Interleukin-4 acts at the locus of the antigen-presenting dendritic cell to counter-regulate cytotoxic CD8+ T-cell responses

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The mechanism underlying suppression of immune responses by interleukin-4 (IL-4) has remained unexplained. Here we show that the antigen-presenting dendritic cell is central to counter-regulation of autoimmune disease by IL-4. IL-4 acts at the locus of the dendritic cell to decrease the cytolytic T-cell response, preventing autoimmunity. Stimulation of cytotoxic precursors by antigen pulsed dendritic cells induces their differentiation but the process is blocked by IL-4. IL-4-influenced DC produce distinct effects on CD8+ T cells depending on their state of activation. The molecular basis for this regulation is the alteration of the expression ratio of the costimulatory ligands B7.1/B7.2 on dendritic cells. Our findings demonstrate that B7.2 induces expansion of CD8+ T cells and B7.1 governs their acquisition of cytolytic activity. IL-4 influences the dendritic cell to elicit qualitative differences in T-cell responses, providing the basis for counter-regulation mediated by IL-4.

Type I or insulin-dependent diabetes mellitus (IDDM) in humans is a chronic autoimmune disease leading to the destruction of the insulin-producing β-cells in the islets of Langerhans in the pancreas. The cytokine interleukin-4 (IL-4) has a potent inhibitory effect on the autoimmune process[1–3]. The mechanism, however, by which IL-4 ameliorates autoimmunity is not understood. To ascertain the mechanism of protection from autoimmune disease by IL-4, a mouse model of IDDM was used in which diabetes is virally induced. In this model, the CD8+ T-cell response to the target self-antigen (lymphocytic choriomeningitis virus nucleoprotein or LCMV-NP), expressed in the β-cells of the pancreas, is inducible by viral infection and has been well-characterized[4]. In these mice, expression of the nucleoprotein transgene in islet β-cells does not typically lead to development of IDDM. However, upon infection with LCMV a CD8+ T cell (cytotoxic T-lymphocyte or CTL)–mediated response is induced that selectively destroys the β-cells, resulting in the three hallmarks of IDDM: hyperglycemia, hypo-insulinemia and a mononuclear cell infiltrate into the pancreatic islets. In contrast to the NOD mouse, this experimental diabetes model has the distinct advantage of providing a unique, β-cell–specific (auto)antigen to which the CD8+ T-cell response can be monitored.

The generation of antigen-specific T cells that express the CD8 coreceptor (that is, CD8+ T cells) is currently thought to require two signals. The first T-cell signal is received during the engagement of the T cell receptor (TCR) by the peptide/major histocompatibility complex (MHC) on the antigen-presenting cell. The second, costimulatory signal can be provided by the interaction between accessory molecules such as CD28 on the surface of CD8+ T cells and the ligands B7.1 (CD80) and B7.2 (CD86) on antigen-presenting cells (APC)(ref. 5, 6). CTL which express the CD8+ coreceptor recognize antigens that are expressed on target cells in the context of the class I MHC (ref. 7). Ligation of CD28 by B7 molecules provokes the release of IL-2 from antigen-specific CD8+ T cells (precursor CTL), which is necessary for their acquisition of cytolytic function[8,9]. Effective CTL responses often require help from T-helper cells—T cells which express the CD4 coreceptor. However, studies using class II MHC-deficient and CD40L-deficient mice call into question the contribution of CD4+ T-cell help to the development of CD8+ CTLs in acute LCMV infection[10,11].

IL-4 has been reported to have divergent effects on the acquisition of cytolytic function in CD8+ T cells. *In vivo* studies utilizing IL-4–deficient mice and IL-4/recombinant vaccinia virus constructs indicated that IL-4 inhibited CTL activity[12,13] or had no effect[14]. Similarly, effector CTL generation and cytolytic activity *in vitro* was either promoted[15–18] or inhibited[19] by IL-4.

Here we demonstrate that transgenic mice that express IL-4 via the human insulin promoter do not develop diabetes following LCMV infection. Our cellular analysis shows that IL-4 suppresses virus-induced diabetes by inhibiting the generation of diabetogenic CTLs that recognize the immunodominant epitope of LCMV nucleoprotein (NP[118–126]). IL-4 acts at the locus of the dendritic cell to increase B7.2 expression and decrease the expression of B7.1. This, in turn, supports the expansion of antigen-specific CD8+ T cells but inhibits their acquisition of cytolytic function.

Pancreatic islet expression of IL-4 prevents LCMV-induced diabetes

To study the mechanism of inhibition of autoimmune diabetes by IL-4, we crossed RIP-NP mice (which are transgenic BALB/c

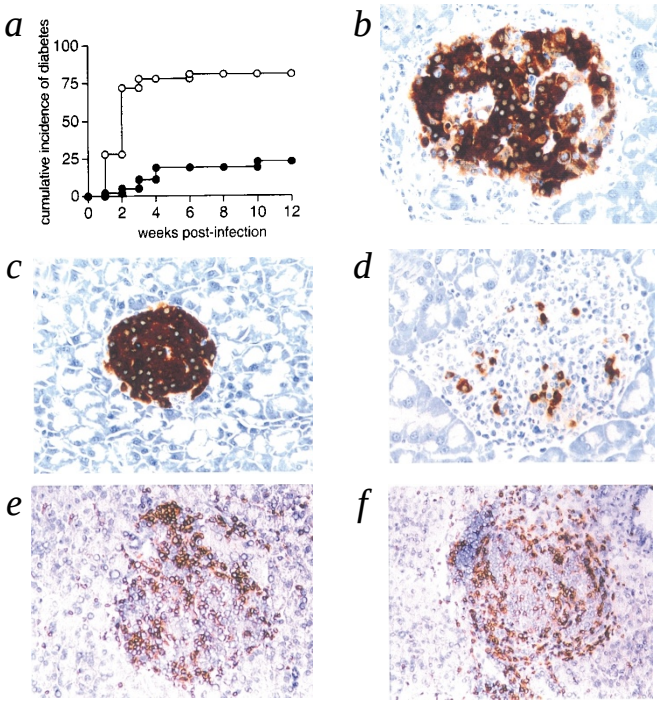

Fig. 1 IL-4 expression prevents IDDM in NP transgenic mice following LCMV infection. ***a***, Islet expression of IL-4 prevents virus-induced diabetes. Cumulative diabetes incidence in NP$^+$/IL-4$^+$ (●) (n = 17), NP$^+$/IL-4$^-$ (○) (n = 21) mice following LCMV challenge. Pancreatic histology at 1 wk and 2 mo following virus challenge. Pancreata were embedded in paraffin and sections were stained with an anti-insulin antibody to reveal the presence of β cells. ***b***, NP$^+$/IL-4$^+$, mouse, 1 wk post-infection (p.i.). ***c***, NP$^+$/IL-4$^+$ mouse, 2 mo p.i. ***d***, NP$^+$/IL-4$^-$ mouse 1 wk p.i., original magnification: ×400. Frozen sections from the pancreas of a representative NP$^+$/IL-4$^+$ mouse ***e***, and NP$^+$/IL-4$^+$ mouse ***f***, at day 5 p.i. were immunostained to reveal the presence of CD8 T cells. Original magnification: ×200.

mice that express LCMV nucleoprotein in pancreatic islets under the control of the rat insulin promoter) with NOD–IL-4 mice (NOD mice that express murine IL-4 in pancreatic islets under the control of the human insulin promoter). At 10 weeks of age, doubly transgenic NP$^+$/IL-4$^+$ offspring and their singly transgenic NP$^+$/IL-4$^-$, singly transgenic NP$^-$/IL-4$^+$ and non-trangenic NP$^-$/IL-4$^-$ littermates were injected with 1×10^5 p.f.u. LCMV, and blood glucose values (BGV) were monitored regularly up to three months after infection (Fig. 1). Mice were scored as diabetic following two consecutive BGV of greater than 300 mg/dL. Uninfected NP$^+$/IL-4$^+$ mice did not develop spontaneous diabetes and exhibited normal glucose metabolism as compared with their singly transgenic and non-trangenic littermates (data not shown).

RIP-NP mice have been reported to developed diabetes within 4 to 8 weeks after LCMV challenge[4]. However, when crossed with NOD mice (NP$^+$/IL-4$^-$ mice [BALB/c × NOD F1]) they developed an accelerated form of the disease as 6 of 21 mice (28.6%) became diabetic within 1 week after virus infection (Fig. 1*a*). By 2 weeks, 71.4% (n = 21) of NP$^+$/IL-4$^-$ mice had become diabetic (Fig. 1*a*). The final cumulative diabetes incidence in NP$^+$/IL-4$^-$ mice was 81.0% (n = 21) at 12 weeks after the infection (Fig. 1*a*). Histological examination revealed that pancreata of both NP$^+$/IL-4$^-$ and NP$^+$/IL-4$^+$ mice were affected with a pronounced inflammation as early as five days following viral infection. In pancreata of NP$^+$/IL-4$^-$ mice, only a few remaining insulin-producing cells could be identified one week after LCMV infection (Fig. 1*d*).

In contrast, most NP$^+$/IL-4$^+$ mice were protected from diabetes (76.5%, n = 17; P = 0.005), their blood glucose values remaining well below 300 mg/dl during the 12 week observation period following the infection (Fig. 1*a*). Indeed, only 23.5% of NP$^+$/IL-4$^+$ mice (n = 17) became hyperglycemic during that time (Fig. 1*a*; P = 0.005). β-cells in pancreata of NP$^+$/IL-4$^+$ mice seemed largely unaffected by the infiltrating cells as indicated by a strong insulin staining both at one week and two months following viral challenge (Fig. 1*b* and *c*, respectively). The islets of both types of mice showed infiltration primarily of CD8$^+$ T cells as early as day five after infection (Fig. 1*e* and *f*).

IL-4 inhibits the acquisition of cytolytic function

The ability of antigen-specific CD8$^+$ T cells, or CTL precursors, to destroy target cells is dependent upon their acquisition of cytolytic function [9]. Islet expression of IL-4 protected against

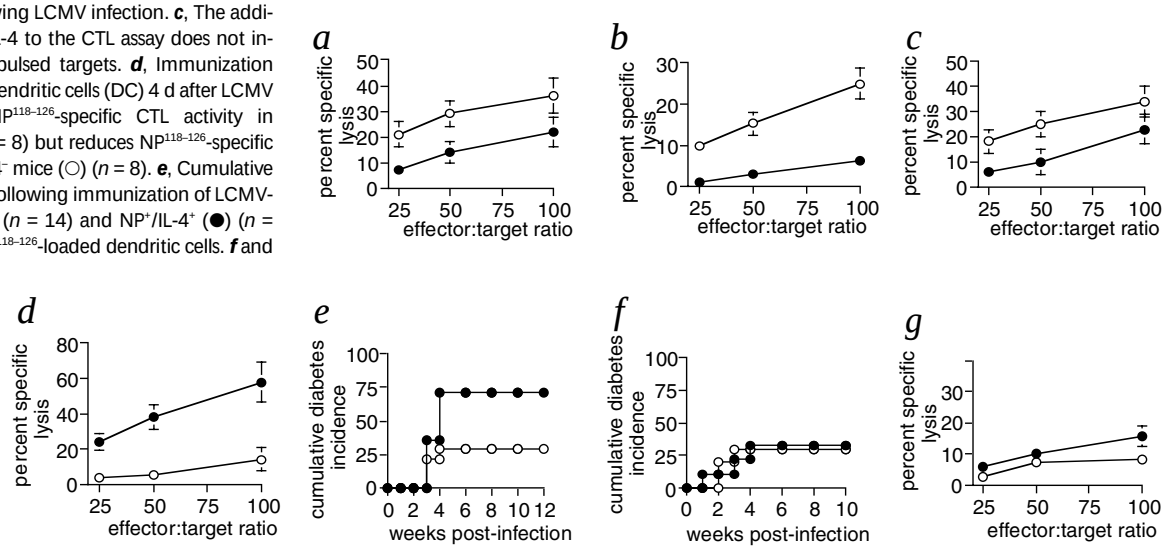

Fig. 2 ***a*** and ***b,*** Islet expression of IL-4 decreases NP$^{118–126}$ CTL activity in (*a*) the spleen and (*b*) the PLN from 10–12-wk-old NP$^+$/IL-4$^-$ (○) (n = 10) and NP$^+$/IL-4$^+$ (●) (n = 10) mice 1 wk following LCMV infection. ***c***, The addition of recombinant IL-4 to the CTL assay does not inhibit lysis of NP$^{118–126}$-pulsed targets. ***d***, Immunization with NP$^{118–126}$-loaded dendritic cells (DC) 4 d after LCMV infection increases NP$^{118–126}$-specific CTL activity in NP$^+$/IL-4$^+$ mice (●) (n = 8) but reduces NP$^{118–126}$-specific CTL activity in NP$^+$/IL-4$^-$ mice (○) (n = 8). ***e***, Cumulative incidence of diabetes following immunization of LCMV-infected NP$^+$/IL-4$^-$ (○) (n = 14) and NP$^+$/IL-4$^+$ (●) (n = 14) recipients with NP$^{118–126}$-loaded dendritic cells. ***f*** and ***g***, Pre-treatment of NP$^{118–126}$-loaded dendritic cells with IL-4 prevents their ability to induce diabetes (*f*) or increase CTL activity in NP$^+$/IL-4$^+$ mice following immunization (*g*; n = 11 for each group).

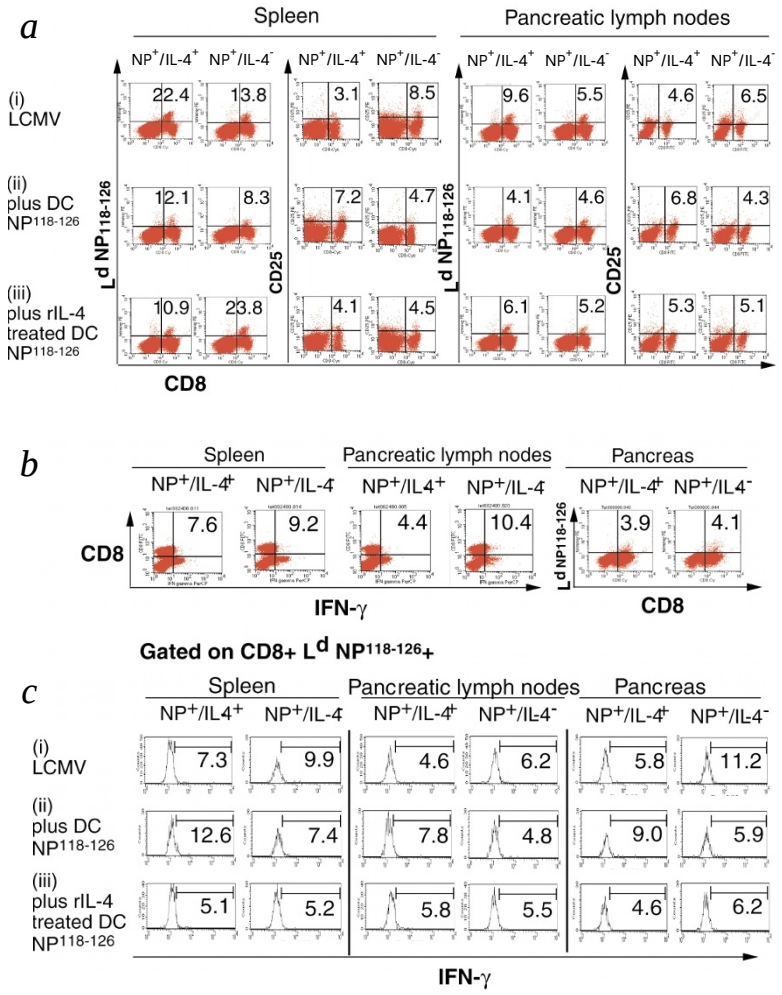

Fig. 3 ***a***, Pancreatic expression of IL-4 increases the percentage of $NP^{118–126}$-specific $CD8^+$ T cells in $NP^+/IL\text{-}4^+$ mice when compared with $NP^+/IL\text{-}4^-$ mice. Flow cytometric analyses of $CD8^+$ T cells from the spleen and PLN of 10–12-wk-old $NP^+/IL\text{-}4^+$ (n = 15) and $NP^+/IL\text{-}4^-$ mice (n = 15) 1 wk after infection with LCMV (top row) 1 wk after LCMV and 3 d after immunization with $NP^{118–126}$-loaded dendritic cells (DC; (middle row) 1 wk after LCMV and 3 d after immunization with IL-4 pre-treated $NP^{118–126}$-loaded DCs (bottom row). Cell suspensions were immunostained with CD8, the tetrameric Ld $NP^{118–126}$ and the activation marker CD25 (IL-2 receptor γ-chain). Representative plots are shown with percentages of Ld $NP^{118–126}/CD8^+$ T cells and percentages of $CD25^+/CD8^+$ T cells from 5 separate experiments with similar results. IL-4 reduces the percentage of IFN-$\gamma^+/CD8^+$ T cells following LCMV infection. ***b***, Intracellular staining for IFN-γ in $CD8^+$ T cells from the spleen and PLN and immunostaining for CD8 T cells recognizing the tetrameric Ld $NP^{118–126}$ in the pancreas. Representative plots are shown with percentages of IFN-$\gamma^+/CD8^+$ T cells from 3 separate experiments with similar results. ***c***, Flow cytometric analyses showing intracellular staining for IFN-γ in $CD8^+$ T cells in the spleen, PLN and pancreas of 10–12-wk-old $NP^+/IL\text{-}4^+$ and $NP^+/IL\text{-}4^-$ mice 1 wk after infection with 1×10^5 p.f.u. LCMV (top row) 1 wk after LCMV infection and 3 d after immunization with $NP^{118–126+}$-loaded DC (middle row) and 1 wk after LCMV infection and 3 days after immunization with IL-4 pre-treated $NP^{118–126}$-loaded DC (bottom row). Histograms show the percentage of Ld $NP^{118–126}/CD8^+$ T cells that immunostained for intracellular IFN-γ. IFN-γ–positive $CD8^+$ T cells were determined by gating against isotype control antibody. Representative plots from three separate experiments with similar results.

LCMV-induced diabetes but it was unclear whether the $CD8^+$ T-cell response toward the target antigen in the islets (NP) was affected. We therefore investigated whether the generation of functional CTL effectors had been affected. We performed CTL assays using splenocytes and pancreatic lymph node (PLN) suspensions from $NP^+/IL\text{-}4^+$ and $NP^+/IL\text{-}4^-$ mice seven days after LCMV infection. Splenocytes from $NP^+/IL\text{-}4^+$ mice showed a reduced ability to lyse class I MHC-matched ($H\text{-}2^d$) target cells pulsed with $NP^{118–126}$ when compared with $NP^+/IL\text{-}4^-$ lymphocytes (P = 0.0075; Fig. 2*a*). The CTL response was even further reduced in the PLN from $NP^+/IL\text{-}4^+$ mice in comparison with the CTL activity in the PLN of $NP^+/IL\text{-}4^-$ mice (Fig. 2*b*).

As it was possible that IL-4 was interfering with the ability of CTL to lyse target cells *in vivo*, we initially investigated whether exogenous recombinant IL-4 would affect CTL activity if added to the five hour CTL assay. The addition of 1 μg/ml recombinant IL-4 did not reduce lysis of $H\text{-}2^d$ targets *in vitro* (Fig. 2*c*).

NP peptide-pulsed DC overturn the IL-4-mediated protection

Our results indicated that IL-4 did not inhibit the effector function of fully differentiated CTL nor did it interfere with the interaction of CTL with their targets *in vitro*; instead, IL-4 inhibited the acquisition of cytolytic function by $CD8^+$ T cells. Because the activation state of antigen-presenting dendritic cells can influence the outcome of autoimmunity[20] and they provide a link between $CD4^+$ T-cell help and the development of effector $CD8^+$ T cells[21–23], they may be involved in mediating IL-4–induced protection from diabetes.

A previous study has shown that anti-viral immunity can be most effectively restored by the administration of antigenic peptides via dendritic cells[24]. Therefore, if IL-4 protects mice from diabetes by its effects on the development of cytolytic capacity in $CD8^+$ T cells, then the suppressive effects of IL-4 might be overturned by providing an exogenous source of dendritic cells that had not been exposed to the NP antigen in the presence of IL-4. To investigate this, we purified dendritic cells from the spleens of uninfected $NP^-/IL\text{-}4^-$ mice, incubated these cells in either the presence or absence of IL-4 and loaded them with $NP^{118–126}$. These cells were injected intravenously into $NP^+/IL\text{-}4^-$ and $NP^+/IL\text{-}4^+$ mice four days after infection with LCMV.

Most (71.4%, n = 14) LCMV-infected $NP^+/IL\text{-}4^+$ recipients of $NP^{118–126}$-loaded dendritic cells developed diabetes within four weeks of infection (Fig. 2*e*). In these mice, transfer of $NP^{118–126}$-loaded dendritic cells also increased CTL activity directed against $NP^{118–126}$-loaded $H\text{-}2^d$ targets (Fig. 2*d*). However, pre-treatment of dendritic cells with IL-4 prevented the ability of those cells to increase CTL activity (Fig. 2*g*) or to overturn suppression of diabetes in $NP^+/IL\text{-}4^+$ mice (30%, n = 10; Fig 2*f*). In contrast, $NP^{118–126}$-pulsed dendritic cells considerably reduced the development of diabetes (28.6%, n = 14) in LCMV-infected $NP^+/IL\text{-}4^-$ mice whether or not they had been pre-treated with IL-4 (30%, n = 10; Fig. 2*e* and *f*). Likewise, the CTL activity was reduced in the

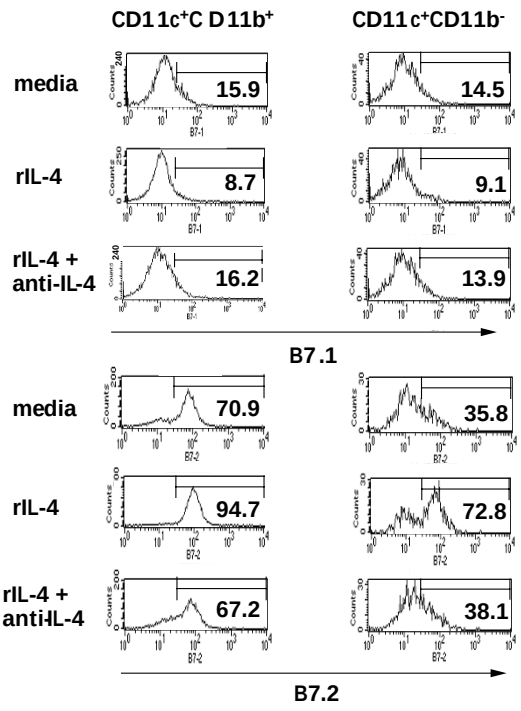

Fig. 4 IL-4 modulates B7 expression on dendritic cells. B7 expression on dendritic cells, enriched from the spleen of non-transgenic mice (n = 9), after overnight incubation in media alone, media plus 0.1 μg/ml rIL-4, media plus 1 μg/ml rIL-4 and 2 μg/ml of anti-IL-4 (11B11). Cells were stained with the APC markers anti-CD11b and anti-CD11c, and either anti-B7.1 or anti-B7.2 monoclonal antibodies and analyzed by flow cytometry. Results are expressed as percentage of B7 positive cells of the $CD11c^+/CD11b^+$ and $CD11c^+/CD11b^-$ populations. The experiment was repeated 3 times with similar results.

$NP^+/IL\text{-}4^-$ mice, following administration of $NP^{118–126}$-loaded dendritic cells, with or without IL-4 treatment (Fig. 2*d* and *g*).

It is unlikely that IL-4 affected dendritic cell function by inhibiting peptide loading as IL-4–treated dendritic cells were not effective whether they were loaded with NP peptide before and during (data not shown) or after incubation with IL-4 (Fig. 2*f* and *g*). In addition, $NP^{118–126}$ or dendritic cells, when administered alone, had little effect on the incidence of LCMV-induced diabetes (data not shown). These results indicate that IL-4 mediated the suppression of viral-induced diabetes by some modulation of the function of antigen-presenting dendritic cells.

IL-4 supports the expansion or survival of $CD8^+$ T cells

Since IL-4 suppressed diabetes development and reduced CTL activity, it is possible that the development of $CD8^+$ T cells specific to the immunodominant epitope of NP were inhibited. Class I epitopes have been classified as dominant or subdominant depending upon the magnitude of the CTL response to the epitope. Previous studies have shown that 90% of the CTL response directed against LCMV in mice with the class I MHC molecule Ld corresponds with amino acid sequence 118–126 from LCMV nucleoprotein[25]. Soluble, tetrameric class I MHC–peptide complexes that bind to the TCR of $CD8^+$ T cells that recognize the epitope $NP^{118–126}$ in the context of Ld have previously been used to quantify the frequency of virus-specific CD8 T cells following LCMV infection[26]. We used Ld-tetramers to determine the percentage of CD8 T cells that recognized $NP^{118–126}$ in both the $NP^+/IL\text{-}4^-$ and $NP^+/IL\text{-}4^+$ mice following infection with LCMV.

Unexpectedly, on day seven after infection, there was a greater fraction of CD8 T cells that recognized $NP^{118–126}$ in the spleen and PLN of protected $NP^+/IL\text{-}4^+$ mice but less activated (CD25) $CD8^+$ T cells than in the diabetes-susceptible $NP^+/IL\text{-}4^-$ mice (Fig. 3*a*, top row). However, the numbers of CD8 T cells that recognized $NP^{118–126}$ in the pancreas were similar in the $NP^+/IL\text{-}4^+$ and $NP^+/IL\text{-}4^-$ mice (Fig. 3*b*). In mice that did not transgenically express NP antigen in the pancreatic islets ($NP^-/IL\text{-}4^+$ mice), IL-4 expression resulted in an increased fraction of CD8 T cells that recognized $NP^{118–126}$ in the PLN (20.3 ± 3.1%, n = 15) when compared with the PLN of $NP^-/IL\text{-}4^-$ mice (3.5 ± 0.4%, n = 15). Pancreatic expression of IL-4 also resulted in an increased number of CD8 T cells specific for $NP^{118–126}$ in the spleens of uninfected $NP^+/IL\text{-}4^+$ mice (5.7%) when compared with $NP^+/IL\text{-}4^-$ mice (1.2%; data not shown).

Following administration of $NP^{118–126}$-loaded dendritic cells, the percentage of $NP^{118–126}$-specific CD8 T cells decreased in the spleen and PLN of both $NP^+/IL\text{-}4^+$ and $NP^+/IL\text{-}4^-$ mice (Fig. 3*a*, middle row). For the $NP^+/IL\text{-}4^-$ mice, there was a reduction in the fraction of CD8 T cells expressing a high density of $NP^{118–126}$-specific TCR (Fig. 3*a*, middle row). When the dendritic cells were pre-treated with IL-4 (Fig. 3*a*, bottom row), the percentage of $NP^{118–126}$-specific CD8 T cells in the spleens of $NP^+/IL\text{-}4^+$ mice was similar to that in recipients of $NP^{118–126}$-loaded, untreated dendritic cells. However, $NP^{118–126}$-loaded dendritic cells pre-treated with IL-4 induced a two-fold increase in the percentage of $NP^{118–126}$-specific CD8 T cells in the spleens of $NP^+/IL\text{-}4^-$ mice (Fig. 3*a*, bottom row) compared with recipients of untreated $NP^{118–126}$-loaded dendritic cells (Fig. 3*a*, middle row). The increased fraction of $NP^{118–126}$-specific CD8 T cells in the spleen and PLN of the mice expressing IL-4 ($NP^+/IL\text{-}4^+$ and $NP^-/IL\text{-}4^+$) suggested that IL-4 was supporting the activation or survival of $NP^{118–126}$-specific CD8 T cells.

A local reduction in antigen-specific CD8 Tc1 cells by IL-4

Previous studies have shown a direct correlation between the percentage of interferon γ (IFN-γ)–producing, antigen-specific CD8 T cells and CTL activity[27]. Since IL-4 is known to cross-regulate the effects of IFN-γ, it is possible that the polarizing influence of islet expression of IL-4 could affect the development of IFN-γ-producing T cells.

Flow cytometric analyses showed that the percentage of IFN-γ–positive CD8 T cells was greater in the $NP^+/IL\text{-}4^-$ mice than in $NP^+/IL\text{-}4^+$ mice (Fig. 4*a*). Moreover, the percentage of IFN-γ–positive CD8 T cells recognizing $NP^{118–126}$ was twice as great in spleen, PLN and pancreas of $NP^+/IL\text{-}4^-$ mice than in $NP^+/IL\text{-}4^+$ mice (Fig. 3*c*, top row). Similarly, $NP^+/IL\text{-}4^-$ mice had greater fractions of IFN-γ–positive $CD4^+$ T cells in the spleen and PLN (data not shown).

Following immunization with $NP^{118–126}$-loaded dendritic cells, LCMV-infected $NP^+/IL\text{-}4^+$ mice showed an increased percentage of $NP^{118–126}$-specific, IFN-γ–producing CD8 T cells in the pancreata and PLN (Fig. 3*c*, middle row). However, pre-treatment of the $NP^{118–126}$-loaded dendritic cells with IL-4 prevented the ability of these dendritic cells to increase the percentage of $NP^{118–126}$-specific, IFN-γ–producing CD8 T cells in $NP^+/IL\text{-}4^+$ mice (Fig. 3*c*, middle row). In contrast, the fraction of IFN-γ-producing CD8 T cells in the $NP^+/IL\text{-}4^-$ mice was reduced following immunization with $NP^{118–126}$-loaded dendritic cells whether or not they had been pre-

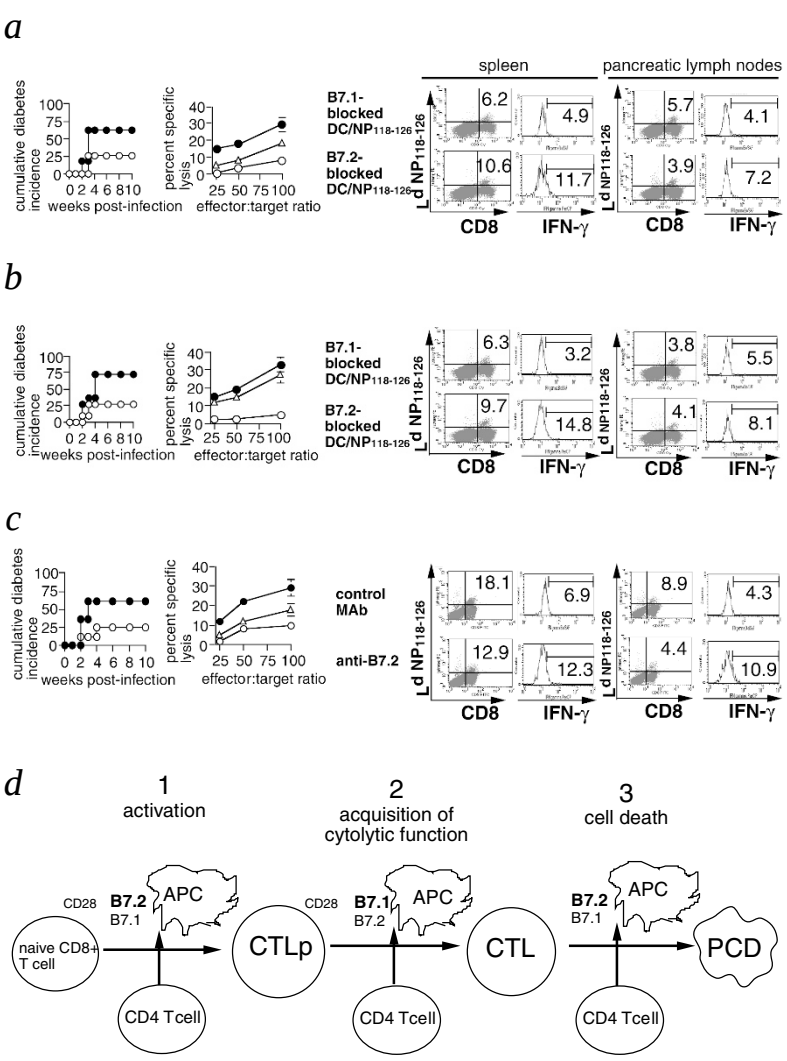

Fig. 5 ***a*** and ***b***, B7.1-blocked $NP^{118–126}$-loaded dendritic cells did not induce diabetes and decreased the percentage of IFN-γ producing $NP^{118–126}$-specific CD8 T cells and CTL activity in 10–12-wk-old (*a*) $NP^+/IL\text{-}4^+$ mice (n = 8) and in (*b*) $NP^+/IL\text{-}4^-$ mice (n = 8). In contrast, B7.2-blocked $NP^{118–126}$-loaded dendritic cells increased diabetes incidence, did not reduce diabetes incidence and increased the percentage of IFN-γ–producing $NP^{118–126}$-specific CD8 T cells and $NP^{118–126}$-specific CTL activity. Similarly, blockade of B7.2 on IL-4 treated $NP^{118–126}$-loaded dendritic cells prevented their ability to suppress CTL activity (n = 8 mice/group). [○, B7.1-blocked DC/$NP^{118-126}$; ●, B7.2-blocked DC/$NP^{118-126}$; △, B7.2-blocked IL-4 treated DC/$NP^{118-126}$.] ***c***, Blockade of B7.2 prevents IL-4–mediated protection from LCMV-induced diabetes. *In vivo* blockade of B7.2 with GL-1 antibody 5 d after LCMV infection induces diabetes in $NP^+/IL\text{-}4^+$ mice (n = 4). This was associated with an increase in CTL activity against $NP^{118–126}$-pulsed $H\text{-}2^d$ target cells and an increase in the percentage of IFN-γ producing $NP^{118–126}$-specific CD8 T cells. Administration of the isotype control rat IgG2a to $NP^+/IL\text{-}4^+$ mice 5 d after LCMV infection had little effect. Blockade of B7.2 with GL-1 antibody before LCMV infection resulted in decreased CTL activity. [●, anti-B7.2 after LCMV; △, isotype control MAb; ○, anti-B7.2 before LCMV.] ***d***, Schematic diagram showing how IL-4 can act at the locus of the APC to modulate $CD8^+$ T-cell–mediated responses. B7 molecules in bold type are the dominant costimulatory molecules for $CD8^+$ T-cell differentiation at each step. IL-4 up-regulates B7.2 expression, and down-regulates B7.1 expression on antigen-presenting dendritic cells, reducing CTL activity and preventing diabetes. IL-4-influenced APC are functional at step 1 and 3 and non-functional at step 2. Anti-B7.2 treatment acts at step 2, resulting in an increase in CTL activity and diabetes incidence. Islet expression of IL-4 acts in place of IL-4-producing Th2 cells to influence APC function.

treated with IL-4 (Fig. 3*c*, bottom row). These findings showed that IL-4 influenced the dendritic cells such that they were unable to induce the acquisition of effector (IFN-γ) function in $CD8^+$ T cells.

IL-4 increases B7.2 and decreases B7.1 expression on DCs

Our results indicated that IL-4 was modulating CD8 T-cell differentiation via its effects on APC function. It is possible that IL-4 was modulating expression of cell surface molecules on APC that are important in CD8 T-cell activation. Optimal CD8 T-cell activation requires TCR recognition of a peptide presented in the context of an MHC class I molecule and additional costimulatory signals provided by the interaction of CD28 on T cells with the B7 molecules on the APC (ref. 5,6).

Immunostaining showed that incubation in the presence of IL-4 increased B7.2 expression and decreased B7.1 expression on the dendritic cells ($CD11c^+$) which were purified from the spleens of uninfected $NP^-/IL\text{-}4^-$ mice before use in our immunization protocol (Fig. 4). These results revealed that IL-4 treatment resulted in both an increased percentage of dendritic cells that expressed B7.2 as well as an increased density of expression of B7.2 (Fig. 4). These results also showed that IL-4 influences dendritic cells by modulating the surface expression of B7 costimulatory molecules in favor of a reduced B7.1/B7.2 ratio.

In addition, we immunostained PLN suspensions from LCMV-infected $NP^+/IL\text{-}4^+$ and $NP^+/IL\text{-}4^-$ mice (n = 7). There was an increased expression of B7.2 on $CD11c^+/CD11b^+$ cells (25%) and $CD11c^+/CD11b^-$ cells (20%) from $NP^+/IL\text{-}4^+$ mice when compared with B7.2 expression on $CD11c^+/CD11b^+$ cells (15%) and $CD11c^+/CD11b^-$ cells (16%) from the PLN of $NP^+/IL\text{-}4^-$ mice (data not shown).

B7.1 supports effector function whereas B7.2 is suppressive

To investigate the molecular mechanisms underlying the ability of $NP^{118–126}$-loaded dendritic cells to induce diabetes in otherwise protected $NP^+/IL\text{-}4^+$ mice, we blocked the costimulatory molecules B7.1 and B7.2 with the monoclonal antibodies 16-10A1 (anti-CD80/B7.1) and GL-1 (anti-CD86/B7.2) on $NP^{118–126}$-loaded dendritic cells prior to transfer.

When B7.2 was blocked on $NP^{118–126}$-loaded dendritic cells before immunization, these cells induced diabetes in otherwise protected LCMV-infected $NP^+/IL\text{-}4^+$ mice (Fig. 5*a*). In addition, administration of B7.2 blocked $NP^{118–126}$-loaded dendritic cells did not change the incidence of disease in the diabetes-susceptible $NP^+/IL\text{-}4^-$ mice (Fig. 5*b*). B7.2-blocked, $NP^{118–126}$-loaded den-

Table 1 Blocking B7.2 prevents the decline in absolute $CD8^+$ T-cell numbers in lymphoid organs from $NP^+/IL\text{-}4^+$ and $NP^+/IL\text{-}4^-$ mice following $DC/NP^{118–126}$ immunization.

	16 h after immunization; Spleen				3 d after immunization; Spleen	PLN	
	$CD8^+$ $CD25^+$	$CD8^+$ Ann-V^+	$CD8^+CD25^+$ Ann-V^+	$CD8^+$T Total ($x10^6$)	$CD8^+$T Total ($x10^6$)	$CD4^+$T Total ($x10^6$)	$CD8^+$T Total ($x10^6$)
$NP^+/IL\text{-}4^+$							
LCMV	2.1	2.5	39.8	8.4	11.6	11.1	4.1
DC/NP118–126	6.5	3.9	47.5	6.8	10.2	15.8	3.3
+ anti-B7.1	3.6	3.2	50.5	6.4	6.2	5.4	0.7
+ anti-B7.2	6.0	5.4	62.5	9.2	12.6	9.4	2.4
IL-4 DC/NP118–126	nd	nd	nd	nd	12.4	13.0	3.6
$NP^+/IL\text{-}4^-$							
LCMV	7.2	3.1	39.4	6.2	13.0	15.4	1.3
DC/NP118–126	15.4	11.5	60.5	5.6	8.4	9.3	1.1
+ anti-B7.1	10.3	8.3	59.3	3.6	5.2	5.4	0.24
+ anti-B7.2	15.11	6.2	34.9	5.2	13.6	12.2	0.68
IL-4 DC/NP118–126	nd	nd	nd	nd	19.2	17.8	1.2

Percentages of activated ($CD25^+$) $CD8^+$ T cells apoptotic (Annexin-V^+) $CD8^+$ T cells and absolute $CD4^+$ and $CD8^+$ T-cell numbers from the spleen, pancreatic lymph nodes and pancreas of $NP^+/IL\text{-}4^+$ ($n = 8$) and $NP^+/IL\text{-}4^-$ mice ($n = 8$) determined by immunostaining and flow cytometry 16 h after immunization (5 d after LCMV infection) and 3 d after immunization (7 d after LCMV infection). The costimulatory molecules B7.1 and B7.2 were blocked on $NP^{118–126}$-loaded dendritic cells prior to transfer with monoclonal antibodies GL-1 and 1G10, respectively. (nd) indicates not done. Results are from a representative flow cytometric analysis that was repeated 4 times with similar results.

dritic cells increased CTL activity as well as the fraction of activated ($CD25^+$) CD8 T cells (Table 1) and $NP^{118–126}$-specific IFN-γ–producing CD8 T cells following immunization in both LCMV-infected $NP^+/IL\text{-}4^+$ or $NP^+/IL\text{-}4^-$ mice (Fig. 5*a* and *b*). Similarly, blockade of B7.2 expressed on $NP^{118–126}$-loaded dendritic cells that had been pre-treated with IL-4 overturned their suppressive ability and enhanced their ability to induce CTL activity (Fig. 5*a* and *b*). This finding indicates that IL-4 mediated its ability to suppress diabetes by increasing B7.2 expression and decreasing B7.1 expression on dendritic cells.

In contrast, when B7.1 was blocked on $NP^{118–126}$-loaded dendritic cells prior to transfer, they failed to increase CTL activity or to induce diabetes in LCMV-infected $NP^+/IL\text{-}4^+$ (Fig. 5*a*) or $NP^+/IL\text{-}4^-$ mice (Fig. 5*b*). Immunostaining of splenocytes 16 hours after administration of B7.1-blocked $NP^{118–126}$-loaded dendritic cells showed a high percentage of apoptotic (Annexin-V^+) CD8 T cells in LCMV-infected mice but less activated CD8 ($CD25^+$) T cells when compared with recipients of B7.2-blocked $NP^{118–126}$-loaded dendritic cells (Table 1). Three days after immunization, there was a reduction in the percentage of $NP^{118–126}$-specific IFN-γ–producing CD8 T cells in those mice that had received B7.1-blocked dendritic cells when compared with mice that had received B7.2-blocked, $NP^{118–126}$-loaded dendritic cells (Fig. 5*a* and *b*). Moreover, blockade of B7.1 expression on $NP^{118–126}$-loaded dendritic cells resulted in a general decline in the total numbers of both CD8 T cells and $CD4^+$ T cells in the spleen and PLN of LCMV-infected mice (Table 1). Pre-blocking $NP^{118–126}$-loaded dendritic cells with isotype control antibodies caused little effect (data not shown). Together, these findings showed that B7.1 expression on $NP^{118–126}$-loaded dendritic cells supported the acquisition of effector and cytolytic function by CTL precursors, reversing IL-4–induced suppression of diabetes.

Blockade of B7.2 prevents IL-4-induced suppression of diabetes

The fraction of dendritic cells that expressed B7.2 was increased in the protected, IL-4–expressing mice and blockade of B7.2 prevented the ability of IL-4–treated dendritic cells to suppress CTL activity. These observations led us to investigate the effects of modulating *in vivo* expression of B7.2 in $NP^+/IL\text{-}4^+$ mice. We used a monoclonal antibody specific for B7.2 (GL-1) to block engagement of the B7.2 molecule in $NP^+/IL\text{-}4^+$ mice *in vivo*.

Blockade of B7.2 overturned IL-4–induced suppression of diabetes in $NP^+/IL\text{-}4^+$ mice but only if we treated these mice after infecting with LCMV (Fig. 5*c*). B7.2 blockade beginning five days after LCMV infection resulted in an increased percentage of $NP^{118–126}$-specific CD8 T cells and increased CTL activity. However, B7.2 blockade starting two days before LCMV infection resulted in decreased CTL activity in $NP^+/IL\text{-}4^+$ mice (Fig. 5*c*). This indicated that B7.2 was required for the early differentiation of CTL precursors but their development into CTL was diminished by B7.2 costimulation (Fig. 5*d*, diagram).

Discussion

The present studies were initiated to investigate the mechanism of IL-4-mediated inhibition of autoimmune diabetes. Our findings show that IL-4 supported the development of antigen-specific CD8 T cells (CTL precursors), which may reflect increased activation or survival of these cells in protected $NP^+/IL\text{-}4^+$ mice. In contrast, IL-4 inhibited the development of effector CTL. This was demonstrated by a decrease in the primary cytotoxic T-cell response, directed toward the immunodominant $NP^{118–126}$-pulsed targets.

The expression of IL-4 in pancreas had a measurable influence on the acquisition of $NP^{118–126}$-specific cytotoxicity as early as day seven post-infection. This was unexpected as presentation of the immunodominant NP epitope occurs systemically following LCMV infection. However, in this accelerated disease model, where these (Balb/c × NOD) mice exhibit early insulitis and diabetes one week following infection, IL-4 in the NP-expressing pancreas most likely plays a prominent role by day seven post-infection. The mechanism(s) explaining this disease acceleration remain unclear but are associated with the NOD genetic background. The specific inhibition of the CTL lysis directed against $NP^{118–126}$-pulsed targets can be explained by exposure of antigen-presenting cells to IL-4 and this notion is supported by the observation that dendritic cells pre-treated with IL-4 did not activate

CTL. An inhibitory effect of IL-4 on the immune system is consistent with previous studies on the ability of IL-4 to downregulate anti-tumor responses[28], anti-viral responses[12,13] and to prevent diabetes[3,19]. However, a molecular mechanism underlying the basis for counter-regulation of autoreactive CD8 T-cell responses by IL-4–influenced antigen-presenting dendritic cells has not been described.

Our findings indicate that IL-4 had modulated dendritic cells so that they were unable to effectively activate NP-specific CTL *in vivo*. CD8 T-cell activation is dependent upon a primary signal delivered through the antigen-specific TCR and costimulatory signals via B7.1 and B7.2 (refs. 5,6). This interaction is important for the acquisition of cytolytic capacity by CD8 cytotoxic T-lymphocyte precursors[29] (CTLp). Here we show that IL-4 inhibited the acquisition of cytolytic capacity in CD8 T cells by decreasing expression of B7.1 and, simultaneously, increasing the expression of B7.2 on dendritic cells. These findings may explain why IL-4 responsiveness in peritoneal macrophages was important for the inhibition of CTL responses *in vitro*[19].

LCMV-infected $NP^+/IL\text{-}4^-$ mice exhibited increased signs of CD8 T-cell activation, but these virally infected mice were rescued from diabetes following $NP^{118–126}$-loaded dendritic-cell administration. Protection from diabetes was mediated by B7.2 expression on the immunizing dendritic cells and associated with a decline in CD8 T-cell numbers, effector function and CTL activity directed against $NP^{118–126}$-loaded target cells. Activated T cells are prone to apoptosis upon restimulation with antigen[30] and high doses of peptide antigen can induce apoptotic death of CTL *in vitro*[31]. In addition, recent studies have shown that repetitive systemic administration of peptide alone can prevent virally-induced diabetes in an analogous transgenic model[32]. It is therefore possible that the transfer of NP-pulsed dendritic cells caused the death of activated CD8 T cells in the spleen of the singly transgenic mice and this resulted in protection from diabetes.

Blockade of B7.2 expression on the $NP^{118–126}$-loaded dendritic cells increased, or supported, CTL development but blockade of B7.1 reduced the percentage of activated CTL and the absolute number of T cells. Whether B7.1-blocked dendritic cells induced activation-induced or passive death of activated T cells is unclear. Infusion of $NP^{118–126}$-loaded dendritic cells into $NP^+/IL\text{-}4^-$ mice also resulted in a decrease in the fraction of activated $CD4^+$ T cells in the spleen. This is unlikely to be due to $CD4^+$ T cells taking up free peptide and serving as CTL targets as $NP^{118–126}$ was loaded onto dendritic cells and excess peptide washed off before transfer. Instead, this finding indicates a bystander or generalized effect resulting in $CD4^+$ T-cell death such as that known to occur via the effects of IL-2 (ref. 33). However, it remains unclear whether the decrease in CTL activity following peptide administration reflected antigen-specific tolerance or some general immunosuppression in the $NP^+/IL\text{-}4^-$ mice.

IL-4–influenced dendritic cells had dual effects on antigen-specific $CD8^+$ T cells depending upon their state of activation. Our data shows that IL-4 supported the expansion of antigen-specific CD8 T cells (CTLp) but simultaneously decreased the fraction of differentiated CTL (IFN-γ–positive CD8 T cells and CTL activity). The fraction of $NP^{118–126}$-specific CD8 T cells in $NP^+/IL\text{-}4^-$ mice increased two-fold after immunization, and this was dependent on IL-4 pre-treatment of the $NP^{118–126}$-loaded dendritic cells. Pancreatic expression of IL-4 also increased the percentage of $NP^{118–126}$-specific CD8 T cells in both infected and uninfected mice, indicating that IL-4 can promote activation of naive CD8 T cells. The effect of IL-4 on CD8 T-cell activation and survival may account for the reports of a positive effect of rIL-4 on IL-2–mediated CTL generation *in vitro*[15–18].

Our results support B7.2 being the major costimulator for T-cell activation[34] and are in turn supported by previous studies showing that naive CD8 T-cell proliferation *in vitro* was upregulated by B7.2/CD28 interactions[35]. IL-4 modulation of B7.2 expression on antigen-presenting cells supported the activation of CD8 T cells but additional effects of IL-4 on T cells may have influenced their survival. In this regard, competitive binding of IL-4 to the common cytokine receptor γ-chain[36] on CD8 T cells can inhibit IL-2–mediated activation induced cell death[37]. However, whether this could also inhibit the acquisition of cytolytic function by CD8 T cells remains unclear.

Protected $NP^+/IL\text{-}4^+$ mice exhibited less IFN-γ–positive, $NP^{118–126}$-specific CD8 T cells in the periphery than in the $NP^+/IL\text{-}4^-$ mice. However, our findings did not support a profound inhibition of IFN-γ–producing T cells by IL-4 but, rather, a local effect of the IL-4 molecule on the development of IFN-γ–producing, $NP^{118–126}$-specific effector CD8 T cells. Our data showed that the ability of $NP^{118–126}$-loaded dendritic cells to mediate the acquisition of effector function and overturn IL-4–induced suppression of diabetes was dependent upon their expression of B7.1. The importance of B7.1 costimulation for the acquisition of cytolytic capacity in CD8 T cells shown here is consistent with previous studies showing a role for B7.1 in CTL responses[38], anti-tumour immunity[39] and the facilitation of diabetes in mice that transgenically express B7.1 in their pancreatic islets[40].

In the $NP^+/IL\text{-}4^+$ mice, their protection by expressed IL-4 was not sufficient to avoid diabetes when peptide-loaded dendritic cells were given without prior IL-4 treatment, that is, when antigen-presenting dendritic cells were already primed (by B7.1 expression) for interaction with CTL precursors. This showed that IL-4 neither masked the antigen-bearing, class I MHC-expressing target cells in the pancreas nor inhibited their lysis by fully differentiated CTL but, instead, influenced CTL generation. These findings are consistent with the long-standing belief that fully differentiated CTL do not proliferate and can act alone when killing target cells[41,42]. Therefore, their cytolytic capacity would not be inhibited by IL-4-influenced antigen-presenting cells in the PLN or pancreas. However, antigen-specific CD8 T cells that require B7.1 costimulation to develop into CTL would be affected by this IL-4 modulation of B7 as would differentiated CTL that may be compelled to interact with NP-loaded B7.2-expressing dendritic cells following immunization. Moreover, the increased fraction of IFN-γ–producing, $NP^{118–126}$-specific CD8 T cells following $NP^{118–126}$-loaded dendritic cell immunization of $NP^+/IL\text{-}4^+$ mice may serve to counter-regulate the effects of IL-4 in the pancreas.

Blockade of B7.2 overturned IL-4–induced suppression of diabetes in $NP^+/IL\text{-}4^+$ mice, but only if we treated these mice with anti-B7.2 after infecting with LCMV. Indeed, B7.2 blockade starting two days before LCMV infection resulted in decreased CTL activity in $NP^+/IL\text{-}4^+$ mice. This result further supports the notion that costimulation by B7.2/CD28 is important in the early differentiation of CD8 CTL precursors but their subsequent acquisition of cytolytic capacity is enhanced by B7.2 blockade, ensuring costimulation via B7.1/CD28 (Fig. 7). This model is also consistent with the kinetics of induced expression of these costimulatory molecules on dendritic cells, as B7.2 is expressed constitutively at low levels and increases with activation before the induced expression of B7.1 (ref. 43).

Central to the prevention of diabetes by IL-4 is its ability to in-

hibit the acquisition of cytolytic function in CTL precursors by modulation of B7 ligand expression on antigen-presenting cells. Our studies invoke a critical qualitative difference provided by B7.1 and B7.2 costimulation depending upon the stage of CD8 T-cell differentiation. These results highlight the potential benefits, as well as the mechanistic complexity, in therapeutic targeting of the B7/CD28 pathway for the treatment of autoimmune disease.

Methods

Transgenic mice. Transgenic NOD–IL-4 mice have been generated in NOD mice using the human insulin promoter and the cDNA sequence of murine IL-4 (ref. 3). This construct led to the expression of IL-4 in the pancreatic β-cells of transgenic mice. RIP-NP mice are transgenic BALB/c mice that express the nucleoprotein of LCMV under the control of the rat insulin promoter (RIP) in their β–cells[4]. To obtain mice, NOD–IL-4 mice were mated with RIP-NP mice. F1 mice were screened for the presence of both transgenes by PCR of tail DNA.

Infection with virus and analysis of IDDM. 10-week-old mice doubly transgenic (NP^+/IL-4^+), their singly transgenic (NP^+/IL-4^- or NP^-/IL-4^+) and non-transgenic (NP^-/IL-4^-) littermates were injected with 1×10^5 p.f.u. of the Armstrong (ARM) strain of LCMV, clone 53b (ref. 44). BGVs were determined weekly following virus infection. Mice with BGV > 300 mg/dL were considered diabetic and used for histology.

Histology and immunocytochemistry. Upon killing of mice, pancreata were fixed in 10% neutral buffered formalin and embedded in paraffin or frozen in OCT in methylbutane (Sigma) on dry ice. Tissue sections were conventionally stained for histological evaluation with H&E, CD8 (Ly2; Pharmingen, San Diego, California) or using guinea pig anti-porcine insulin (DAKO, Carpenteria, California).

CTL assays. CTL activity was determined in a 5 hr *in vitro* [^{51}Cr]-release assay as described[4]. Briefly, H-2^d (Balb/c17) fibroblasts were used as target cells, either alone or loaded with $NP^{118–126}$ (RPQASGVYM). Effectors were in the form of splenocytes, isolated from 10-12-wk-old mice inoculated intraperitoneally with 1×10^5 p.f.u. of LCMV (ARM 53b) 8 days earlier or from unimmunized control mice. Effector to target (E:T) ratios were 100:1, 50:1 and 25:1, respectively.

Purification of dendritic cells for immunization. Dendritic cells were enriched from the spleens of uninfected non-transgenic NOD × Balb/c(F1) mice by a combination of plastic adherence and a Metrizamide gradient. Briefly, splenocytes were depleted of red blood cells by hypotonic lysis and resuspended in $RPMI^+$ 10% FCS at 1×10^7 cells/ml. Cells were incubated on petri dishes for 3 h (37 °C, 5% CO_2). Adherent cells were then further incubated for 16 h in media alone or media containing 1 μg/ml of rIL-4 (Boehringer) with or without the homegrown neutralizing monoclonal antibody against IL-4 (11B11)[45]. Non-adherent cells were collected and spun on a 20 ml Metrizamide (Sigma) gradient for 10 min at 600*g*, and collected at the interface. Purity of cells was assessed by flow cytometric analysis of immunostained populations and viability by trypan blue exclusion. $CD11c^+$ dendritic cells repeatedly constituted about 83 ± 4% of all viable cells recovered. Cells were incubated in protein-free PBS for 4 h prior to loading with an excess of $NP^{118–126}$ peptide (200 μg/ml, that is, 20 μg/1×10^5 dendritic cells in PBS) for 1–2 h at 37 °C, washed once with ample PBS and transferred intravenously (1×10^5 dendritic cells per mouse) 4 d after LCMV infection.

Immunostaining for flow cytometric analysis. 1 wk post-infection with 1×10^5 p.f.u. of LCMV (ARM 53b) and/or 3 d after $NP^{118–126}$-pulsed dendritic cell immunization, mice were killed and spleens and PLN were collected. Aliquots of a single cell suspension (1×10^6 cells) were stained for surface markers using directly conjugated antibodies; anti-CD4, anti-CD8, anti-CD11b (Mac-1), anti-CD11c, anti-CD25 (BD Pharmingen) for 30 min on ice. The tetrameric Ld $NP^{118–126}$ (PE) was prepared as described[26] and used to stain cell suspensions as above. Aliquots of cells were pre-blocked antibody directed against the αβ-TCR (BD Pharmingen) to establish specificity of tetramer staining. For intracellular cytokine staining, aliquots of 1×10^6 cells were stained for intracellular IFN-γ using standard procedures[46]. We used the antibody against IFN-γ (homegrown biotinylated XMG), the isotype control rat IgG1 (Pharmingen) and streptavidin FITC or perCP. Samples were analyzed by flow cytometry.

Monoclonal antibody blocking studies *in vitro*. After peptide loading, dendritic cells were incubated on ice for 30 min in the presence of 20 μg/ml of either purified 1G10 (anti-B7.1), GL-1 (anti-B7.2) or control monoclonal antibody rat IgG2a (BD Pharmingen). Dendritic cells were then washed in cold PBS and 1×10^5 cells in 200 μl of PBS were injected (IV) into each mouse. For *in vivo* blocking of B7.2, anti-B7.2 (GL-1) or control rat IgG2a antibody (100 μg in 200 μl PBS) were injected (IP) either before infection with 1×10^5 p.f.u. of LCMV (ARM 53b; days 4, 2 and 0) or starting 5 d after infection with 1 x 10^5 p.f.u. of LCMV (ARM 53b; days 5, 7 and 9).

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