DiLorenzo et

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al., 1998) and recognize the same mimotope (NRP-A7) (Anderson et al., 1999). These T-cells are already a significant component of the earliest NOD islet CD8+ infiltrates (DiLorenzo et al., 1998; Anderson et al., 1999; Amrani et al., 2001), are diabetogenic (Verdaguer et al., 1996; Verdaguer et al., 1997), target a peptide from islet-specific glucose 6 phosphatase catalytic subunit-related protein (IGRP₂₀₆₋₂₁₄) (Lieberman et al., 2003), and are unusually frequent in the periphery (~1/200 circulating CD8+ cells) (Lieberman et al., 2003; Trudeau et al., 2003). Notably, progression of insulinitis to diabetes in NOD mice is invariably accompanied by cyclic expansion of the circulating IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cell pool (Trudeau et al., 2003), and by avidity maturation of its islet-associated counterpart (Amrani et al., 2000). When considered together, these data strongly support the idea that IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells play a key role in the initiation and/or progression of murine T1D. Intriguingly, the human *igrp* gene, which maps to chromosome 2q28-32 (Martin et al., 2001), overlaps a T1D susceptibility locus, IDDM7 (2q31) (Pociot and McDermott, 2002), raising the possibility that IGRP may also be target of the human diabetogenic response.

Administration of soluble peptides (without adjuvant) is an effective way of inducing antigen-specific T-cell tolerance (Aichele et al., 1994; Toes et al., 1996). Previously, we showed that repeated treatment of pre-diabetic NOD mice with soluble NRP-A7 peptide blunted avidity maturation of the IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cell subset by selectively deleting clonotypes expressing TCRs with the highest affinity for peptide/MHC (Amrani et al., 2000). These observations suggested that avidity maturation of pathogenic T-cell populations is a key event in the progression of benign inflammation to overt disease in autoimmunity. However, they also raised the possibility that NRP-A7's anti-diabetogenic activity was mediated by occupation of the "high avidity clonotype niche" (emptied by NRP-A7 treatment) by "low avidity" (and potentially antidiabetogenic) clonotypes. To test this hypothesis, we here identified APLs with partial, full or super agonistic activity for IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells and compared their anti-diabetogenic activity *in vivo* over a wide dose-range.

Our data show that wild-type NOD mice spontaneously mount highly prevalent CD8+ T-cell responses against numerous IGRP epitopes, and that peptide therapy aimed at selectively targeting the IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cell subset is effective if it does not delete non-diabetogenic, low avidity clonotypes. Complete depletion of the prevalent IGRP₂₀₆₋₂₁₄-reactive T-cell niche with high doses of IGRP₂₀₆₋₂₁₄ or high affinity APLs was associated with enhanced recruitment of sub-dominant epitope-specific IGRP-reactive T-cell specificities and did not blunt diabetes progression. These data raise important considerations for the design of antigen-specific immunotherapies in autoimmunity.

Materials and Methods

Mice, cell lines and antibodies. 8.3-NOD mice, expressing the TCR $\alpha\beta$ -rearrangements of the NRPA7/IGRP₂₀₆₋₂₁₄-reactive, H-2K^d-restricted beta cell-reactive CD8+ T-cell clone NY8.3 have been described (Verdaguer et al., 1997). NOD mice were purchased from Taconic Farms (Germantown,

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NY). Anti-Lyt-2 (CD8 α) (53-6.7), anti-L3T4 (IM7), anti-V β 8.1/8.2 (MR5-2), anti-H-2K^d (SF1-1.1), and anti-H-2D^b (KH95) mAbs were from PharMingen (San Diego, CA).

Peptides and peptide libraries. The peptide libraries used to identify APLs for this study were prepared using multipin synthesis technology and standard Fmoc chemistry (Chiron Technologies, San Diego, CA) (Amrani et al., 2001). The first screen was done using NRP-based dipeptide libraries. Libraries capable of eliciting responses were deconvoluted by probing single amino acid variants of NRP. Representative APLs displaying partial, full or super-agonistic activities (FIG. 1) were chosen for *in vivo* experimentation. Specific single custom peptides were purified through rpHPLC to >80% purity and sequenced by ion spray mass spectrometry (Chiron Technologies, San Diego, CA).

Peptides were resuspended at 10 mg/ml in 0.1M HEPES (Sigma, St. Louis, MO) in 40% acetonitrile (Fisher Scientific, Fair Lawn, NJ) at pH 7.4, aliquoted at -80°C, and further resuspended in PBS prior to use. This resuspension strategy differs from that used in (Amrani et al., 2000), in that it obviates concentration of diluted stocks of peptide by vacuum centrifugation immediately prior to resuspension in PBS and injection into mice. The latter strategy results in loss of material owing to difficulties in re-solubilization of dried peptide. H-2K^d or H-2D^b-binding IGRP peptide libraries were designed by screening the IGRP amino acid sequence with Rankpep (<http://mif.dfci.harvard.edu/cgi-bin/rankpep.cgi>) and SYFPEITHI (<http://syfpeithi.bmiheidelberg.com>). Pepsets comprised of predicted MHC-binders with scores >39 (rankpep) or >25 (syfpeithi) were synthesized, resuspended as described above, and used for *in vitro* assays at 10 μ M.

Generation of NOD islet-derived CD8+ T-cell lines. Islet-derived CD8+ T-cells from peptide-treated or unmanipulated NOD mice were generated by culturing pancreatic islets in complete media (CM: RPMI 1640 media containing 10% fetal bovine serum), containing 0.5 U/ml Takeda rhIL-2 (10-50 islets/well in 24-well plates). The *in vivo*-activated, IL-2R+ lymphocytes migrating from islets into the culture media were used in functional assays within 6-9 days of islet isolation (Amrani et al., 2000; Anderson et al., 1999).

Proliferation Assays. Naïve or NRP-A7-differentiated (see below) splenic CD8+ T-cells from 8.3-NOD mice (2×10^4 /well) were incubated, in duplicate, with peptide-pulsed (0.01, 0.1 and 1 μ M), γ -irradiated (3,000 rad) NOD splenocytes (10^5 /well) for 3 days at 37°C in 5% CO₂. Cultures were pulsed with 1 μ Ci of (³H)-thymidine during the last 18 hours of culture and harvested.

Cytokine secretion. Naïve splenic CD8+ T-cells from 8.3-NOD mice (2×10^4 /well) were incubated with peptide-pulsed (0.001, 0.01, 0.1, 1 and/or 10 μ M) γ -irradiated NOD splenocytes (10^5 /well) in 96-well plates for 48 hours at 37°C. Short-term islet-derived T-cell lines from peptide-treated or unmanipulated NOD mice (adjusted at 2×10^4 CD8+ T-cells/well) were tested the same way, but using 10 μ M of peptide. The supernatants (100 μ l/well) were assayed in duplicate for IL-2, IL-4, and/or IFN- γ content by ELISA using commercially available kits (Genzyme Diagnostics,

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Cambridge, MA).

Tetramer staining. Tetramers were prepared and used as described in (Amrani et al., 2000). Hand-picked pancreatic islets from individual mice were cultured in complete media supplemented with 0.5 U/ml of Takeda rhIL-2 for 7-9 days. T-cells (10^6 per 20 μ l) were then stained for 45 minutes on ice in 20 μ l of wash media (0.2% sodium bicarbonate, 0.1% sodium azide and 2% FBS in RPMI-1640) containing anti-CD8-FITC (clone YTS169.4; 0.5 μ g) and tetramer (85.5 nM). After washing, the cells were resuspended in wash media and analyzed with a flow cytometer (Becton Dickinson). For the analysis of tetramer staining at equilibrium, T-cells were stained for 2 hours at room temperature with 20 μ l of wash media (0.2% sodium bicarbonate, 0.1% sodium azide and 2% FBS in RPMI-1640) containing anti-CD8 α -FITC (clone YTS169.4; 0.5 μ g) and different concentrations of tetramers (8.55, 17.1, 42.75 and 85.5 nM). After washing, cells were resuspended in 100 μ l of wash media, fixed in 0.4% paraformaldehyde and analyzed with a flow cytometer. The apparent K_d values were determined by plotting the negative reciprocal of the slope of the line fit to Scatchard plots of fluorescence units (median of CD8+ population tetramer staining)/nM versus fluorescence units.

H-2K^d-stabilization assay. RMA-SK^d cells that had been cultured overnight at 26°C were seeded, in quadruplicate, at 10^4 cells/well in 96-well plates, pulsed with peptides in RPMI-1640, 0.25% BSA for 1 hour at 26°C, incubated at 37°C for 3 hours, washed, stained with anti-H-2K^d-FITC or anti-H-2D^b-FITC, and the mean fluorescence intensity (mfi) for MHC class I expression analyzed by flow cytometry (Anderson et al., 1999). Controls used included TUM (H-2K^d-binder), LCMV-GP33 (H-2D^b-binder) and no peptide. The dissociation constant (K_d) was measured by repeating the experiments described above except using different concentrations of peptides (10, 1, 0.1, 0.01, 0.001 μ M). The K_d values were calculated as the concentration of peptide required to rescue 50% of the H-2K^d molecules on RMA-SK^d cells (100 % at 10 μ M).

Peptide treatment. Cohorts of 3-to-4 week-old female NOD mice were injected with 1-100 μ g of peptide in PBS intraperitoneally. This was repeated every 2 weeks until the 7-8th week, and every 3 weeks thereafter. Mice were monitored for development of hyperglycemia until at least the 28th week of age. The 8.3-CD8+ T-cell tolerogenic activity of APLs was determined by treating 8-15 wk-old 8.3-NOD mice with one i.p. injection of 100 μ g of peptide. Peptide-treatment experiments employing older mice (28-30 wk-old) and NRP-A7 yielded results statistically similar to those seen in younger mice, indicating that reductions in transgenic T-cell numbers resulting from peptide treatment were independent of the age of mice. Mice were sacrificed a week after treatment, and their spleens analyzed for presence of 8.3-CD8+ T-cells by flow cytometry.

Statistical analyses. Data were compared using linear regression and variance analysis, Mann-Whitney U test or χ^2 .

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Results

APLs for IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells. We first searched for NRP APLs capable of engaging the 8.3-TCR with different affinity, as compared to NRP-A7. This work, which was initiated well before the discovery of IGRP206-214, involved generating numerous single amino acid mutants of NRP and testing their ability to elicit 8.3-CD8+ T-cell responses (Amrani et al., 2001). NRP-I4 was chosen as a potential “low avidity” APL candidate, since it behaved as a partial agonist; it was quite efficient at triggering IFN- γ secretion by naïve 8.3-CD8+ T-cells, but significantly less efficient than NRP-A7 at inducing other responses (FIG. 5). NRP-V7 was chosen as a potential “very high avidity” APL because it had superior agonistic activity on 8.3-CD8+ T-cells than NRP-A7 (agonist) (FIG. 5). To confirm that these peptides were in fact recognized with different avidity by 8.3-CD8+ cells (they all bound to K^d with similar avidity, as determined by the RMA-SK^d stabilization assay; data not shown), we tested the ability of the corresponding peptide/MHC tetramers to stain naïve 8.3-CD8+ cells. As shown in FIG. 6A, the tetramer staining intensities were consistent with the functional avidities of the different peptides (FIG. 5). The functional avidity of these peptides also correlated well with their tolerogenic activity *in vivo*. Treatment of 8.3-NOD mice, expressing a TCR that recognizes NRP-V7 with intermediate-to-high avidity (our unpublished data), with a single injection of NRP-V7 (100 μ g in PBS) contained fewer 8.3-CD8+ splenocytes than 8.3-NOD mice treated with NRP-A7 or NRP-I4 (FIG. 6B).

Experiments employing CD8+ cells derived from pancreatic islets of wild-type NOD mice indicated that differential binding of each of these peptide/K^d complexes by IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells was not a peculiarity of cells expressing the 8.3-TCR. Whereas the NRP-I4 tetramer could not stain T-cells derived from islets of 20 wk-old wild-type NOD mice above the threshold for detection by flow cytometry (data not shown), the NRP-V7 tetramer generally did so with higher fluorescence intensity than the NRP-A7 tetramer (FIG. 6C). Quantitative measurements of avidity confirmed these results. NRP-V7 tetramers bound CD8+ cells derived from pancreatic islets of 9 wk-old NOD mice with significantly lower K^d (higher avidity) than NRP-A7 (FIG. 6D). As a whole, these data demonstrate that IGRP₂₀₆₋₂₁₄-reactive CD8+ cells recognize NRP-I4, NRP-A7 and NRP-V7 with increasing affinity, both *in vitro* and *in vivo*.

IGRP₂₀₆₋₂₁₄ APLs can curb the progression of T1D in NOD mice but only within a narrow range of dose and affinity. We next investigated whether the anti-diabetogenic activity of the above APLs was a function of “avidity”, as defined not only by the affinity of individual peptide/MHC complexes for specific TCRs, but also by the absolute number of peptide/MHC complexes that are available for recognition (i.e. dose of peptide). This was done by treating cohorts of female NOD mice with repeated injections of TUM (negative control), NRP-I4, NRP-A7 and NRP-V7 (in PBS) over a wide dose range, and by following the mice for development of diabetes (FIG. 7A, top).

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Starting at 3-4 weeks of age, mice received one injection of peptide i.p. every 2 (first three doses) or 3 weeks after initiation of treatment. Mice were sacrificed at diabetes onset or at the end of the follow-up period (28-32 weeks), to investigate the effects of treatment on the size and tetramer-binding avidity of the islet-associated IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T-cell subpopulation (FIG. 7A, bottom).

5 NRP-I4 was anti-diabetogenic in a dose-dependent manner: it was not protective at all when given at <20 µg/injection, but was highly anti-diabetogenic when given at 100 µg/injection (FIG. 7A, top). Strikingly, the protective effect of NRP-I4 (at 100 µg/injection) was associated with recruitment of low avidity IGRP₂₀₆₋₂₁₄-reactive CD8⁺ cells into islets, rather than with massive deletion of the IGRP₂₀₆₋₂₁₄-reactive CD8⁺ cell subset. Thus, the percentages (FIG. 7A, bottom, right y axis) and
10 absolute numbers (FIG. 7B) of NRP-V7-reactive CD8⁺ cells contained in the islets of these mice were similar to those seen in TUM-treated controls. Furthermore, the islet-associated CD8⁺ cells of NRP-I4-treated mice bound NRP-V7 tetramers with significantly lower avidity than those derived from islets of TUM-treated mice (left y axis) (the values shown in FIG. 7A reflect differences between these two groups of mice). The most logical interpretation of these results is that the
15 protective effect of high-dose NRP-I4 treatment resulted from selective deletion of high-avidity IGRP₂₀₆₋₂₁₄-reactive CD8⁺ cells. In agreement with this view, NRP-A7, a higher affinity ligand of IGRP₂₀₆₋₂₁₄-reactive CD8⁺ cells, protected mice from diabetes at a lower dose than NRP-I4 (50 µg/injection), and this, too, coincided with accumulation of low avidity IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T-cells within islets (FIG. 7A, bottom). The absolute number of NRP-V7-reactive T-cells in the islets of
20 these mice were reduced, but not significantly, as compared to TUM-treated mice ($0.6 \times 10^5 \pm 0.3$ vs. $1 \times 10^5 \pm 0.3$, respectively).

However, this interpretation that protection by high doses of NRP-I4 and intermediate doses of NRP-A7 was due solely to deletion of high avidity clonotypes was at odds with two unexpected observations. First, high doses of NRP-A7 (100 µg/injection) were ineffective. It should be noted
25 that here we prepared the inoculum by diluting concentrated stocks of peptide with vehicle, as opposed to by dissolving material dried from diluted stocks (Amrani et al., 2000). As the latter strategy results in peptide loss (our observations), it should not be a surprise that in a previous study NRP-A7 was protective when given at the “100 µg” dose (Amrani et al., 2000). The second unexpected observation was that NRP-V7 (an even higher affinity ligand of IGRP₂₀₆₋₂₁₄-reactive
30 CD8⁺ T-cells than NRP-A7) was not significantly protective at any dose (FIG. 7A, top). This was puzzling because the islets of NRP-A7- (100 µg/dose) and NRP-V7-treated mice (over a range of doses) either contained significantly reduced numbers of NRP-V7-reactive CD8⁺ cells, or were almost completely devoid of these T-cells, respectively (FIGS. 7A, top, and 7B). Thus, the anti-diabetogenic activity of NRP-I4 (at 100 µg) and NRP-A7 (at 50 µg) could not be attributed to deletion
35 of (high avidity) IGRP₂₀₆₋₂₁₄-reactive CD8⁺ cells alone. Rather, the data suggested that protection

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required the recruitment of low avidity IGRP₂₀₆₋₂₁₄-reactive CD8⁺ cells to islets. Presumably, these low avidity cells would promote a state of chronic, yet non-pathogenic inflammation.

Wild-type NOD mice spontaneously mount CD8⁺ T-cell responses against numerous IGRP epitopes. Treatment of NOD mice with high doses of IGRP₂₀₆₋₂₁₄ (75-100 µg/injection) yielded results very similar to those obtained in NRP-V7-treated mice: this peptide delayed the age at onset of diabetes, but did not significantly reduce its incidence (FIG. 8A). Notably, the ineffectiveness of IGRP₂₀₆₋₂₁₄ treatment was also associated with near complete deletion of the intra-islet IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T-cell subpopulation, as determined by both flow cytometry (FIG. 8B and C, left panels) and functional assays employing islet-derived T-cells (FIGS. 8B and C, right panels).

These results prompted us to consider the possibility that near complete deletion of the IGRP₂₀₆₋₂₁₄-reactive CD8⁺ subset (by NRP-V7 or IGRP₂₀₆₋₂₁₄ treatment) might have somehow fostered the creation of a “niche” for subdominant autoreactive T-cell specificities in islets. Conceivably, recruitment and/or accumulation of these clonotypes could have counteracted any protection afforded by deletion of the dominant IGRP₂₀₆₋₂₁₄-reactive pool. Since staining of islet-derived T-cells of TUM- versus NRP-V7- or IGRP₂₀₆₋₂₁₄-treated mice with high avidity insulin 15-23/K^d (InsL) tetramers (the only other known target of beta cell autoreactive CD8⁺ cells (Wong et al., 1999)), did not reveal significant differences (data not shown), we wondered whether the anti-IGRP-CD8⁺ cell response in diabetes involved multiple epitopes. To investigate this, we designed an IGRP-based peptide library comprised of 76 MHC class I-binding nonamers (33 K^d- and 43 D^b-binding) (Table I, ordered based on their location in IGRP; from amino to carboxy termini). Only one of these peptides (IGRP₂₀₇₋₂₁₅; peptide “6”) was cross-reactive with IGRP₂₀₆₋₂₁₄ (peptide “IG”) as determined by its ability to elicit 8.3-CD8⁺ T-cell responses (data not shown). We then tested the ability of each of these IGRP peptides, as well as NRP-V7 and TUM (at 10 µM) to elicit IFN-γ secretion by CD8⁺ cells propagated from islets of pre-diabetic (21 ± 1 weeks) or acutely diabetic NOD mice (18 + 1 weeks) (Table II and FIG. 9A). Although most mice, regardless of diabetes status, contained IGRP₂₀₆₋₂₁₄ (and IGRP₂₀₇₋₂₁₅)-reactive CD8⁺ T-cells in islets (peptides “IG” and “6”, respectively) Table II and FIG. 9A, left panel), cells derived from the diabetic mice mounted more vigorous responses against these two epitopes than cells derived from non-diabetic animals (FIG. 9A, right panel, which excludes non-responder mice (<50 pg/ml)). Experiments employing IGRP₂₀₆₋₂₁₄ tetramers confirmed the existence of a correlation between the magnitude of IFN-γ secretion by islet-associated CD8⁺ T-cells and the percentage of tetramer-positive cells (p<0.001), suggesting that the above differences in IFN-γ secretion reflected differences in cell numbers (data not shown).

Interestingly, a significant number of mice also mounted responses against other IGRP epitopes, particularly peptides 72, 7, 8 and 39. Limited studies with tetramers confirmed the presence of peptide 72 and peptide 39-reactive CD8⁺ T-cells in at least some animals (FIG. 9B). Comparison of

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the magnitude of the total anti-IGRP responses further indicated that whereas the response against IGRP₂₀₆₋₂₁₄ was significantly higher in diabetic animals than in non-diabetic ones, the combined response against other IGRP epitopes (excluding IGRP₂₀₆₋₂₁₄) was not (FIG. 9C). This is consistent with the hypothesis that progression to overt diabetes involves the recruitment of IGRP₂₀₆₋₂₁₄-reactive clonotypes, as we have previously proposed (Amrani et al., 2000; Lieberman et al., 2003).

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TABLE I. IGRP-Specific Peptide Library

	#	SEQUENCE	RESIDUES	#	SEQUENCE	RESIDUES
	34	DFLHRSGVL	2-10	61	LFALGFYLL	216-224
5	18	FLHRSGVLI	3-11	68	FALGFYLLL	217-225
	65	SGVLIHHL	7-15	55	LGFYLLRL	219-227
	73	DYRTYYGFL	18-26	7	LRLFGIDLL	225-231
	72	TYYGFLNFM	21-29	50	FGIDLLWSV	228-236
	75	GFLNFMSNV	24-32	47	WSVFWLIQI	234-242
10	32	SNVGDPRI	30-38	8	KWCANPDWI	241-249
	4	GDPRNIFSI	33-41	30	CANPDWIHI	243-251
	35	IYFPLWFQL	41-49	74	PFAGLVRNL	255-263
	37	LWFQLNQNV	45-53	1	GLVRNLGVL	258-266
	14	QLNQNVGTK	48-56	58	RNLGVLFGL	261-269
15	42	LNQNVGTKM	49-57	69	VLFGLGFAI	265-273
	17	NQNVGTKMI	50-58	56	LGFAINSEM	269-277
	59	WFNLIFKWI	66-74	5	GFAINSEMF	270-278
	52	FGHRPYWWI	76-84	3	FAINSEMFL	271-279
	71	IYPNHSSPC	89-97	23	CQGENGTKP	282-290
20	9	YPNHSSPCL	90-98	12	GTKPSFRL	287-295
	31	GHAMGSSCV	114-122	44	SFRLLCALT	291-299
	45	VWYVMVTAA	122-130	41	RLLCALTSL	293-301
	76	WYVMVTAAL	123-131	2	CALTSLTTM	296-304
	11	ALSYTISRM	130-138	26	LTSLTMMQL	298-306
25	28	YTISRMEES	133-141	16	TSLTMMQLY	299-307
	25	ISRMEESSV	135-143	19	MQLYRFIKI	304-312
	29	SRMEESSVT	136-144	36	RFIKIPHTA	308-316
	15	RMEESSVTL	137-145	13	KIPHTAEPL	311-319
	20	ESSVTLHRL	140-148	46	THAEPLFYLL	314-322
30	57	SFLWSVFWL	151-159	70	HAEPLFYLL	315-323
	66	FLWSVFWLI	152-160	22	LSFCKSASI	323-331
	67	VFWLIQISV	156-164	39	SFCKSASIP	324-332
	48	SRVFIATHF	167-175	10	CKSASIPLM	326-332
	24	ATHFPHQVI	172-180	43	SIPLMVVAL	330-338
35	27	THFPHQVIL	173-181	63	IPLMVVALI	331-339
	54	FEHTPGVHM	193-201	51	ALIPYCVHM	337-345
	21	TPGVHMASL	196-204			
	38	HMASLSVYL	200-208			
	60	LSVYLKTNV	204-212			
40	6	YLKTNVFLF	207-215			

Table II, shown on the following two pages, illustrates peptides ordered from the amino to the carboxy terminus of IGRP. Only peptides that elicited a response in at least one mouse are shown.

Symbols: M# = mouse number; IU = diabetic, non-peptide-treated mice; N = non-diabetic, non-peptide-treated mice; IT = diabetic, TUM peptide-treated mice; T = non-diabetic, TUM peptide-treated mice; IIG = diabetic IGRP₂₀₆₋₂₁₄ peptide-treated mice; IG = non-diabetic, IGRP₂₀₆₋₂₁₄ peptide-treated mice; nd = not determined. Significant responses are shaded.

TABLE II. Amounts of $\text{H}^+ \text{J}$ (pg/ml) secreted by B16-galactam DB-1 cells																											
M#	Age wk	34 (Kd)	38 (Db)	73 (Kd)	72 (Kd)	4 (Db)	35 (Kd)	14 (Db)	17 (Db)	59 (Kd)	71 (Kd)	9 (Db)	31 (Db)	11 (Db)	28 (Db)	29 (Db)	15 (Db)	20 (Db)	57 (Kd)	67 (Kd)	48 (Db)	24 (Db)	27 (Db)	54 (Db)	38 (Kd)	60 (Kd)	
I1	12																										
I2	13				105					95						63		604			134	66	110				82
I3	20				252	nd																					
I4	17				161																						
I5	23	69	68			94	61	63	78			52		122	54	76	61										
I6	21				267																100		125	72			
I7	19												282									468					
I8	17																										
N1	17	201	168		100			141			90						144										
N2	21				253						337			52													
N3	25																										
N4	25																										
N5	21				477								50														
N6	21				996																						
N7	25				255																						
N8	25				1418			55	72		377																
N9	19										54																
N10	19																										
N11	19				150																						
N12	19				193																						
N13	21																										
N14	21				243																						
N15	21				1491																						
N16	20																										
N17	20				84																						
N18	19				229						1947																
N19	20																										
N20	20																										
N21	19				96	65																					
N22	16	nd	nd		181	nd	nd	nd	nd	nd	227	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
N23	24	nd	nd		725	nd	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
N24	18	nd	nd			nd	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
N25	23	nd	nd		358	nd	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
I1	16				223		62			57									202		243			50		360	
I2	16				210														190		231					288	
I3	15				498																						
I4	20				498																						
T1	21		70	77	1920	96	61		124		71	77	51		85			70									
T2	20																										
T3	21																										
I1	20	713	650	359	481	431	124	621	615		234	556	279	364	629	225	662	291	114								
I5	26				246								494				165	80									
I6	27																										
IG1	>27	150	97		67		73		80					83	157		207	73									
IG3	>27																										
IG4	>27	75	83			106			83			64		57	66	60											
IG8	>27																										
IG9	>27								80																		

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Table II - continued

M#	Age wk	IG (Kd)	6 (Db)	55 (Db)	7 (Db)	47 (Db)	8 (Db)	30 (Db)	1 (Db)	58 (Kd)	56 (Db)	5 (Db)	5 (Db)	23 (Db)	12 (Db)	2 (Db)	26 (Db)	16 (Db)	19 (Db)	36 (Kd)	13 (Db)	46 (Db)	70 (Kd)	22 (Db)	39 (Kd)	16 (Db)	51 (Db)
IU1	12	395	384																								
IU2	13	481	513					162	584													308					
IU3	20	273	9239					52	nd			nd				nd											
IU4	17	3597	1811			56								592	1450								70				
IU5	23	4997	332	59	1393		125	67	74			138	96	74	121						51		71	506	61	-	
IU6	21	5313	2102		1069		67							149	105								76				
IU7	19	2433	1180		1304		194	211						60	71		910					943		341			
IU8	17	252	86		1304																						
NI1	17	413	1705		278												154			111						90	
N2	21	222	343		2230		163														51						
N3	25	1051	909		58																						
N4	25	344	140																								
N5	21	375	157		360		414				59	80															
N6	21	255	516		457																						
N7	25	354	930		254		86					79						73					52		156		
N8	25	293	96		1372		617					157															
N9	19	270	203																83			56					
NI0	19	38																									
NI1	19	188	220																								
NI2	19	188	466		50																						
NI3	21	3																									
NI4	21	863	944		242		77					70						64									
NI5	21	335	535		1431		621					46															
NI6	20	123	94																								
NI7	20	646	611		63																	66					
NI8	19	255	1775		426																						
NI9	20	222	27		168							75															
N20	20	1281	1551		889		129																				
N21	19	1498			656		246					65							378								
N22	16	215	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
N23	24	255	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
N24	18	491	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
N25	23	275	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
TI1	16	1609	1302		131	275																					
TI2	16	1667	1343		120	263																					
TI3	15	1324			258																						
TI4	20	157	52		162	171																					
T1	21	2781	529		2540		118				243	61		103	72	94	51	75	98	53	74		121	52		64	248
T2	20	454	267		91																						
T3	21	1																									
IG1	>27	163	97				83	77			80	73	80	127	83		57	107					107		940		70
IG3	>27	1																									
IG4	>27	26	60					68					53	64		62	70	128					55			87	
IG8	>27	1																									
IG9	>27	1					87															51					

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Enhanced T-cell responses against subdominant epitopes of IGRP in mice depleted of IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells. We next investigated whether near complete deletion of the IGRP₂₀₆₋₂₁₄-reactive CD8+ subset by IGRP₂₀₆₋₂₁₄ treatment might have failed to protect mice from diabetes because it fostered the creation of a “niche” for T-cell clones recognizing sub-dominant epitopes of IGRP in islets. To that end, we evaluated the presence of anti-IGRP CD8+ T-cells in untreated, TUM-treated and IGRP₂₀₆₋₂₁₄-treated NOD mice (Table II, FIG. 10A). TUM treatment did not significantly increase the frequency or magnitude of anti-IGRP responses. In contrast, whereas IGRP₂₀₆₋₂₁₄ treatment reduced both the frequency and magnitude of the anti-IGRP₂₀₆₋₂₁₄ response, it increased the frequency and magnitude of T-cell responses against other IGRP epitopes (Table II and FIG. 10A). As a result, the magnitude of the total intra-islet anti-IGRP response in IGRP₂₀₆₋₂₁₄-treated NOD mice was similar to those calculated for untreated or TUM-treated animals (FIG. 10B). Importantly, presence of subdominant anti-IGRP-reactive CD8+ T-cell responses in islets of IGRP₂₀₆₋₂₁₄-treated NOD mice was most obvious in mice that had progressed to diabetes (FIG. 6C and D). Taken together, these observations provide an explanation for the paradoxical ineffectiveness of IGRP₂₀₆₋₂₁₄ (and NRP-V7) peptide treatment for T1D prevention.

Discussion

Recent years have witnessed the emergence of CD8+ cells as major effectors of tissue damage in organ-specific autoimmunity (Liblau et al., 2002). We have previously shown that a significant fraction of islet-associated CD8+ cells in NOD mice recognize a peptide from the islet-specific protein IGRP (IGRP₂₀₆₋₂₁₄) (Lieberman et al., 2003). We have also shown that this T-cell subset undergoes a process of avidity maturation that results from the competitive outgrowth of a small pool of high avidity clonotypes at the expense of a larger pool of non-diabetogenic, low avidity ones (Amrani et al., 2000). The present work was initiated to investigate the conditions under which manipulation of the IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cell pool with APLs had therapeutic significance. Our data show that chronic treatment of mice with intermediate doses of an intermediate affinity APL (NRP-A7) or high doses of a low affinity APL (NRP-I4) afforded near complete protection from diabetes. Disease protection was associated with local accumulation of nondiabetogenic, low avidity IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells at the expense of their high avidity counterparts, which were deleted. Unexpectedly, repeated treatment of mice with low or high doses of a very high affinity APL (NRP-V7) or high doses of the natural peptide ligand (IGRP₂₀₆₋₂₁₄) only afforded marginal protection from disease. Strikingly, our detailed systematic analyses of the islet-associated CD8+ T-cells of these mice revealed that the islets of these mice contained very few IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells, but increased populations of CD8+ T-cells recognizing numerous other IGRP epitopes. These results strongly argue against the usefulness of high avidity APL therapy for the prevention of organ-specific autoimmunity via elimination of prevalent subpopulations of autoreactive T-lymphocytes.

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An important new observation that has emerged from this work is that NOD islet-associated CD8+ T-cells mount responses to numerous epitopes of IGRP, in addition to IGRP₂₀₆₋₂₁₄. If we assume that the magnitude of IFN- γ that is secreted by islet-derived T-cells in response to different IGRP epitopes correlates with the number of reactive clonotypes (a situation that is true for IGRP₂₀₆₋₂₁₄-reactive cells), IGRP-reactive CD8+ T-cells would account for ~40% of all NOD islet-associated CD8+ T-cells. Since only about half of these T-cells recognize IGRP₂₀₆₋₂₁₄, these observations suggest that high prevalence of the anti-IGRP-specific CD8+ T-cell response in diabetes is not simply due to the high peripheral frequency of V α 17-J α 42+, IGRP₂₀₆₋₂₁₄-specific CD8+ T-cells in pre-diabetic animals (Trudeau et al., 2003). Rather, these new data point to the existence of something unique about IGRP that makes it a preferred target of autoreactive CD8+ T-cell responses in diabetogenesis. Since IGRP is expressed in beta cells at levels comparable to those of other beta cell proteins that are not target of autoimmune attack (Martin et al., 2001; Arden et al., 1999), it is tempting to speculate that its immunogenicity is related to its molecular nature and/or predicted intracellular topology (i.e. the endoplasmic reticulum membrane). Recently, it has been shown that a large fraction of MHC class I/peptide complexes in cells originate from defective ribosomal products (DRiPs), which are rapidly degraded by the proteasome after synthesis (Princiotta et al., 2003). The hydrophobicity and unusual transmembrane structure of IGRP may promote a high rate of DRiP formation in beta cells and/or dendritic cells. Alternatively, the location of IGRP in the endoplasmic reticulum membrane may foster its accumulation in beta cell apoptotic bodies, membrane-bound particles containing packed nuclear material (Cocca et al., 2002). Since the nuclear membrane is part of the ER system, IGRP might segregate with nuclear fragments into apoptotic bodies, which are readily phagocytosed by immature DCs (Sauter et al., 2000). Although this hypothesis is speculative, two lines of evidence indirectly support it. First, capture of apoptotic NIT-1 (IGRP+) and IGRP-transfected P815 cells by dendritic cells in vitro results in 8.3-T cell activation (our unpublished observations). Second, induction of beta cell apoptosis in vivo enhances cross-presentation of IGRP₂₀₆₋₂₁₄ to 8.3-T-cells in the PLNs (Zhang et al., 2002).

Since soluble peptides are usually cleared within 2 days (Metzler et al., 2000), they induce a weak and short-lived activation state that, in the absence of costimulatory signals (such as those induced on dendritic cells by adjuvants), leads to anergy and deletion (Aichele et al., 1994; Toes et al., 1996). Since this tolerogenic stimulus must reach an undefined threshold of TCR occupancy, the effectiveness of soluble peptides for induction of tolerance should be a function of dose, as well as affinity for TCR and MHC. Accordingly, the observation that the effectiveness of NRP-I4 and NRP-A7 therapy increased with dose was not unexpected, nor was the fact that NRP-A7 reached maximum protective activity at a lower dose than NRP-I4 (NRP-A7 is recognized with much higher avidity than NRP-I4). Notably, the islets of mice treated with protective doses of NRP-A7 and NRP-I4 contained

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normal or only slightly reduced numbers of IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells, and these cells bound NRP-V7 tetramers with lower avidity than those isolated from TUM-treated controls. These observations suggest that protective doses of NRP-A7 and NRP-I4 selectively delete pathogenic, high-avidity clonotypes, sparing their low-avidity counterparts.

5 Since the efficacy of vaccination protocols for induction of anti-tumor immunity requires the recruitment of high avidity CTL (Alexander-Miller et al., 1996; Perex-Diaz et al., 2002; Zeh et al., 1999), it is safe to assume that deletion of prevalent high avidity clonotypes by NRP-I4 and NRP-A7 does protect mice from T1D. However, the loss of NRP-A7's antidiabetogenic potential with increased doses of peptide despite significant depletion of the total IGRP₂₀₆₋₂₁₄-reactive T-cell pool
10 implied that protection was not solely due to deletion of high avidity clonotypes. This surprising outcome was clearly not a peculiarity of NRP-A7, because mice treated with the natural ligand (IGRP₂₀₆₋₂₁₄) or NRP-V7 (a higher affinity APL than NRP-A7) were only slightly protected from disease despite the fact that their islets barely contained IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cells. Therefore, the protective effect of NRP-I4 and NRP-A7 must have also required the recruitment of
15 low avidity clonotypes. This concept is supported by mathematical modeling of T-cell avidity maturation in untreated and APL-treated mice (A.F.M. Maree, P. Santamaria, and L. Edelstein-Keshet, in preparation). This work forecasts that APL-induced protection from diabetes would require occupation of the intra-islet T-lymphocyte niche by non-pathogenic low avidity clonotypes. Whether these cells afford diabetes protection by denying access of other autoreactive specificities to
20 the site, or by secreting immunoregulatory factors remains to be determined. Autoreactive T-cells with immunoregulatory properties have been found in normal individuals (Arif et al., 2004), and APLs can induce specific immunoregulatory T-cells (Kappos et al., 2000; Nicholson et al., 1995).

A fundamental observation of this study is that near-complete deletion of the IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cell subset was associated with increased responses of islet-derived CD8+ T-cells
25 against subdominant epitopes of IGRP, especially in mice that had become diabetic. This suggests that depletion of the IGRP₂₀₆₋₂₁₄-reactive CD8+ T-cell niche created a "vacuum" that somehow promoted the expansion of diabetogenic subdominant epitope-specific clonotypes. These subdominant epitope-specific (and potentially high avidity) CTLs may be highly effective at destroying their cellular targets because they readily evade mechanisms of tolerance, as proposed
30 recently (Gross et al., 2004). Conceivably, these two opposing phenomena (tolerance of dominant epitope-specific T-cells and recruitment of subdominant epitope-specific T-cells) might account for the ineffectiveness of human trials using full-length protein autoantigens (Weiner, 1993; Trentham et al., 1993; McKown et al., 1999; Pozzalli et al., 2000; Group, D.P.T.-T.D.S., 2002; Kappos et al., 2000; Bielekova et al., 2000).

35 In sum, our findings suggest that complete elimination of a dominant T-cell subpopulation by

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using high doses of high affinity APLs is an inefficient way to halt the progression of cellularly complex, polyclonal autoimmune responses. Rather, we argue that effective prevention of such diseases with APLs requires the selective elimination of high avidity clonotypes and the unopposed recruitment of their low avidity, non-pathogenic counterparts. The fact that this outcome occurs only within a narrow range of dose and functional avidity bears an important lesson that may aid in the design of APL- or self antigen-based vaccines as “tolerogens” in autoimmunity. Careful examination of peptide affinity for MHC and TCR and dose are therefore warranted in the design of clinical trials.

Example 4. An Additional T-cell –reactive IGRP Peptide

Using procedures described in Example 1, we have cultured T-cells from the islets of 12-week-old female NOD mice. T-cells were assayed for recognition of several different IGRP peptides by interferon- γ ELISpot. Reactivity was found to peptide 152-160 of murine IGRP (having the sequence FLWSVFWLI).

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the above description and shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

All references cited in this specification are hereby incorporated by reference. The discussion of the references herein is intended merely to summarize the assertions made by the authors and no admission is made that any reference constitutes prior art. Applicants reserve the right to challenge the accuracy and pertinence of the cited references.

Appendix - Mouse and human IGRP amino acid sequences

SEQ ID NO:1 - Mouse IGRP, from NP 067306. The peptide reactive to 8.3-like T cells is bold-underlined; other peptides identified herein are underlined.

1 mdfllhrsgvl iihhlqedyr tyygflnfms nvgdprnifs iyfplwfqln qnvgtkmiwv
 61 avigdwnfli fkwilfghrp ywwiqeteiy pnhsspcl eq fpttcetgpg spsghamgss
 121 cvwyvmvtaa lsytismee ssvtlhrltw sflwsvfwli qisvcisrvf iathfphqvi
 181 lgviggm lva eafehtpgvh masls **vyllkt** **nvflfl**alg fyllrlf gi dllwsvpiak
 241 kwcanpdwih idstpfa glv rnlgvlf glg fainsemflr scqgengtkp sfrllcalts
 301 lttmqlyrfi kipthaep lf yllsfcksas iplmvvalip ycvhmlmrpg dkktk

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SEQ ID NO:2 - Human IGRP, from NP 066999. The peptide reactive to 8.3-like T cells is bold-underlined.

1 mdfllhrngvl iiqhlqkdyr ayytflnfms nvgdprniff iyfplcfqfn qtvgtkmiwv
61 avigdwnli fkwilfghrp ywwvqetqiy pnhsspcleq fpttcetgpg spsghamgas
5 121 cvwyvmvtaa lshtvcgmdk fsitlhrltw sflwsvfwli qisvcisrvf iathfphqvi
181 lgviggmiva eafehtpgiq tasl**tylkt nlf**flfavgy fyllrvlni dllwsvpiak
241 kwcanpdwih idtppfaglv nlgvlfglg fainsemfll scrngnnytl sfrllcalts
301 ltilqlyhfl qiptheehlf yvlsfcksas ipltvvafip ysvhmlmkqs gkksq

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What is claimed is:

1. An isolated and purified oligopeptide or polypeptide less than 355 amino acids comprising a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP.

5

2. The oligopeptide or polypeptide of claim 1, completely homologous to a mammalian IGRP having at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2.

3. The oligopeptide or polypeptide of claim 1, comprising 100 amino acids or less.

10

4. The oligopeptide or polypeptide of claim 1, comprising 25 amino acids or less.

5. The oligopeptide or polypeptide of claim 1, comprising 13-25 amino acids.

15

6. The oligopeptide or polypeptide of claim 1, comprising 8-10 amino acids.

7. The oligopeptide or polypeptide of claim 1, comprising VYLKTNVFL.

8. The oligopeptide or polypeptide of claim 1, comprising TYLKTNLFL.

20

9. The oligopeptide or polypeptide of claim 1, comprising FLWSVFWLI.

10. The oligopeptide or polypeptide of claim 1, comprising TYYGFLNFM.

25

11. The oligopeptide or polypeptide of claim 1, comprising AYYTFLNFM.

12. The oligopeptide or polypeptide of claim 1, comprising LRLFGIDLL.

13. The oligopeptide or polypeptide of claim 1, comprising LRVLNIDLL.

30

14. The oligopeptide or polypeptide of claim 1, comprising SFCKSASIP.

15. The oligopeptide or polypeptide of claim 1, further comprising an antigenic carrier.

35

16. The oligopeptide of claim 6, further comprising a detectable label.

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17. The oligopeptide of claim 16, wherein the detectable label is a fluorescent molecule.

18. The oligopeptide of claim 16, wherein the detectable label is a radioactive molecule.

5 19. The oligopeptide of claim 16, wherein the detectable label is an enzyme.

20. The oligopeptide of claim 16, further comprising an MHC class I molecule that is capable of binding the oligopeptide.

10 21. The oligopeptide of claim 6, further comprising an MHC class I molecule that is capable of binding the oligopeptide.

22. The oligopeptide of claim 21, wherein the MHC class I molecule is mouse H-2K^d.

15 23. The oligopeptide of claim 21, further comprising a cytotoxic molecule.

24. The oligopeptide of claim 23, wherein the cytotoxic molecule is radioactive.

20 25. The oligopeptide of claim 5, further comprising an MHC class II molecule that is capable of binding the oligopeptide.

26. The oligopeptide of claim 25, further comprising a cytotoxic molecule.

25 27. The oligopeptide or polypeptide of claim 1, in a sterile pharmaceutical preparation.

28. The oligopeptide or polypeptide of claim 27, comprising a sequence selected from the group consisting of YLKTNVFL, TYLKTNLFL, TYYGFLNFM, AYYTFLNFM, LRLFGIDLL, LRVLNIDLL, SFCKSASIP, and KWCANPDWI.

30 29. The oligopeptide or polypeptide of claim 28, wherein the oligopeptide or polypeptide is a mammalian IGRP polypeptide having at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2.

30. The polypeptide of claim 29, wherein the mammalian IGRP is a mouse IGRP or a human IGRP.

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31. The oligopeptide or polypeptide of claim 28, capable of tolerizing a mammal to reduce CD8⁺ T cells reactive to IGRP.

5 32. An isolated and purified oligopeptide 8-10 amino acids in length, completely homologous with a mammalian IGRP having at least 90% homology to SEQ ID NO:1 or SEQ ID NO:2, wherein the oligopeptide is capable of binding an MHC class I molecule of the mammal.

10 33. The isolated and purified oligopeptide of claim 32, comprising a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP.

34. The oligopeptide of claim 25, further comprising a detectable label.

15 35. The oligopeptide of claim 34, wherein the detectable label is a fluorescent molecule.

36. The oligopeptide of claim 34, wherein the detectable label is a radioactive molecule.

37. The oligopeptide of claim 34, wherein the detectable label is an enzyme.

20 38. The oligopeptide of claim 34, further comprising an MHC class I molecule that is capable of binding the oligopeptide.

25 39. The oligopeptide of claim 32, further comprising an MHC class I molecule that is capable of binding the oligopeptide.

40. The oligopeptide of claim 39, further comprising a cytotoxic molecule.

41. The oligopeptide of claim 40, wherein the cytotoxic molecule is radioactive.

30 42. An antisense molecule complementary to at least a portion of an mRNA encoding a mammalian IGRP, wherein the mammalian IGRP is at least 90% homologous to SEQ ID NO:1 or SEQ ID NO:2, and wherein the antisense molecule is capable of inhibiting translation of a mammalian IGRP.

35 43. A ribozyme specific for a portion of an mRNA encoding a mammalian IGRP, wherein

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the mammalian IGRP at least 90% homologous to SEQ ID NO:1 or SEQ ID NO:2, and wherein the ribozyme inhibits translation of a mammalian IGRP.

44. An RNAi molecule homologous to a portion of an mRNA encoding a mammalian IGRP,
5 wherein the mammalian IGRP is at least 90% homologous SEQ ID NO:1 or SEQ ID NO:2, and
wherein the RNAi molecule is capable of inhibiting translation of a mammalian IGRP.

45. A method of treating a mammal, wherein the mammal is at risk for or has type 1 diabetes,
the method comprising administering an oligopeptide to the mammal in a manner sufficient to reduce
10 CD8⁺ T cells reactive to IGRP, wherein the oligopeptide is 8-10 amino acids in length, and comprises
a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI,
(T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL.

46. The method of claim 45, wherein the oligopeptide further comprises a cytotoxic
15 molecule.

47. The method of claim 45, further comprising treating the mammal with a second
oligopeptide 8-10 amino acids in length, and comprising a sequence selected from the group
consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM,
20 LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL.

48. A method of treating a mammal, wherein the mammal is at risk for or has type 1 diabetes,
the method comprising administering the oligopeptide of claim 32 to the mammal in a manner
sufficient to reduce CD8⁺ T cells reactive to IGRP.
25

49. The method of claim 48, wherein the oligopeptide further comprises a cytotoxic
molecule.

50. The method of claim 48, further comprising administering a second oligopeptide of claim
30 32 to the mammal.

51. A method of treating a mammal, wherein the mammal is at risk for or has type 1 diabetes,
the method comprising administering an oligopeptide 8-10 amino acids in length to the mammal,
wherein the oligopeptide is a medium- or low-affinity ligand to an IGRP₂₀₆₋₂₁₄-reactive CD8⁺ T cell.
35

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52. The method of claim 51, wherein the oligopeptide is KYNIANWFL or KYNKANAFL.

53. A method of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a β -cell, the method comprising treating the β -cell with a compound capable of specifically binding the oligopeptide of claim 32.

54. The method of claim 53, wherein the oligopeptide-binding compound is an antibody or an aptamer.

55. The method of claim 53, wherein the oligopeptide comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, YNIANWFL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, and SFCKSASIP.

56. The method of claim 53, wherein the pancreatic islet β -cell is part of a pancreas of a mammal at risk for or having type 1 diabetes.

57. The method of claim 56, wherein the mammal is a mouse.

58. The method of claim 56, wherein the mammal is a human.

59. The method of claim 53, further comprising treating the β cell with an MHC class I molecule that is capable of binding the oligopeptide.

60. The method of claim 59, wherein the oligopeptide further comprises a cytotoxic molecule.

61. The method of claim 59, wherein the pancreatic islet β -cell is part of a pancreas of a mammal at risk for or having type 1 diabetes.

62. The method of claim 61, wherein the mammal is a mouse.

63. The method of claim 61, wherein the mammal is a human.

64. A method of preventing a CD8⁺ T cell that is cytotoxic to pancreatic islet β -cells from destroying a mammalian β -cell, the method comprising treating the CD8⁺ T cell with an oligopeptide

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8-10 amino acids in length to the β -cell in a manner sufficient to reduce CD8⁺ T cells reactive to IGRP, wherein the oligopeptide comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL.

5

65. The method of claim 64, wherein the oligopeptide further comprises a cytotoxic molecule.

10 66. The method of claim 64, further comprising treating the CD8⁺ T cell with a second oligopeptide of claim 39.

67. The method of claim 64, wherein the pancreatic islet β -cell is part of a pancreas of a mammal at risk for or having type 1 diabetes.

15 68. A method of treating a mammal at risk for or having type 1 diabetes, the method comprising administering to the mammal the antisense molecule of claim 42, wherein the nucleic acid is administered in a manner sufficient to decrease expression of IGRP in the mammal.

20 69. A method of treating a mammal at risk for or having type 1 diabetes, the method comprising administering to the mammal the ribozyme of claim 43, wherein the nucleic acid is administered in a manner sufficient to decrease expression of IGRP in the mammal.

25 70. A method of treating a mammal at risk for or having type 1 diabetes, the method comprising administering to the mammal the RNAi molecule of claim 44, wherein the nucleic acid is administered in a manner sufficient to decrease expression of IGRP in the mammal.

30 71. A method of treating a mammal having type 1 diabetes, the method comprising transplanting a pancreatic islet β -cell into the pancreas of the mammal, wherein the β -cell is transfected with a vector that expresses an antisense molecule, a ribozyme, or an RNAi molecule that is capable of specifically inhibiting translation of a mammalian IGRP.

72. A method for determining whether a mammal is at risk for or has type 1 diabetes, the method comprising determining the presence of CD8⁺ T cells reactive to IGRP in the mammal by

- a. obtaining a sample of lymphocytes comprising CD8⁺ T cells from the mammal;
- b. combining the lymphocytes with an oligopeptide and an MHC class I molecule

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that is capable of binding the oligopeptide, wherein the oligopeptide or the MHC molecule further comprises a detectable label and wherein the oligopeptide is 8-10 amino acids in length and comprises a sequence selected from the group consisting of YLKTN(A/I/L/V)FL, FLWSVFWLI, (T/A)YY(G/T)FLNFM, LR(L/V)(F/L)(G/N)IDLL, KWCANPDWI, SFCKSASIP, and YNIANWFL;

5 and

c. determining whether any CD8⁺ T cells specifically bind to the oligopeptide, wherein CD8⁺ T cell binding to the oligopeptide indicates that the mammal is at risk for or has type 1 diabetes.

10 73. The method of claim 72, wherein the determination step is performed by counting labeled CD8⁺ T cells using a cell sorter or labeled cell counter.

74. The method of claim 72, wherein the determination step is performed by microscopic observation of the lymphocytes under conditions where the label can be observed.

15

75. The method of claim 72, wherein the determination step is performed by measuring activation of the CD8⁺ T cells.

20 76. The method of claim 75, wherein activation of the CD8⁺ T cells is measured by measuring interferon- γ production.

77. The method of claim 76, wherein interferon- γ production is measured using an ELISpot assay.

25 78. A method for determining whether a mammal is at risk for or has type 1 diabetes, the method comprising determining the presence of CD8⁺ T cells reactive to IGRP in the mammal by

a. obtaining a sample of lymphocytes comprising CD8⁺ T cells from the mammal;

b. combining the lymphocytes with an oligopeptide and an MHC class I molecule that is capable of binding the oligopeptide, wherein the oligopeptide or the MHC molecule further comprises a detectable label and wherein the oligopeptide is 8-10 amino acids and is completely homologous with a mammalian IGRP; and

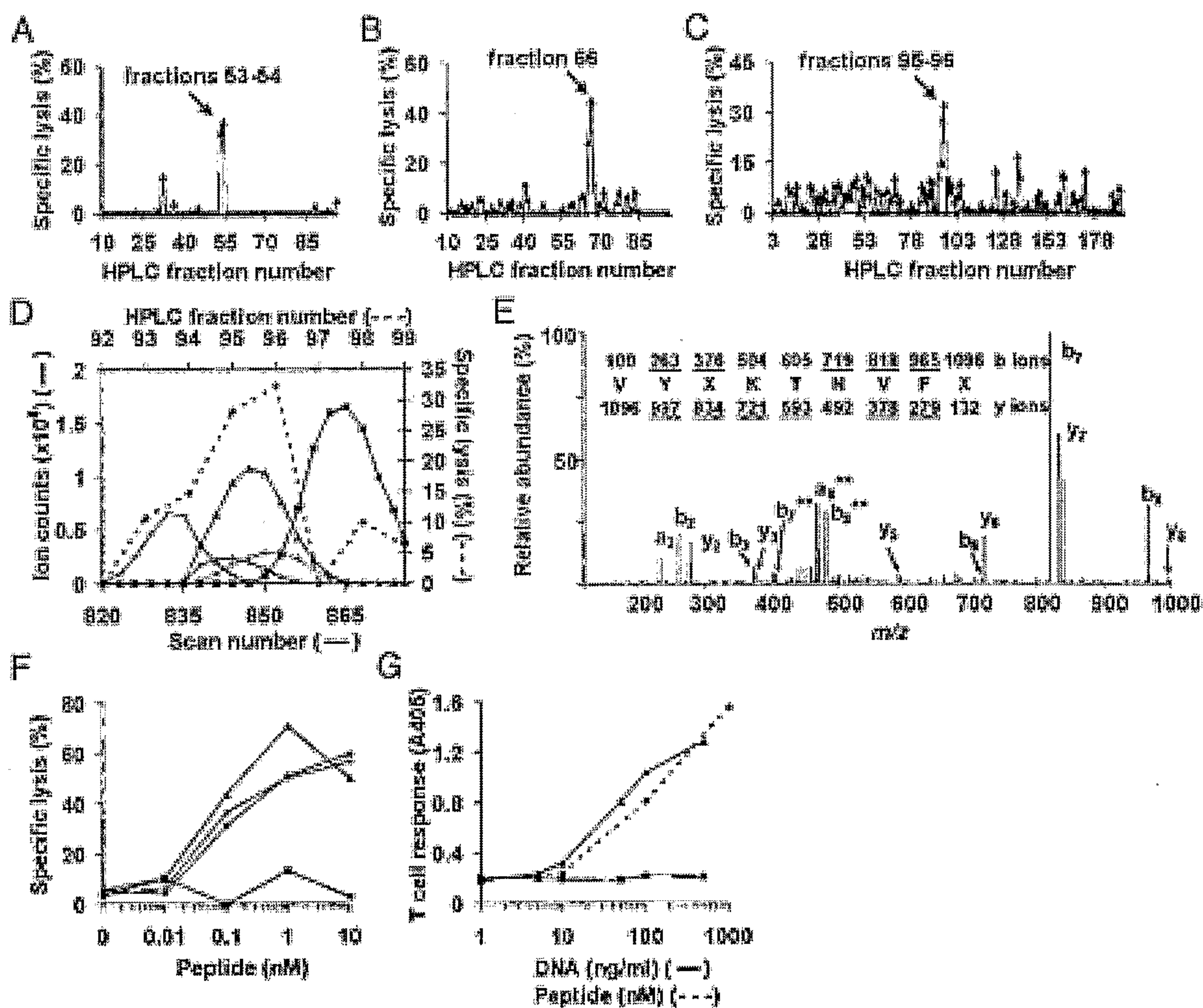
30

c. determining whether any CD8⁺ T cells specifically bind to the oligopeptide,

wherein CD8⁺ T cell binding to the oligopeptide indicates that the mammal is at risk for or has type 1 diabetes.

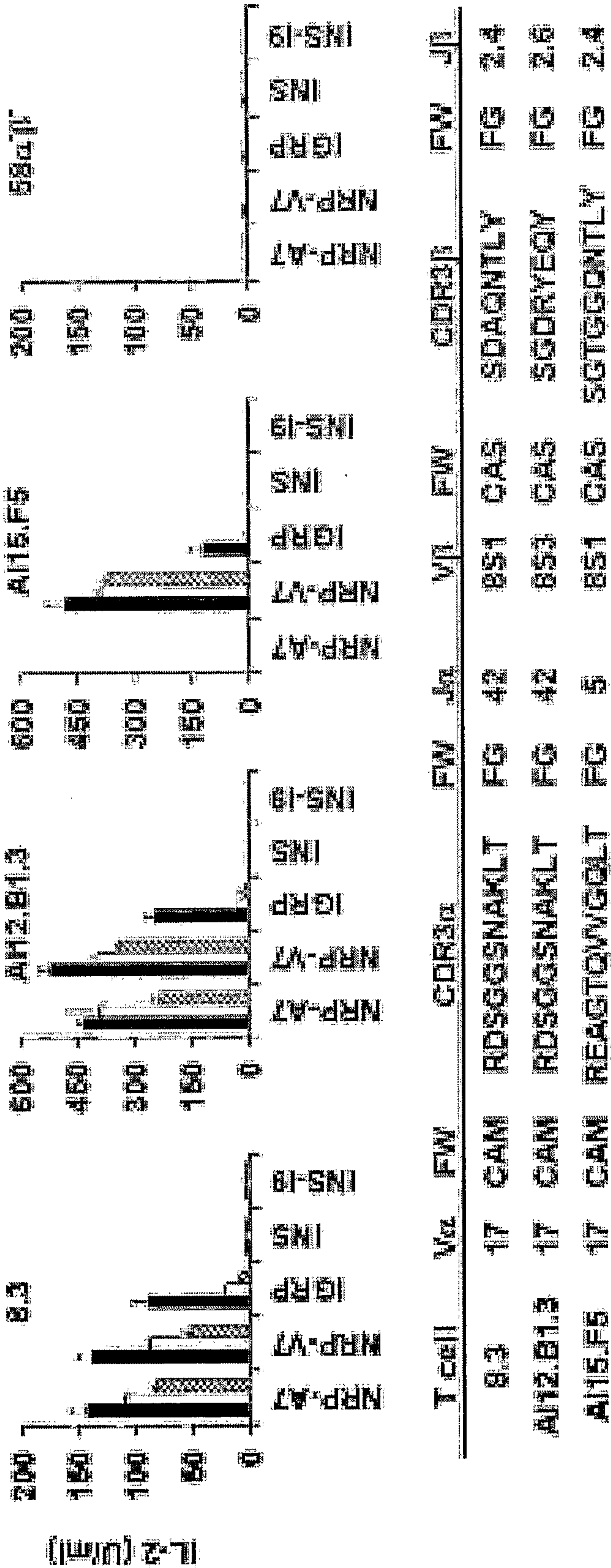
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FIG. 1



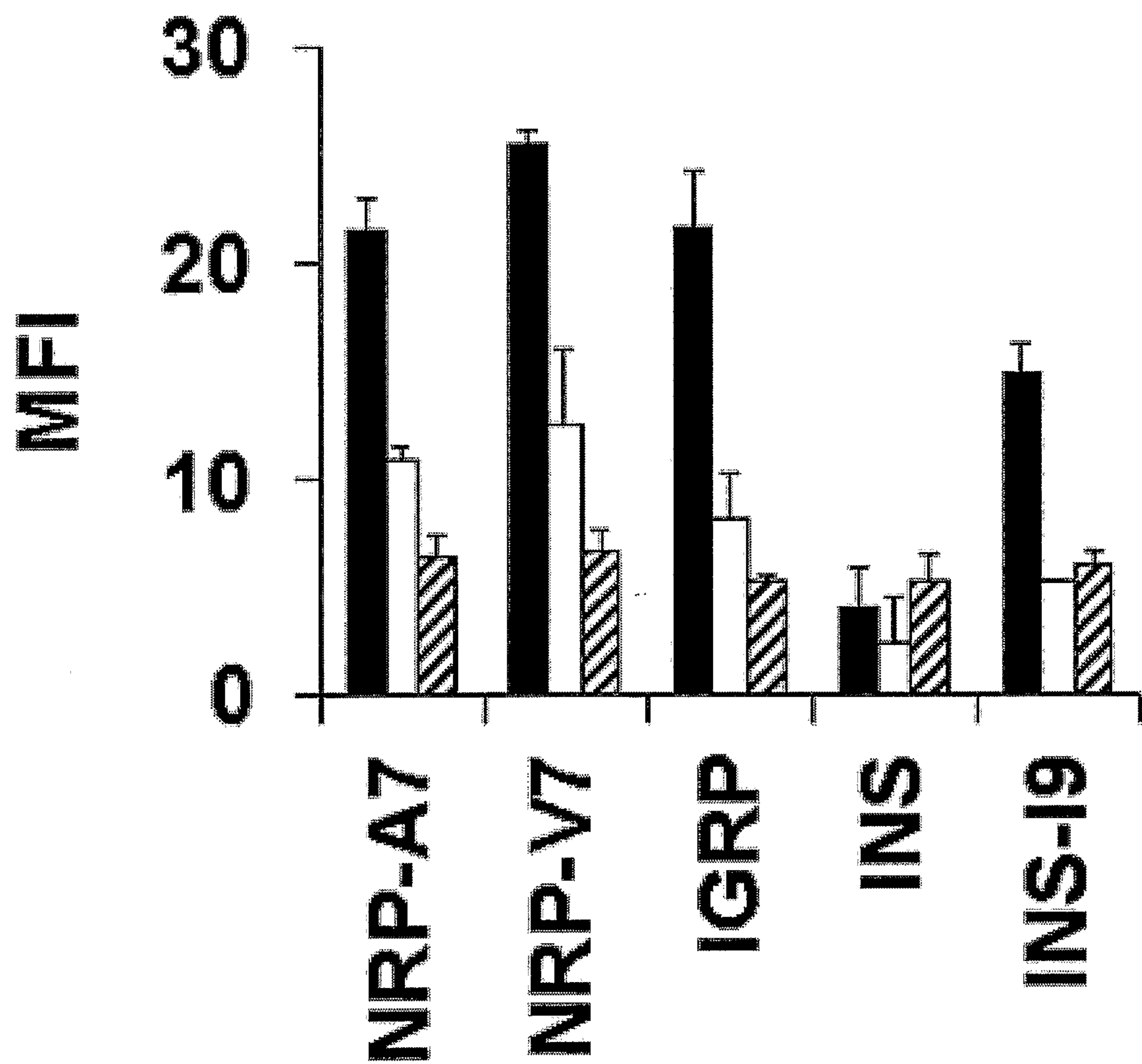
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FIG. 2



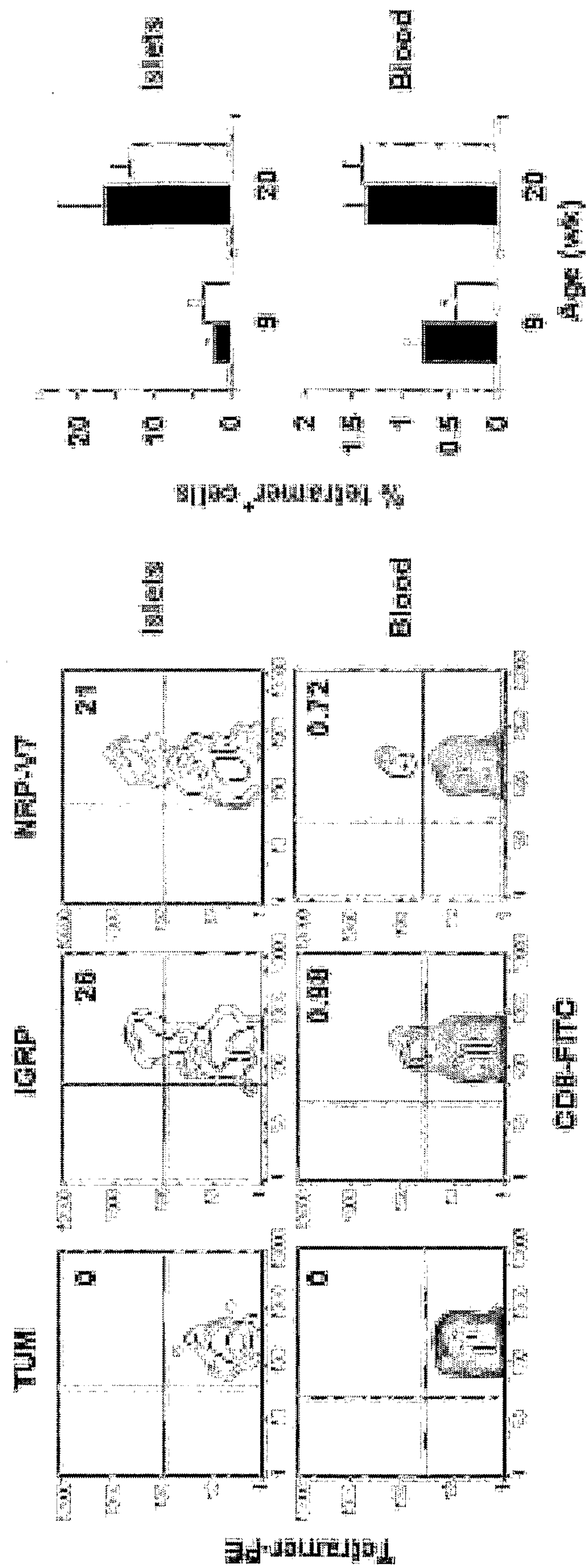
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FIG. 3



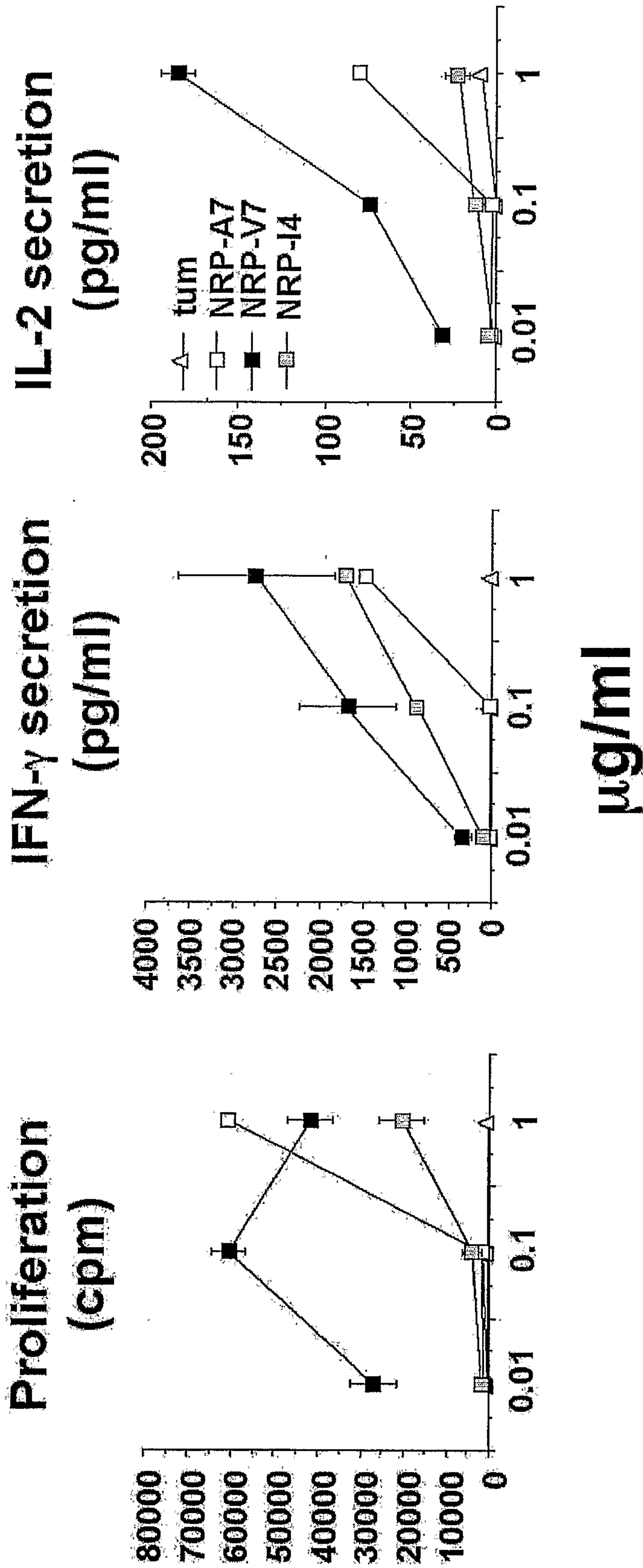
4/10

FIG. 4



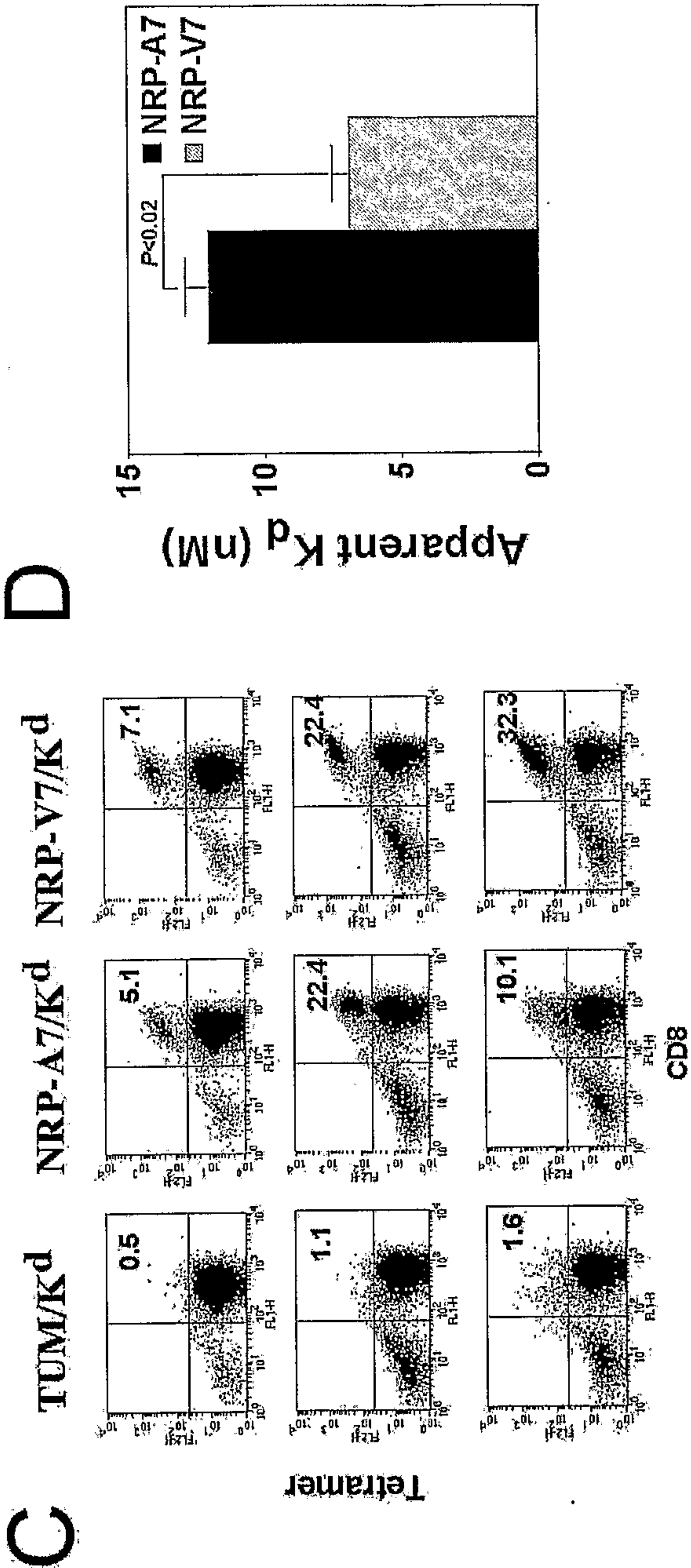
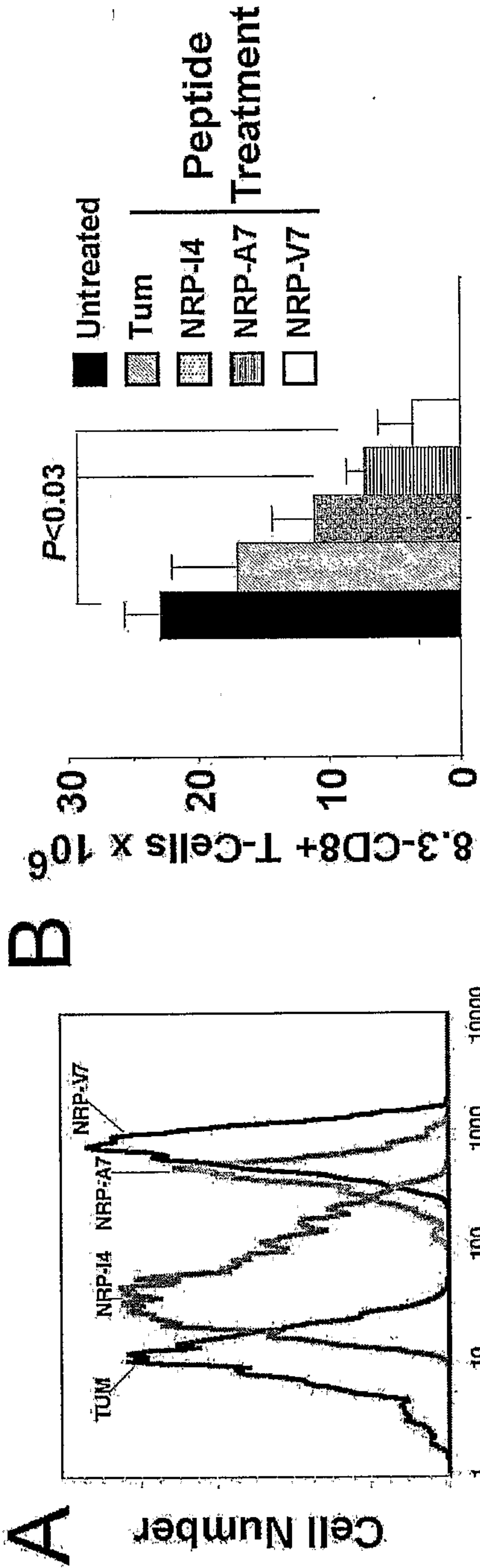
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FIG. 5

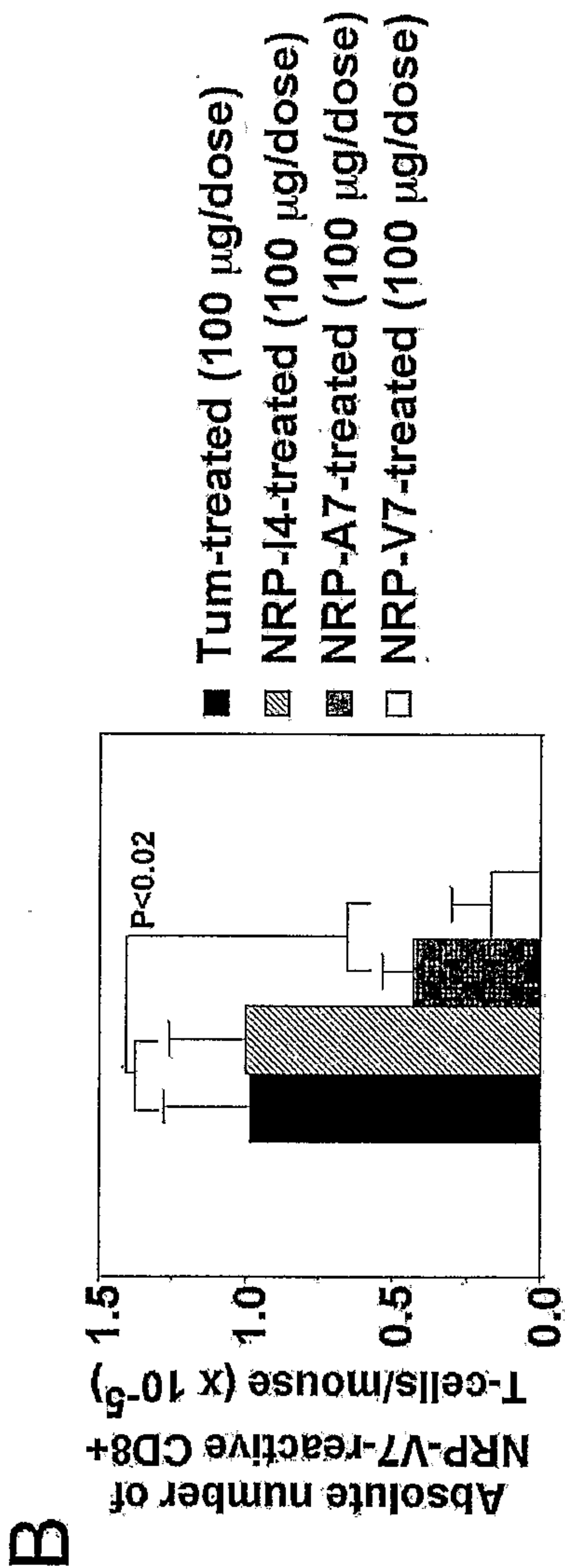
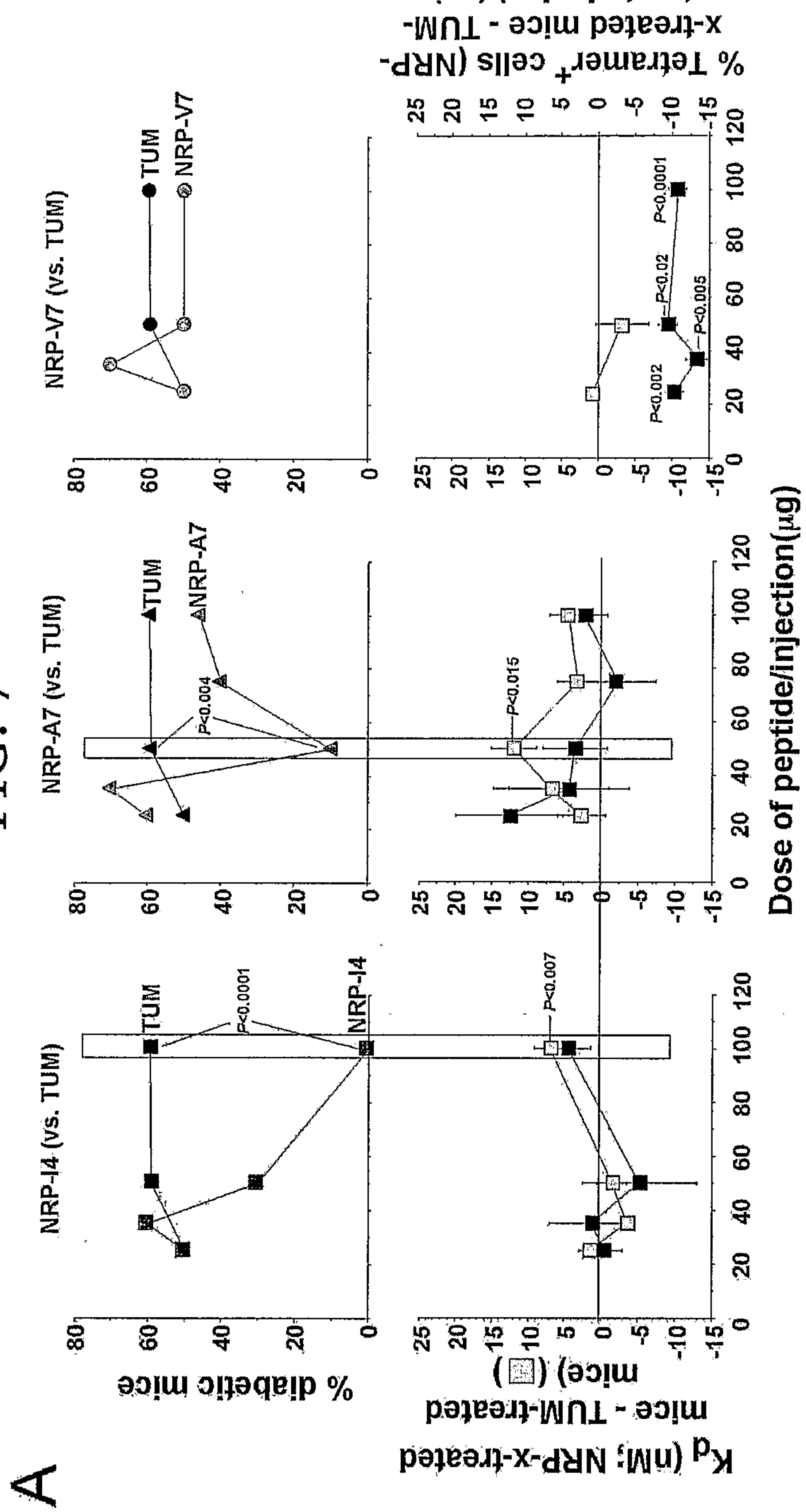


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FIG. 6

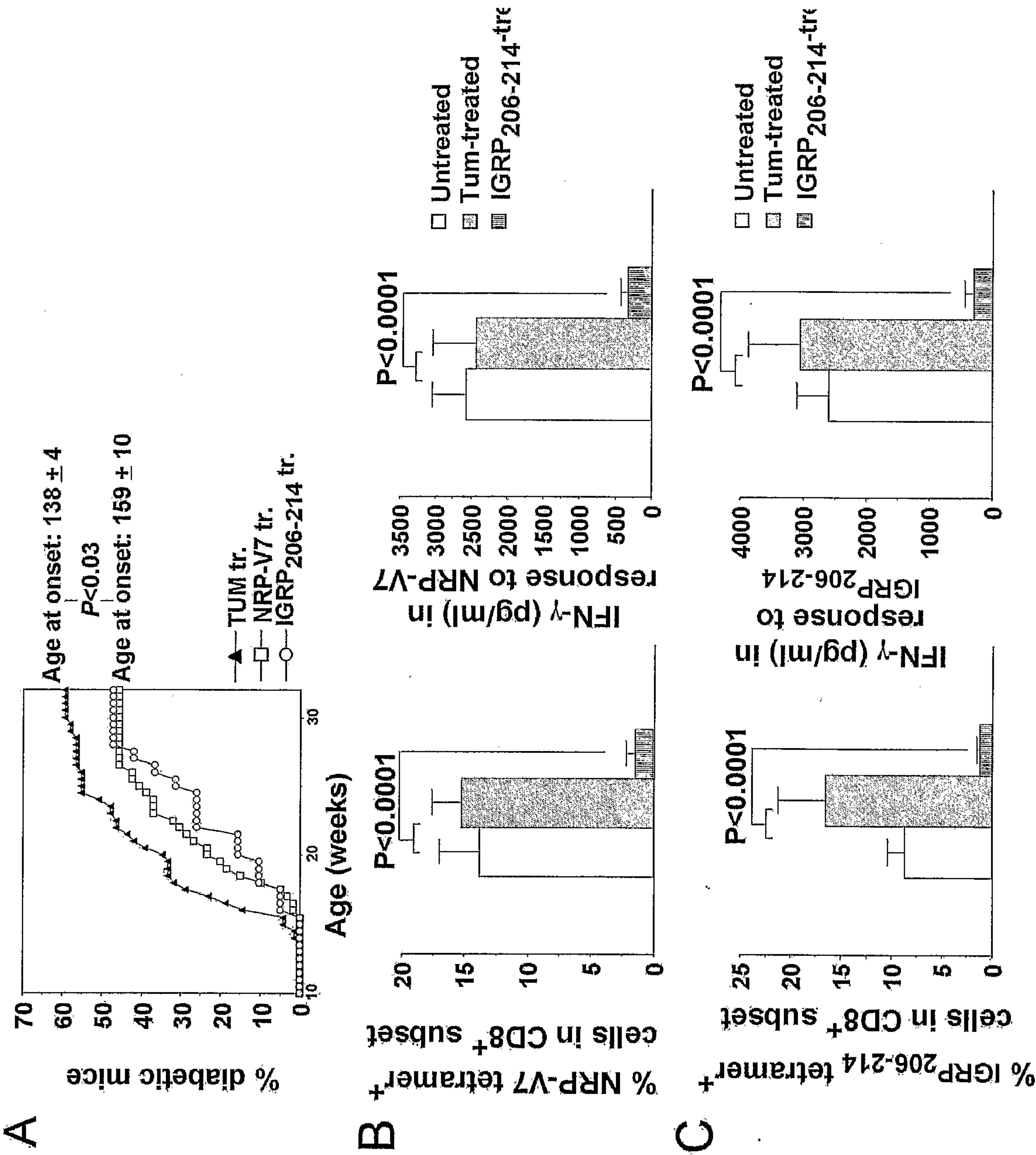


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FIG. 7



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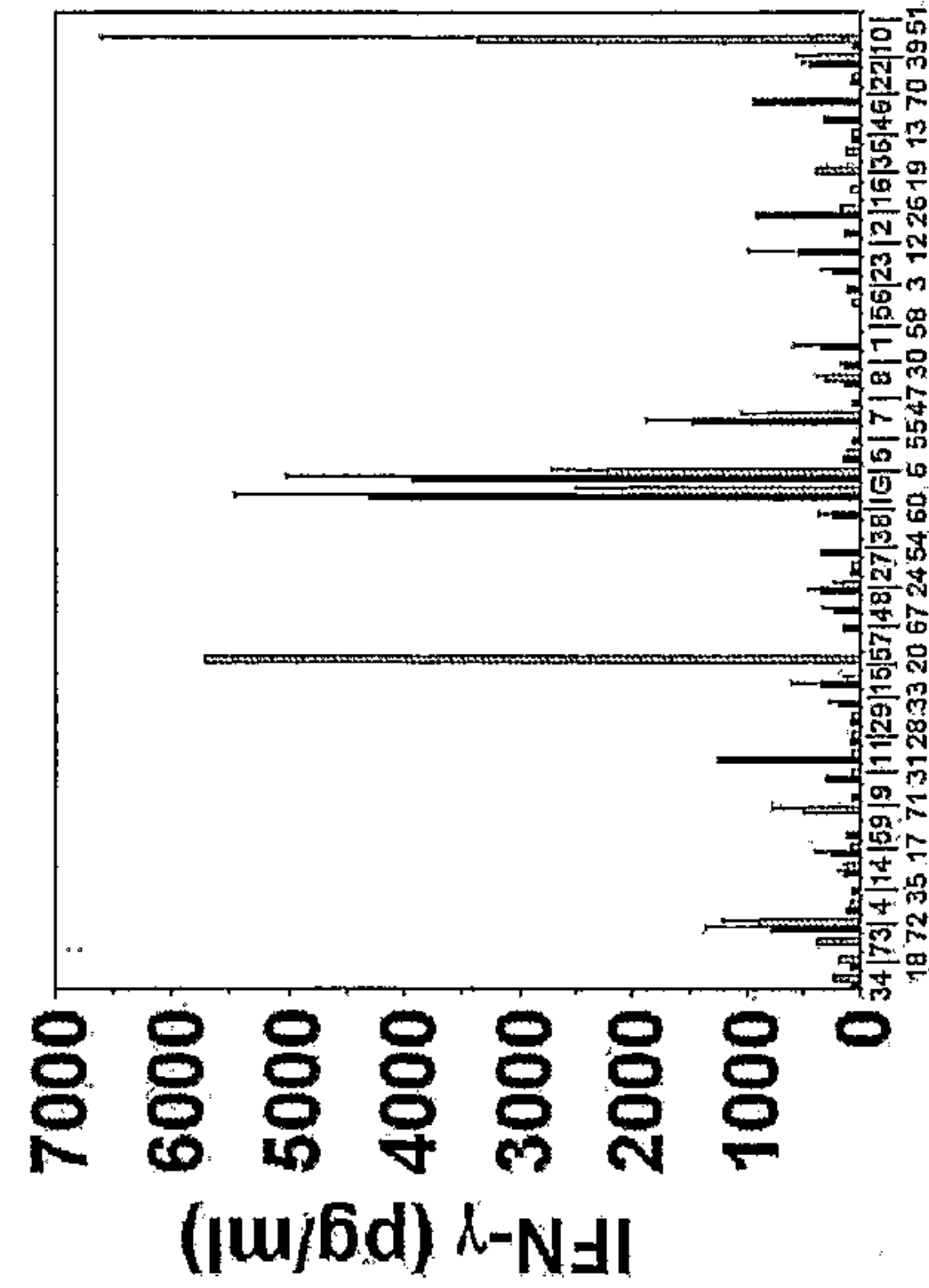
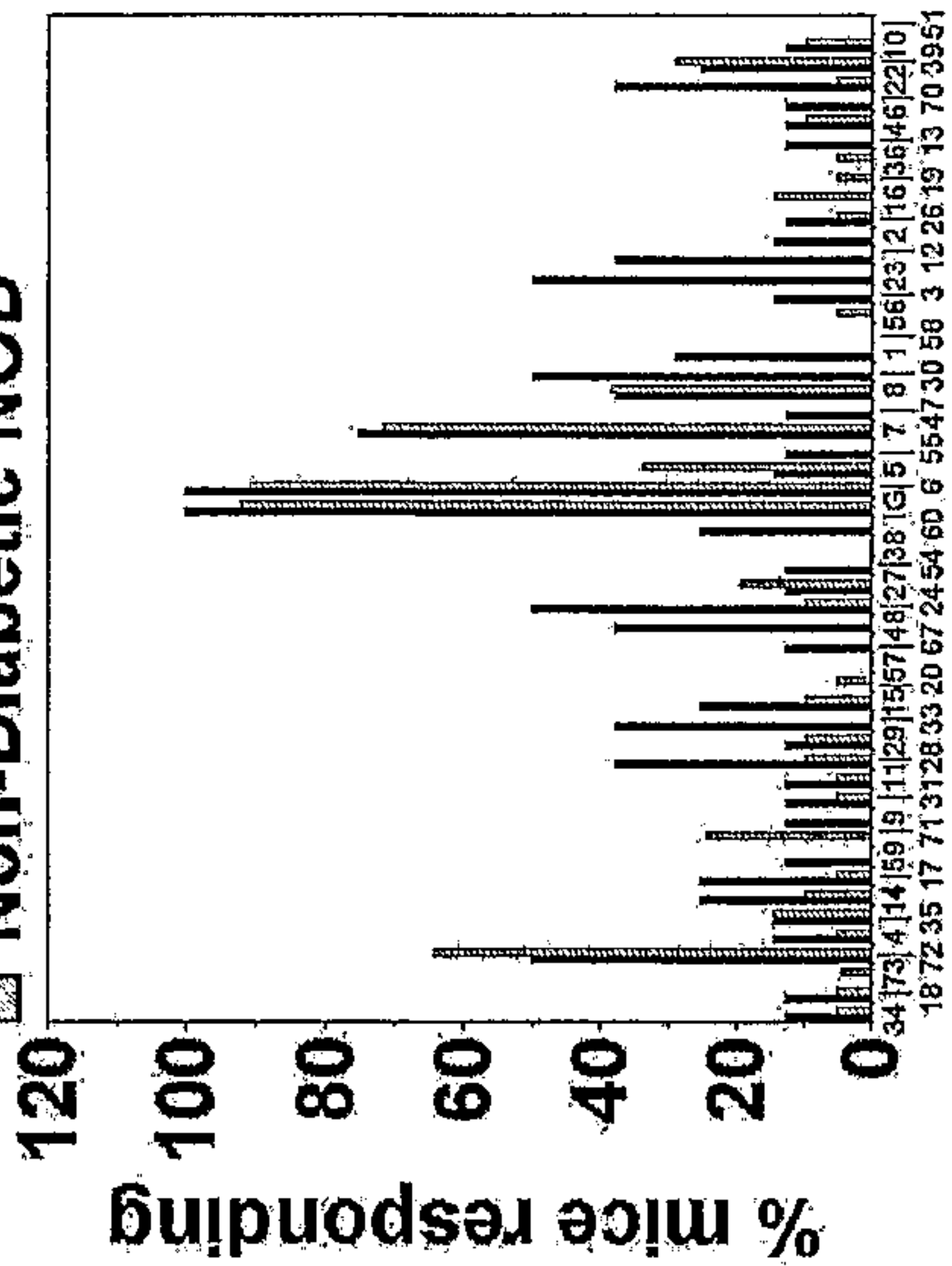
FIG. 8



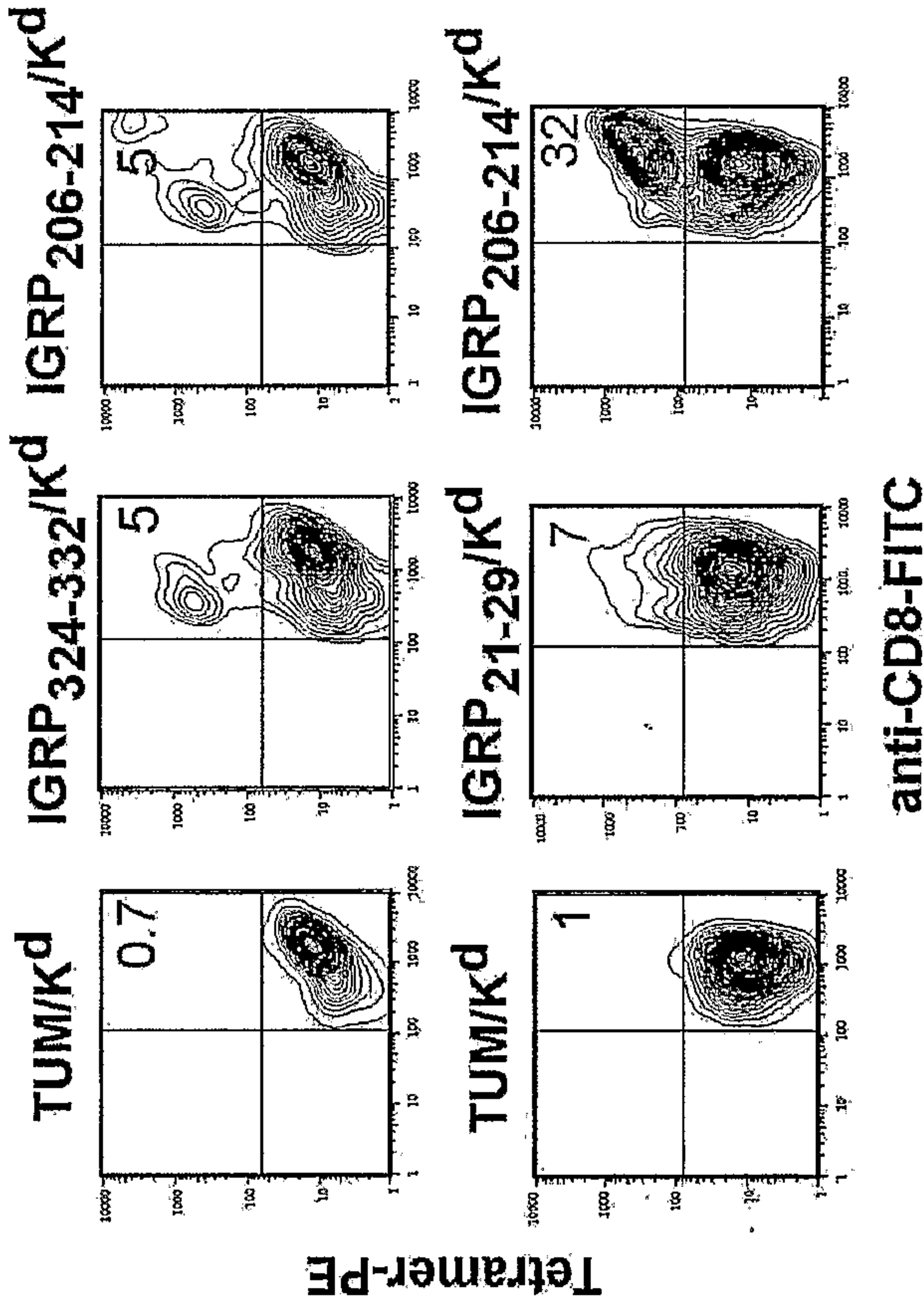
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FIG. 9

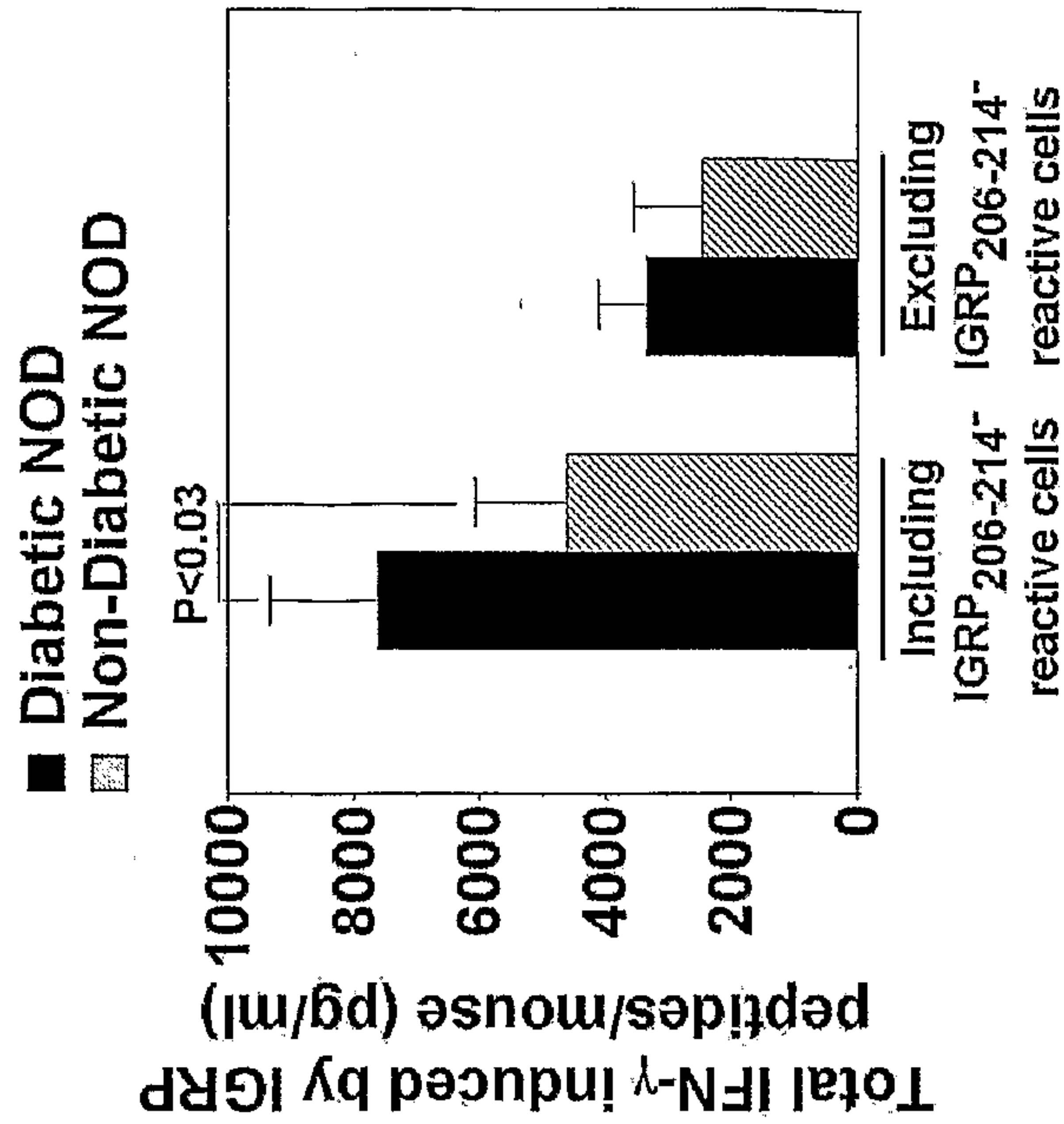
■ Diabetic NOD
▨ Non-Diabetic NOD



B



C



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FIG. 10

