Brief Definitive Report

Interleukin 12 Administration Induces T Helper Type 1 Cells and Accelerates Autoimmune Diabetes in NOD Mice

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Summary

T cells play a major role in the development of insulin-dependent diabetes mellitus (IDDM) in nonobese diabetic (NOD) mice. Administration of interleukin 12 (IL-12), a key cytokine which guides the development of T helper type 1 (Th1) $CD4^+$ T cells, induces rapid onset of IDDM in NOD, but not in BALB/c mice. Histologically, IL-12 administration induces massive infiltration of lymphoid cells, mostly T cells, in the pancreatic islets of NOD mice. $CD4^+$ pancreas-infiltrating T cells, after activation by insolubilized anti T cell receptor antibody, secrete high levels of interferon γ and low levels of IL-4. Therefore, IL-12 administration accelerates IDDM development in genetically susceptible NOD mice, and this correlates with increased Th1 cytokine production by islet-infiltrating cells. These results hold implications for the pathogenesis, and possibly for the therapy of IDDM and of other Th1 cell–mediated autoimmune diseases.

Based on the repertoire of lymphokine production, mouse (1) and human (2) $CD4^+$ T cells can be subdivided into two subsets, Th1 and Th2, characterized by secretion of IFN-γ and IL-4, respectively. The generation of Th1 and Th2 subsets is influenced by the cytokines present during the initial phase of the immune response, and a major role is played by IL-12 and IL-4 (3, 4). IL-12, a heterodimeric cytokine (5, 6) produced by activated monocytes and B cells (7), promotes Th1 cell development. It also enhances proliferation and cytolytic activity of NK and T cells (8), and stimulates production of IFN-γ by these cell types (9). IFN-γ appears to play a role in the development of insulin-dependent diabetes mellitus (IDDM), as demonstrated by the prevention of disease by administration of anti-IFN-γ mAb to nonobese diabetic (NOD) mice (10, 11), or by IDDM induction in mice expressing genes encoding IFN-γ under the control of the insulin promoter (12). It has been proposed that the pathological immune response of NOD mice to islet β cells is initiated by $CD4^+$ T cells that recognize glutamic acid decarboxylase (13, 14), and splenic $CD4^+$ T cells from NOD mice secrete IFN-γ when stimulated with this enzyme (13). Conversely, the Th2-derived lymphokines IL-4 and IL-10 appear to inhibit progression to IDDM in NOD mice (15–17). These findings suggest that T cells that recognize pancreatic β cell antigens cause IDDM only if they develop into Th1 cells.

To test this assumption, we treated prediabetic NOD mice with IL-12, which has been shown to induce the development of IFN-γ–producing Th1 cells in vitro (18, 19) and in vivo (20). Results in the present paper demonstrate that administration of IL-12 to prediabetic NOD female mice induces rapid onset of IDDM. This is associated with enhanced production of Th1-type cytokines by islet-infiltrating lymphocytes, and with selective destruction of islet β cells.

Materials and Methods

Mice. 10-wk-old female BALB/c mice were obtained from Charles River (Calco, Italy). NOD/Lt mice from The Jackson Laboratory (Bar Harbor, ME) were bred and kept in conventional housing conditions in our animal facility. Mice were diagnosed diabetic after two sequential measurements of blood glucose levels >200 mg/dl. The incidence of IDDM in NOD female mice from our colony was ~60%.

Recombinant Mouse IL-12. Recombinant mouse IL-12 was produced in serum-free medium by transfected CHO cells and purified by sequential chromatography, as described (21). The IL-12 used in this study was >95% pure, as assessed by SDS-PAGE analysis, and the endotoxin content was <5 endotoxin U/mg IL-12, as determined by the *Limulus* amebocyte assay. IL-12 was diluted in PBS containing 1% syngenic mouse serum or 100 μg/ml mouse serum albumin (Sigma Chemical Co., St. Louis, MO) and injected intraperitoneally in a 0.2-ml volume. IL-12 has a half-life in mice of ~5 h (Gately, M., and R. Nadeau, unpublished observations).

Isolation of Pancreas-infiltrating Cells. Individual pancreata were perfused with PBS. After removal of all visible pancreatic lymph nodes, the pancreata were digested in HBSS containing 5 mg/ml collagenase IV and 2.5 mg/ml DNase (Sigma Chemical Co.), by shaking (200 rpm) at 37°C for 12 min. Single cell suspensions were

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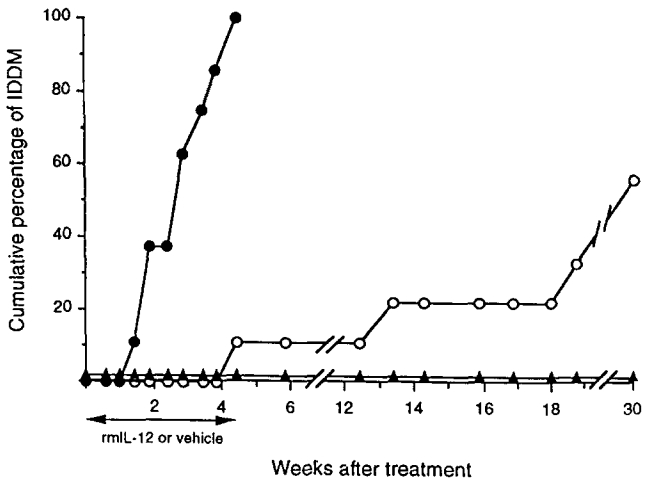
collected after diluting the enzymes with ice-cold HBSS containing 5% FCS and removal of the aggregates by settling 5 min on ice. Aggregates were further digested with collagenase IV (2.5 mg/ml) and DNase (1.25 mg/ml) for 5 min. Single cell suspensions from three to four mice were pooled, washed three times, and $CD4^+$ and $CD8^+$ cells were sorted by positive selection on Mini-MACS® (Miltenyi Biotec Inc., Sunnyvale, CA). The mean number of $CD4^+$ and $CD8^+$ cells recovered per pancreas in three separate experiments was, respectively, 51 and 20 × 10^4 for vehicle-injected mice, 82 and 66 × 10^4 for IL-12–treated littermates.

Induction of Lymphokine Production. $CD4^+$ and $CD8^+$ cells were cultured (2.5 × 10^5 cells/well) in 96-well round bottom plates coated with 5 μg/ml anti-TCR mAb (American Type Culture Collection [ATCC] HB 218; Rockville, MD) in serum-free HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mM L-glutamine and 50 μg/ml gentamicin (Sigma Chemical Co.). Supernatants were collected after 48 h of culture. Sera were collected ~15 h after the last IL-12 injection and assayed for ELISA for circulating IFN-γ.

Immunohistology. For hematoxylin and eosin staining, pancreata were fixed in 10% formalin and embedded in paraffin. For immunoperoxidase staining, pancreata were snap-frozen in Tissue-Tek (Miles Laboratories Inc., Elkhart, IN), and consecutive 5 μm sections stained for 45 min with rabbit anti-glucagon and mouse anti-insulin antibodies (Ortho Diagnostic Systems, Raritan, NJ). After washing, sections were incubated for 30 min with secondary antibodies conjugated to horseradish peroxidase, which was visualized using 3 amino-9 ethyl-carbazole (AEC, Sigma Chemical Co.) as chromogen and hematoxylin as counterstain. In addition, pancreas cryostat sections were stained by biotinylated mAb directed against CD4, CD8, B220 (PharMingen, San Diego, CA), p150/90 leucocyte β2 integrin (N418, ATCC), and $I\text{-}A^{g7}$ (10.3.62, ATCC), followed by streptavidin–peroxidase conjugate. AEC was used as a chromogen and hematoxylin as a counterstain.

Cytokine Assays. IFN-γ and IL-4 concentrations were determined by ELISA using, respectively, mAb AN-18.17.24 (22) or BVD4-1D11 (PharMingen) for capture, and peroxidase-conjugated XMG1.2 (23) or biotinylated BVD6-24G2 (PharMingen) followed by avidin-peroxidase (Sigma Chemical Co.) for detection. The substrate was 3,3′-5,5′-tetramethylbenzidine (Fluka Chemie AG, Buchs, Switzerland). Dilutions of culture supernatant or serum were assayed in the linear portion of the dose–response curve. Standard curves were generated in each assay using purified recombinant mouse IFN-γ or IL-4.



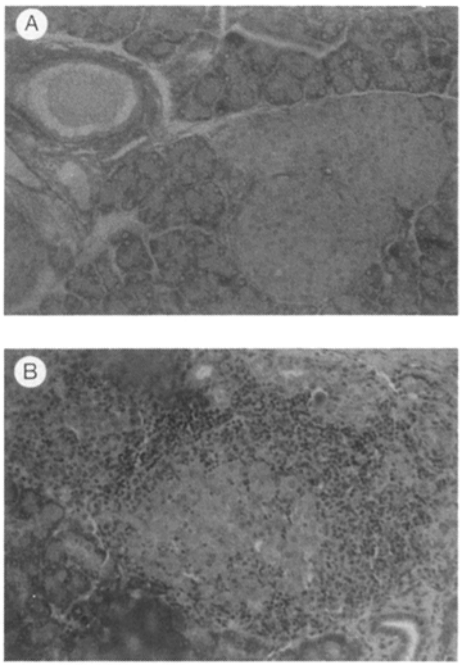


Figure 2. Insulitis is observed in NOD but not in BALB/c mice after administration of IL-12. Islets from a 10-wk-old BALB/c mouse after 32 injections of IL-12 are intact (*A*). In contrast, islets from a 10-wk-old NOD mouse after 20 injections of IL-12 show a severe mononuclear cell infiltration (*B*). ×250.

Results and Discussion

Administration of recombinant mouse IL-12 to 8–10-wk-old prediabetic NOD female mice induced rapid onset of IDDM (Fig. 1). Overt diabetes began after about 10 IL-12 injections, and after 30 injections it was present in 100% of the mice. The same treatment did not induce IDDM in BALB/c mice. In the NOD female littermates injected with vehicle only (PBS-1% normal NOD serum), IDDM began only at 15 wk of age, and by week 40, hyperglycemia was present in 55% of the mice.

Histological analysis of pancreatic sections revealed insulitis in NOD mice injected with IL-12, but not in BALB/c mice, even after 32 injections of IL-12 (Fig. 2). The normal appearance of pancreatic islets and the absence of IDDM in BALB/c mice indicates a lack of direct toxic effects of IL-12 administration on islet β cells in vivo. Quantification of islet infiltrates showed a higher score in NOD mice injected with IL-12 as compared with those injected with vehicle only (not

Figure 1. Acceleration of IDDM onset by IL-12 administration to NOD mice. 10 female NOD/Lt prediabetic mice (8–10 wk old, randomized from four different litters) were injected daily with 0.3 μg i.p. recombinant mouse IL-12 for the first 7 d, then with 0.15 μg for the following 24 d (●), or with vehicle (PBS containing 1% NOD serum) only (○). As control, 10 BALB/c female mice (10 wk old) received the same IL-12 treatment (▲). Four other independent experiments, performed with 10–14-wk-old NOD females injected with 0.15 μg IL-12 daily, gave similar results, IDDM onset starting after 5–10 IL-12 injections.

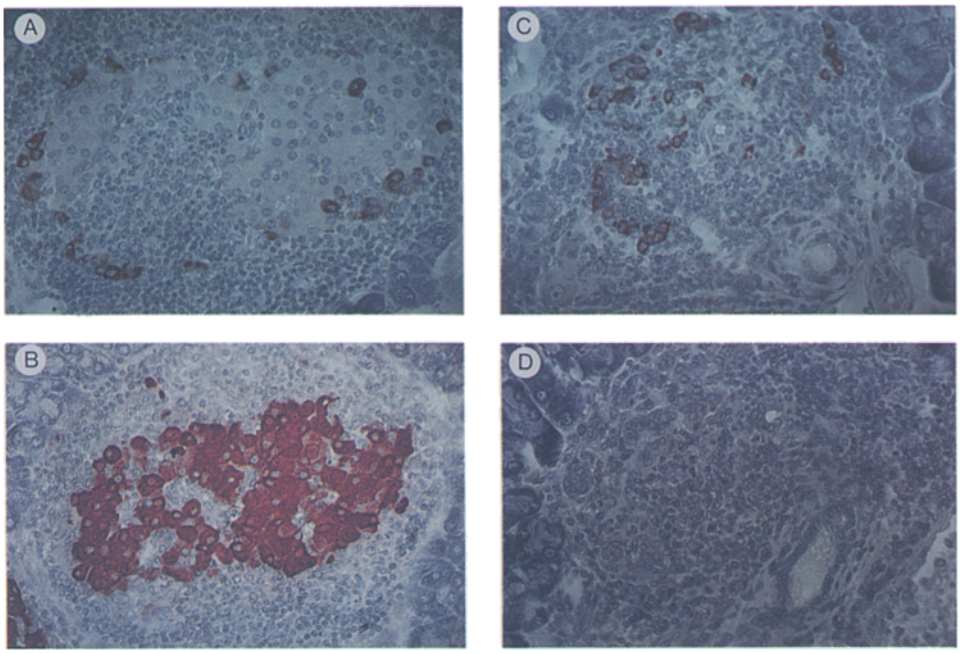


Figure 3. Selective loss of islet β cells in NOD mice injected with IL-12. Pancreas from a 10-wk-old NOD female mouse injected 14 times with vehicle only displays insulitis, but glucagon- (*A*) and insulin- (*B*) containing islet cells are well represented. In contrast, the NOD littermate injected 14 times with IL-12 (0.15 μg/day) had disorganized but relatively normal numbers of glucagon-containing cells (*C*), whereas insulin-containing cells (*D*) are absent. The latter finding correlates with hyperglycemia (>500 mg/dl) at the time of pancreas removal. ×400.

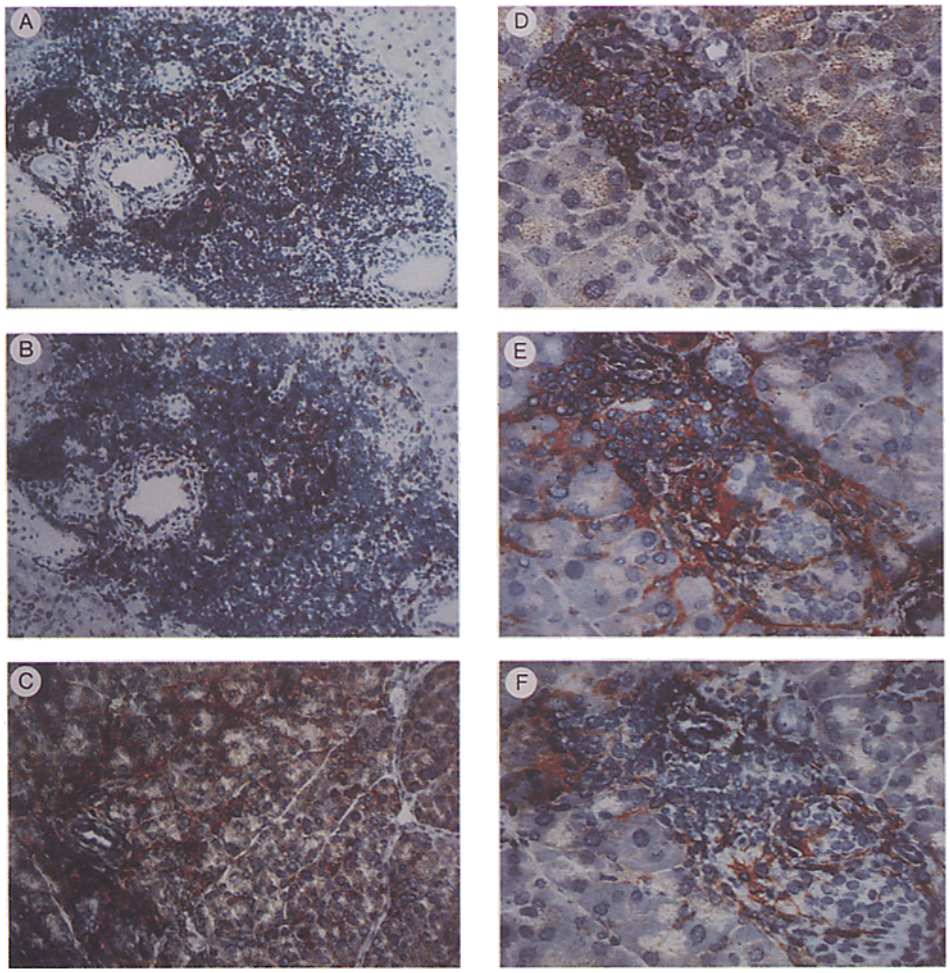


Figure 4. Immunostaining of pancreas-infiltrating cells in NOD mice injected with IL-12. Massive infiltration containing high numbers of CD4$^+$ (*A*), and CD8$^+$ (*B*) T cells is observed in the pancreas from a 10 wk-old NOD female injected 14 times with IL-12 (0.15 μg/day). An infiltrated islet from this mouse shows abundant B220$^+$ (*D*), class II$^+$ (*E*) and N418$^+$ (*F*) cells. N418$^+$ cells are also present in the exocrine pancreas (*C*). *A–C*, ×200; *D–F*, ×400.

Table 1. *Production of IFN-γ and IL-4 by Pancreas-infiltrating CD4+ or CD8+ T Cells from NOD Mice*

	IFN-γ						IL-4			
			Pancreas-infiltrating cells				Pancreas-infiltrating cells			
	Serum		CD4+		CD8+		CD4+		CD8+	
	Day 7	Day 14	–	α-TCR	–	α-TCR	–	α-TCR	–	α-TCR
Vehicle	<5	<5	<15	9,241	<15	9,200	148	2,268	50	107
IL-12	303	125	<15	37,226	<15	17,721	59	471	<15	<15

Pancreas-infiltrating CD4+ or CD8+ cells from 9-wk old NOD mice injected with IL-12 (0.15 μg/mouse/d for 13–14 d) or with vehicle only were stimulated with insolubilized anti-TCR mAb (5 μg/ml). After 48 h of culture, IFN-γ and IL-4 production (pg/ml) were quantified by ELISA. Sera were assayed by ELISA for circulating IFN-γ (pg/ml). Results are means of two to four experiments.

shown). IL-12–treated mice had profoundly reduced numbers of insulin-secreting β cells, whereas glucagon-producing cells were not affected (Fig. 3). These results demonstrate that the diabetes induced by IL-12 administration is of an autoimmune nature, selectively destroying insulin-producing β cells. Surface markers of pancreas-infiltrating cells were analyzed by immunohistochemistry and representative sections from IL-12–injected NOD mice are shown in Fig. 4. Islet infiltrates are dominated by CD4+ and CD8+ T cells and tend to extend into the exocrine pancreas (*A* and *B*). IL-12 administration induces an increase in the number of CD4+ cells, and a marked increase of CD8+ cells (see Materials and Methods). The infiltrates contain class II+ cells (*E*) which include B cells (*D*) and N418+ cells (*F*). The latter cells are abundant also in the exocrine pancreas (*C*). The N418 antibody recognizes CD11c, an integrin expressed on dendritic cells and at a low level on macrophages in normal lymphoid tissues (24). Thus, all the cell types necessary for an immune response are present in the islets of IL-12–treated mice.

To determine whether IL-12 administration induces Th1 cells we tested IFN-γ and IL-4 production by islet-infiltrating T cells after stimulation by insolubilized anti-TCR mAb. Islet-infiltrating CD4+ cells from NOD mice injected with IL-12 produced high amounts of IFN-γ, about fourfold higher than T cells from NOD mice injected with vehicle only (Table 1). Conversely, IL-4 production was decreased in pancreas-infiltrating CD4+ cells from IL-12 treated as compared with control NOD mice. These results indicate that CD4+ cells infiltrating the lesion site in IL-12–injected NOD mice mostly exhibit a Th1 lymphokine profile. Pancreas-infiltrating CD8+ cells, which participate in IDDM induction (25), also produce high levels of IFN-γ and little IL-4, indicating a Th1-like phenotype (26). IFN-γ could also be detected in the serum of NOD mice injected with IL-12 (Table 1). In agreement with previous results, it peaked 1 wk after beginning of IL-12 administration (21).

These results show that administration of IL-12 accelerates the onset of IDDM and induces disease in all NOD female mice tested, whereas only ~60% of control littermates eventually develop IDDM. Acceleration of IDDM is accompanied by expansion of Th1 cells and by selective destruction of islet β cells, suggesting a causal link between IL-12, Th1 cell induction, and development of IDDM. The spontaneous development of IDDM may also involve IL-12–dependent generation of Th1 cells. This possibility will be tested by administration of IL-12 antagonists (27) to NOD mice, a potential therapeutic approach for the treatment of IDDM and of other Th1 cell–mediated autoimmune diseases. The present results also suggest that IL-12, a possible therapeutic agent against tumors (28) and infectious diseases (29), should be administered cautiously to patients with Th1-mediated autoimmune diseases, or with a known predisposition to them.

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References

1. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffmann. 1986. Two types of murine helper T cell clone. I Definition according to profile of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348–2357.
2. Del Prete, G., M. De Carli, C. Mastromauro, R. Biagiotti, D. Macchia, P. Falagiani, M. Ricci, and S. Romagnani. 1991. Purified protein derivative of mycobacterium tuberculosis and excretory-secretory antigen(s) of toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. *J. Clin. Invest.* 88:346–350.
3. Trinchieri, G. 1993. Interleukin-12 and its role in the generation of Th1 cells. *Immunol. Today.* 14:335–338.
4. Paul, W.E., and R.A. Seder. 1994. Lymphocytes responses and cytokines. *Cell.* 76:241–251.
5. Wolf, S.F., P.A. Temple, M. Kobayashi, D. Young, M. Dicig, L. Lowe, R. Dzialo, L. Fitz, D. Ferenz, R.M. Hewick, et al. 1991. Cloning of cDNA for natural killer stimulatory factor, a heterodimeric cytokine with multiple biological effects on T and natural killer cells. *J. Immunol.* 146:3074–3081.
6. Gubler, U., A.O. Chua, D.S. Schoenhaut, C.M. Dwyer, W. McComas, R. Motyka, N. Nabavi, A.G. Wolitzky, P.M. Quinn, P.C. Familletti, and M.K. Gately. 1991. Coexpression of two distinct genes is required to generate secreted, bioactive cytotoxic lymphocyte maturation factor. *Proc. Natl. Acad. Sci. USA.* 88:4143–4147.
7. D'Andrea, A., M. Rengaraju, N.M. Valiante, J. Chehimi, M. Kubin, M. Aste, S.H. Chan, M. Kobayashi, D. Young, E. Nickbarg, et al. 1992. Production of natural killer cell stimulatory factor (interleukin 12) by peripheral blood mononuclear cells. *J. Exp. Med.* 176:1387–1398.
8. Kobayashi, M., L. Fitz, M. Ryan, R.M. Hewick, S.C. Clark, S. Chan, R. Loudon, F. Sherman, B. Perussia, and G. Trinchieri. 1989. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J. Exp. Med.* 170:827–845.
9. Chan, S.H., B. Perussia, J.W. Gupta, M. Kobayashi, M. Pospíšil, H.A. Young, S.F. Wolf, D. Young, S.C. Clark, and G. Trinchieri. 1991. Induction of interferon γ production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J. Exp. Med.* 173:869–879.
10. Debray-Sachs, M., C. Carnaud, C. Boitard, H. Cohen, I. Gresser, P. Bedossa, and J.-F. Bach. 1991. Prevention of diabetes in NOD mice treated with antibody to murine IFN-γ. *J. Autoimmun.* 4:237–248.
11. Campbell, I.L., T.W. Kay, L. Oxbrow, and L.C. Harrison. 1991. Essential role for interferon-gamma and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. *J. Clin. Invest.* 87:739–742.
12. Sarvetnick, N., D. Liggitt, S.L. Pitts, S.E. Hansen, and T.A. Stewart. 1988. Insulin-dependent diabetes mellitus induced in transgenic mice by ectopic expression of class II MHC and interferon-gamma. *Cell.* 52:773–782.
13. Kaufman, D.L., M. Clare-Salzler, J. Tian, T. Forsthuber, G.S.P. Ting, P. Robinson, M.A. Atkinson, E.E. Sercarz, A.J. Tobin, and P.V. Lehmann. 1993. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. *Nature (Lond.).* 366:69–72.
14. Tisch, R., X.-D. Yang, S.M. Singer, R.S. Liblau, L. Fugger, and H.O. McDevitt. 1993. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. *Nature (Lond.).* 366:72–75.
15. Rapoport, M.J., A. Jaramillo, D. Zipris, A.H. Lazarus, D.V. Serreze, E.H. Leiter, P. Cyopick, J.S. Danska, and T.L. Delovitch. 1993. Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. *J. Exp. Med.* 178:87–99.
16. Pennline, K.J., E. Roquegaffney, and M. Monahan. 1994. Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. *Clin. Immunol. Immunopathol.* 71: 169–175.
17. Scott, B., R. Liblau, S. Degermann, L.A. Marconi, L. Ogata, A.J. Caton, H.O. McDevitt, and D. Lo. 1994. A role for non-MHC genetic polymorphism in susceptibility to spontaneous autoimmunity. *Immunity.* 1:1–20.
18. Manetti, R., P. Parronchi, M.G. Giudizi, M.-P. Piccinni, E. Maggi, G. Trinchieri, and S. Romagnani. 1993. Natural killer cell stimulatory factor (interleukin 12 [IL-12]) induces T helper type 1 (Th1)-specific immune responses and inhibits the development of IL-4–producing Th cells. *J. Exp. Med.* 177:1199–1204.
19. Hsieh, C.-S., S.E. Macatonia, C.S. Tripp, S.F. Wolf, A. O'Garra, and K.M. Murphy. 1993. Development of Th1 $CD4^+$ T cells through IL-12 produced by *Listeria*-induced macrophages. *Science (Wash. DC).* 260:547–549.
20. Afonso, L.C.C., T.M. Scharton, L.Q. Vieira, M. Wysocka, G. Trinchieri, and P. Scott. 1994. The adjuvant effect of interleukin-12 in a vaccine against *Leishmania major. Science (Wash. DC).* 263:235.
21. Gately, M.K., R.R. Warrier, S. Honasoge, D.M. Carvajal, D.A. Faherty, S.E. Connaughton, T.D. Anderson, U. Sarmiento, B.R. Hubbard, and M. Murphy. 1994. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-γ in vivo. *Int. Immunol.* 6:157–167.
22. Prat, M., G. Gribaudo, P.M. Comoglio, G. Cavallo, and S. Landolfo. 1984. Monoclonal antibodies against murine γ interferon. *Proc. Natl. Acad. Sci. USA.* 81:4515–4519.
23. Cherwinski, H., J.H. Shumacher, K.D. Brown, and T.R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166: 1229–1244.
24. Crowley, M.T., K. Inaba, M.D. Witmer-Pack, S. Gezelter, and R.M. Steinman. 1990. Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. *J. Immunol. Methods.* 133:55–66.
25. Bendelac, A., C. Carnaud, C. Boitard, and J.-F. Bach. 1987. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both $L3T4^+$ and $Lyt\text{-}2^+$ T cells. *J. Exp. Med.* 166:823–832.
26. Erard, F., M.-T. Wild, J.A. Garcia-Sanz, and G. Le Gros. 1993. Switch of CD8 T cells to noncytolytic $CD8^-CD4^-$ cells that make T_H2 cytokines and help B cells. *Science (Wash. DC).* 260:1802–1805.
27. Mattner, F., S. Fischer, S. Guckes, S. Jin, H. Kaulen, E. Schmitt, E. Rude, and T. Germann. 1993. The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *Eur. J. Immunol.* 23:2202–2208.
28. Brunda, M.J., L. Luistro, R.R. Warrier, R.B. Wright, B.R. Hubbard, M. Murphy, S.F. Wolf, and M.K. Gately. 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med.* 178:1223–1230.
29. Heinzel, F.P., D.S. Schoenhaut, R.M. Rerko, L.E. Rosser, and M.K. Gately. 1993. Recombinant interleukin 12 cures mice infected with *Leishmania major. J. Exp. Med.* 177:1505–1509.