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Invariant NKT Cells Exacerbate Type 1 Diabetes Induced by CD8 T Cells

Thibault Griseri,* Lucie Beaudoin,* Jan Novak,* Lennart T. Mars,† Françoise Lepault,* Roland Liblau,‡ and Agnès Lehuen2*

Invariant NKT (iNKT) cells have been implicated in the regulation of autoimmune diseases. In several models of type 1 diabetes, increasing the number of iNKT cells prevents the development of diabetes. Because CD8 T cells play a crucial role in the pathogenesis of diabetes, we have investigated the influence of iNKT cells on diabetogenic CD8 T cells. In the present study, type 1 diabetes was induced by the transfer of CD8 T cells specific for the influenza virus hemagglutinin into recipient mice expressing the hemagglutinin Ag specifically in their β pancreatic cells. In contrast to previous reports, high frequency of iNKT cells promoted severe insulitis and exacerbated diabetes. Analysis of diabetogenic CD8 T cells showed that iNKT cells enhance their activation, their expansion, and their differentiation into effector cells producing IFN-γ. This first analysis of the influence of iNKT cells on diabetogenic CD8 T cells reveals that iNKT cells not only fail to regulate but in fact exacerbate the development of diabetes. Thus, iNKT cells can induce opposing effects dependent on the model of type 1 diabetes that is being studied. This prodiabeticogenic capacity of iNKT cells should be taken into consideration when developing therapeutic approaches based on iNKT cell manipulation. The Journal of Immunology, 2005, 175: 2091–2101.

Invariant NKT (iNKT)3 cells are unconventional αβ T cells that are restricted by a nonpolymorphic molecule, CD1d (1, 2). CD1d is a nonclassical MHC class I molecule that presents self and foreign glycolipids (3, 4). iNKT cells and CD1d molecule have been well characterized in mice and humans, and they are quite conserved between both species. iNKT cells express an invariant TCRα-chain, Vα14-Jα18 (Vα14) in mice and Vα24-Jα18 in humans, paired with a limited set of TCRβ-chains (5). iNKT cells have a unique property to promptly secrete, upon TCR triggering, copious amounts of various cytokines such as IL-4 and IFN-γ, thereby contributing to both innate and acquired immunity (1–3, 6). iNKT cells have been involved in the protection against tumors, pathogens (viruses, bacteria, and parasites), as well as in the maintenance of self-tolerance (7). Indeed, many studies have shown that iNKT cells are able to inhibit the development of various autoimmune diseases such as type 1 diabetes (8).

Type 1 diabetes is characterized by an inflammatory infiltrate in the pancreatic islets leading to the specific destruction of insulin-producing β cells. The study of type 1 diabetes has been greatly facilitated by the availability of good animal models such as the NOD mice, which develop spontaneously a disease largely similar to the human ailment (9, 10). A crucial role for T cells has been demonstrated by transfer experiments that revealed the necessity of both CD4 and CD8 T cells in the disease pathogenesis (11, 12). We and other laboratories have shown that other particular T cell populations such as iNKT cells can inhibit the development of type 1 diabetes (8). Increasing iNKT cell numbers by transgenesis or by iNKT cell transfer prevents diabetes onset in NOD mice (13, 14). Protection against diabetes was also obtained by specific iNKT cell stimulation with an exogenous ligand, α-galactosylceramide (α-GalCer) (15, 16).

Analysis of NOD mice protected against diabetes by iNKT cells revealed that Th1 responses against islets were decreased as compared with control NOD mice (16, 17). This protection was associated with a significant reduction in the level of IFN-γ mRNA inside islets, IFN-γ production by autoreactive T cells, and level of IgG2c autoantibodies. Recently, we have set up a transfer system of islet-specific CD4 T cells to determine at a cellular level how iNKT cells interfere with the development of autoreactive pathogenic T cells. Our results showed that iNKT cells impaired the differentiation of anti-islet CD4 T cells into Th1 effector cells, in a cell contact-dependent manner, and that eventually these autoreactive CD4 T cells became anergic (18, 54). Up to now, no data are available on the effect of iNKT cells on diabetogenic CD8 T cells.

Many observations suggest that CD8 T cells play an important role in type 1 diabetes (19). In humans, insulin-dependent diabetes mellitus is associated with certain MHC class I alleles (20) and CD8 T cells are abundant among islet-infiltrating cells at the clinical onset of the disease and in pancreas grafts (21). Studies performed with NOD mice and genetically modified murine models of insulin-dependent diabetes mellitus confirmed the important contribution of CD8 T cells in the development of diabetes. CD8 recognition of MHC class I-self-epitopes on β cells is crucial for the development of insulitis (22–24) and effector CD8 T cells contribute to the progression from insulitis to diabetes (25–27).

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could prevent diabetes onset, and iNKT cells could be a means of blocking disease process.

The aim of this study was to explore the role of iNKT cells on the development of diabetes induced by CD8 T cells. To approach this question, we took advantage of a model of diabetes induction by the transfer of hemagglutinin (HA)-specific CD8 T cells into recipient mice expressing HA under the control of the rat insulin promoter (Ins-HA) (28). Anti-HA CD8 T cells were obtained from clone-4 TCR transgenic mice (CL4-TCR mice) (29). To analyze the role of iNKT cells on diabetogenic CD8 T cells, CL4-TCR T cells were transferred into Ins-HA mice or into Vα14 transgenic Ins-HA mice containing an elevated frequency of iNKT cells. This approach enabled us not only to analyze the role of iNKT cells on the development of diabetes but also to follow the fate of the transferred diabetogenic CD8 T cells in various lymphoid organs of both types of recipient mice. We found that, in contrast to the inhibitory role of iNKT cells on diabetes induced by diabetogenic CD4 T cells, iNKT cells provided help to diabetogenic CD8 T cells, resulting in a higher incidence of diabetes in Vα14 transgenic Ins-HA mice.

Materials and Methods

Mice

The CL4-TCR transgenic mouse line (29) expresses a H-2Kd-restricted TCR (Vα10, Vβ8.2) specific for an influenza virus HA peptide (HASY152-S202, YTVSTVASSL). Ins-HA mice, used as recipient in adoptive transfer experiments, express HA specifically in the pancreatic β cells (28). The CL4-TCR and Ins-HA transgenic mice had been backcrossed >10 times onto the BALB/c background. The Vα14 transgenic mouse line, expressing the Vα14-Jα18 TCRα-chain, corresponds to line A14-86 (14) generated by microinjections directly into NOD eggs. NOD Ly5.2 were generated by >10 backcrosses of the CD45.2 locus from C57BL/6 onto NOD background. All mice used in the present study, CL4-TCR mice, Ins-HA, Vα14 transgenic, and Ins-HA control mice, were on an identical (BALB/c C57BL/6) background. To analyze iNKT cell function in vitro on CL4-TCR CD8 T cell response, these CD8 T cells (5 × 10⁴/well) were incubated with 5 μM α-GalCer (generous gift from Kirin Brewery). To analyze in vivo proliferation, CL4-TCR T cells were incubated for 8 min at 37°C with 5 μM CFSE (Molecular Probes) diluted in PBS-0.5% BSA before injection into recipient mice. Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences).

Intracytoplasmic stainings

Cell suspensions were prepared from popliteal and pancreatic lymph nodes of Ins-HA control or Ins-HA Vα14 recipient mice. Cells were stimulated in vitro at 1 × 10⁶ cells/ml by 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of 10 μg/ml brefeldin A in RPMI 1640, 10% FCS for 4 h at 37°C. The cells were harvested and surface stained for CD8 and Ly5.1, and following fixation and permeabilization, intracytoplasmic staining for IFN-γ (XMG1.2; BD Pharmingen) was performed. The same protocol were used to analyzed cells at the end of in vitro cultures. Staining was also performed for TNF-α (MP6-XT22; BD Pharmingen).

In vitro cultures

To analyze iNKT cell status, cell suspensions were prepared from popliteal and pancreatic lymph nodes. In some experiments, CD8 T cells were depleted before culture. Cells (2 × 10⁶ cells/well) were stimulated in vitro during 48 h with 100 ng/ml α-GalCer. IL-4 and IFN-γ released in the supernatant were measured by ELISA as previously described (14), and proliferation was determined by [3H]thymidine incorporation over the last 4 h.

To analyze iNKT cell function in vitro on CL4-TCR CD8 T cell response, these CD8 T cells (5 × 10⁴/well) were incubated with 5 × 10⁴ APC/well (obtained from the peritoneal cavity of F1 mice and irradiated with 3000 rad) in complete IMDM for 4 days at 37°C. Cells were incubated with 10 U/ml recombinant mouse IL-2 and with or without 40 ng/ml peptide HA152-S202, and iNKT cells (5 × 10⁴/well) were added to some wells at the beginning of the culture period.

Results

Characterization of transferred diabetogenic CD8 T cells and Ins-HA recipient mice

To analyze the effect of iNKT cells on CD8 diabetogenic T cells, HA-specific CD8 T cells were injected into recipient mice expressing HA Ags in β cells. Anti-HA CD8 T cells were obtained from the spleen of CL4-TCR BALB/c × NOD)F1 mice. As shown in Fig. 1A, purified CL4-TCR CD8 T cells expressed high levels of CD62L and CD45RB molecules, a phenotype characteristic of naïve CD8 T cells. Two types of recipient mice were transferred with CL4-TCR T cells: Vα14 and control Ins-HA BALB/c × NOD)F1 mice containing, respectively, an elevated or natural frequency of iNKT cells. Immunofluorescence stainings with CD1d:α-GalCer tetramers showed that control Ins-HA mice contain very few (~0.1%) iNKT cells in popliteal lymph node and ~1% in pancreatic lymph nodes (Fig. 1B). In contrast, in Vα14 Ins-HA mice, iNKT cells represented 10% of popliteal lymph node cells and ~40% of pancreatic lymph node cells. This increase in iNKT cell frequency in popliteal and pancreatic lymph node cells from Vα14 ins-HA mice was associated with their increased proliferation in presence of α-GalCer in vitro (Fig. 1B). One could note that iNKT cells in Vα14 Ins-HA (BALB/c × NOD)F1 mice, were present at a similar frequency as in both Vα14 NOD and Vα14 C57BL/6 mice, and they exhibit similar cytokine profiles in these three types of mice (Fig. 1C). To determine whether Vα14 Ins-HA mice harbor normal CD4⁺CD25⁺ T reg pool, this subset was quantified by immunofluorescence. Both control and Vα14 Ins-HA mice contain similar frequencies and absolute numbers of CD4⁺CD25⁺ T cells (Fig. 1D and data not shown).

iNKT cells exacerbate diabetes induced by CL4-TCR T cells

To induce diabetes in Ins-HA recipient mice, CL4-TCR T cells should be activated before encountering HA Ags expressed by
pancreatic β cells (30–32). In the present study, we set up a new model of CD8-mediated diabetes using an in vivo priming of the pathogenic HA-specific CD8 T cells. Ins-HA recipient mice that received naive CL4-TCR T cells were stained with anti-αβ TCR, anti-CD8, anti-CD45RB, and anti-CD62L mAbs. R. Analysis of iNKT cells from both types of recipient mice. Popliteal and pancreatic lymph node cells from control and Vα14 Ins-HA mice were stained with anti-αβ TCR mAb and CD1d tetramers loaded with α-GalCer. The numbers represent the percentage of double positive iNKT cells. The lower panel represents iNKT cell response of popliteal and pancreatic lymph nodes after in vitro stimulation with α-GalCer during 48 h. Proliferation was measured by [3H]thymidine incorporation. The values represent the means ± SD of the results obtained from three individual mice per group.

FIGURE 1. Characterization of the transferred diabetogenic CL4-TCR T cells and of both types of recipient mice: control and Vα14 Ins-HA mice. A. Dot plots represent the phenotype of purified CL4-TCR T cells. After positive selection of CD8+ T cells, cells were stained with anti-αβ TCR, anti-CD8, and CD45RB mAbs. B. Analysis of iNKT cells from both types of recipient mice. Proliferation after α-GalCer stimulation.
iNKT cells exacerbate diabetes induced by CD8 T cells

To determine whether the enhanced proliferation of CL4-TCR T cells in Vra14 Ins-HA recipient mice, led to an increased number of diabetogenic CD8 T cells, the kinetic of CL4-TCR T cell expansion was analyzed in both types of Ins-HA recipient mice. The percentage and the absolute numbers of CL4-TCR T cells, present in popliteal and pancreatic lymph nodes after HA512–520 peptide immunization, were increased in Vra14 recipient mice as compared with control mice. At the peak of the response, occurring at day 4 in popliteal lymph nodes, CL4-TCR T cells have divided in control and Vra14 Ins-HA recipient mice, as compared with 24% in control recipient mice. A similar increase was observed for CL4-TCR T cell proliferation measured by CFSE dilution: 61 vs 79% of CL4-TCR T cells have proliferated in Vra14 Ins-HA recipient mice, as compared with 50% of the CL4-TCR cells are blasts in Vra14 recipient mice, as compared with 24% in control recipient mice. A similar increase was observed for CL4-TCR T cell activation in response to HA512–520 peptide immunization. T cell activation is usually associated with loss of CD62L expression, blast formation, and cell division. In popliteal lymph nodes, 2 days after immunization with the HA512–520 peptide, 60% of CL4-TCR T cells from control recipient mice have down-regulated CD62L (Fig. 3B). Interestingly, a higher percentage (85%) of CL4-TCR T cells from Vra14 Ins-HA recipient mice have down-regulated CD62L. Analysis of CL4-TCR T cell size, forward light scatter parameter in the dot plots of Fig. 3B, showed that 50% of the CL4-TCR cells are blasts in Vra14 recipient mice, as compared with 24% in control recipient mice. A similar increase was observed for CL4-TCR T cell proliferation measured by CFSE dilution: 61 vs 79% of CL4-TCR T cells have divided in control and Vra14 recipient mice, respectively. The same parameters were analyzed in pancreatic lymph nodes. At day 4 in these lymph nodes, 57 and 74% of CL4-TCR T cells have lost CD62L expression, respectively, in control and Vra14 recipient mice, observed at day 4 in pancreatic lymph nodes, was in line with the data obtained at day 2 in popliteal lymph nodes. Thus, in both lymphoid organs where CL4-TCR T cells were stimulated by the presence of their specific Ag, high numbers of iNKT cells helped the activation and the proliferation of CL4-TCR T cells.
cells were more frequent in Vα14 than in control recipient mice, 2.9 vs 1.2% at day 6. This increased frequency of CL4-TCR T cells in Vα14 recipient mice was associated with increases in absolute numbers of CL4-TCR T cells (Fig. 4A). In both lymphoid organs in which CL4-TCR T cells were activated by either exogenous or endogenous HA Ags, respectively, the presence of a high number of iNKT cells increased the magnitude of diabetogenic CD8 T cell expansion.

**iNKT cells help the differentiation of CL4-TCR T cells**

Because type 1 diabetes is due to the presence of islet-specific Th1/Te1 T cells, it was important to analyze the extent of CL4-TCR T cell differentiation into Te1 effector cells in both types of Ins-HA recipient mice. Te1 differentiation is characterized by IFN-γ production; therefore, intracytoplasmic stainings were performed for this cytokine. As shown in Fig. 5, A and B, at day 6 after immunization, ~60% of CL4-TCR T cells from splenic and pancreatic lymph nodes of control recipient mice produced IFN-γ. In Vα14 recipient mice, the frequency of CL4-TCR T cells producing IFN-γ reached 71 and 82% in popliteal and pancreatic lymph nodes, respectively. The assessment of absolute numbers of IFN-γ+ CL4-TCR T cells in both types of Ins-HA recipient mice further documents the Te1-promoting effect of iNKT cells (Fig. 5B). Indeed, there was a 2.5-fold increase of Te1 CL4-TCR T cells

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**FIGURE 3.** iNKT cells enhance the activation and the proliferation of diabetogenic CL4-TCR T cells. CFSE-labeled Ly5.1+ CL4-TCR T cells (3 × 10⁶) were injected i.v. into control and Vα14 Ins-HA Ly5.1+ mouse. Popliteal and pancreatic lymph node cells from both types of recipient mice were stained with anti-CD8, anti-Ly5.1, and anti-CD62L mAbs. A, Homing before immunization. Analysis of the homing of Ly5.1+ CD8+ CL4-TCR T cells in the two types of recipient mice were performed 24 h after CL4-TCR T cell transfer in absence of immunization. Values correspond to the percentage of Ly5.1+ CD8+ CL4-TCR T cells. B, Analysis of CL4-TCR T cells in popliteal lymph nodes 2 days after immunization. C, Analysis of CL4-TCR T cells in pancreatic lymph nodes 4 days after immunization. B and C, Dot plots are gated on donor Ly5.1+ CD8+ CL4-TCR T cells. Values in the dot plots correspond to the percentage of activated Ly5.1+ CD8+ CL4-TCR T cells (CD62Llow and CFSElow). The graphs represent the mean values ± SD obtained with three individual mice per group and per time point.
enlargement of inflamed popliteal lymph nodes (Fig. 6, A and B). The frequency and number of iNKT cells in the pancreatic lymph nodes of Vα14 recipient mice did not change following immunization. Because several studies have suggested that CD4+ iNKT cells exhibit regulatory function, the frequencies of both iNKT cell subsets, CD4+ and double negative, were determined before and after immunization (Fig. 6A). The ratio between CD4+ and double negative iNKT cells remained stable during CL4-TCR T cell immune response.

Functional analysis of iNKT cells were also performed before and after immunization. As shown in Fig. 6C, iNKT cells present in popliteal lymph nodes at day 4 after immunization were highly responsive as compared with iNKT cells from nonimmunized mice upon restimulation with α-GalCer in vitro. This enhance response is even more marked when normalized for the number of iNKT cells. Day 4 iNKT cells exhibited an increased proliferative capacity (5.5-fold) and IL-4 production (5.7-fold) than day 0 iNKT cells. More strikingly, day 4 iNKT cells produced much larger amount of IFN-γ (77-fold) than day 0 iNKT cells (Fig. 6C). In contrast, the activity status of iNKT cells from pancreatic lymph nodes remained relatively stable between days 0 and 4. These data reveal that iNKT cells present in popliteal lymph nodes, after CL4-TCR T cell transfer and immunization with the HA512–520 peptide, are activated and biased toward a Th1 profile. The massive production of IFN-γ by these iNKT cells might favor the activation and the differentiation of CL4-TCR CD8 T cells into diabetogenic effectors.

Promoting effect of iNKT cells on CL4-TCR T cell response in the absence of immunization

Even though immunization with HA512–520 peptide in the presence of CFA is required to induce diabetes and iNKT cells increased this immune response, it was interesting to analyze the impact of iNKT cells on CL4-TCR T cells response to their endogenous Ags in the absence of immunization. The expansion of CL4-TCR T cells were analyzed between days 4 and 6 in the absence of immunization (Fig. 7A). There was a very limited expansion of CL4-TCR T cells in control Ins-HA mice; these cells represented 0.34% on day 4 and 0.51% on day 6 in pancreatic lymph nodes. However, the presence of a high number of iNKT cells significantly increased CL4-TCR T cell expansion: 0.35% on day 4 and 1.3% on day 6. The production of IFN-γ by CL4-TCR T cells were also analyzed at day 6 after their transfer in the absence of immunization. As shown for their expansion, high frequency of iNKT cells promoted the differentiation of CL4-TCR T cells into Th1 effectors (Fig. 7B). Similar results were obtained in pancreatic and popliteal lymph nodes probably due to the recirculation of CL4-TCR T cells once activated in pancreatic lymph nodes. These data showed that iNKT cells promoted CL4-TCR T cell expansion and differentiation even in the absence of immunization. Although, it is important to note that in the absence of immunization the levels of CL4-TCR T cell response, expansion, and differentiation remained lower than those obtained after immunization (see Figs. 4 and 5 vs Fig. 7). These difference between these data could reflect why immunization is required to induce diabetes.

InNKT cells promote CL4-TCR CD8 T cell response in vitro

To examine the in vitro effect of iNKT cells on CL4-TCR CD8 T cell response, cultures were performed. Addition of HA512–520 peptide induced some IFN-γ and TNF-α production by CL4-TCR CD8 T cells. As shown in Fig. 8, the presence of iNKT cells in the cultures enhanced (4-fold) the production of both cytokines by CL4-TCR T cells. These results showed that iNKT cells exhibit a similar promoting effect on CL4-TCR T cell responses.

**FIGURE 4.** iNKT cells increase the expansion of diabetogenic CL4-TCR T cells. Ly5.1− CL4-TCR T cells (3 × 106) were injected i.v. into control and Vα14 Ins-HA Ly5.1− mice. Cells from popliteal and pancreatic lymph nodes were stained with anti-CD8 and anti-Ly5.1 mAbs. A. Percentage and absolute number of Ly5.1− CD8+ CL4-TCR T cells in popliteal and pancreatic lymph nodes from both types of recipient mice. Results represent the mean values ± SD obtained from three individual mice per group and per time point. These kinetics are representative of two sets of independent experiments. B. Dot plots represent popliteal and pancreatic lymph node cells from both types of recipient mice on days 4 or 6 after immunization. Values correspond to the percentage of Ly5.1− CD8+ CL4-TCR T cells.

In pancreatic lymph nodes of Vα14 recipient mice as compared with control recipient mice. Taken together, these data suggest that iNKT cells promote the generation of diabetogenic CD8 T cells that migrate toward pancreatic islets, leading to exacerbated insulitis and diabetes onset in Vα14 Ins-HA recipient mice.

**iNKT cells accumulated in popliteal lymph nodes produce large amount of IFN-γ**

To better understand the role of iNKT cells on CL4-TCR T cell activation and differentiation, iNKT cells were analyzed in popliteal and pancreatic lymph nodes of Vα14 recipient mice before and after CL4-TCR T cell transfer and immunization with the HA512–520 peptide. iNKT cell frequency was determined by CD1d: α-GalCer tetramer staining (Fig. 6A). Despite a decrease in the frequency of iNKT cells in popliteal lymph nodes between day 0 and day 4 postimmunization, there was a dramatic increase (12-fold) in the absolute number of iNKT cells due to the massive
lymph nodes (34). Moreover, Vaccinia of CFA that promotes a strong local response in popliteal mice, even though this disease is induced by immunization in pres-

Discussion

The present study reveals for the first time that iNKT cells can exacerbate the development of type 1 diabetes. Increased diabetes incidence was observed either by increasing iNKT cell frequency or by specifically stimulating them with α-GalCer. In the present model, diabetes is induced by anti-HA CD8 CL4-TCR T cells that are primed in popliteal lymph nodes in the presence of their specific Ag, HA_{512-520} peptide, and CFA. The comparison of Vα14 transgenic mice to control mice shows that the prodiabetogenic effect of iNKT cells is observed at several levels. In popliteal lymph nodes, diabetogenic CL4-TCR T cells exhibit increased activation, proliferation, and differentiation into Tc1 effector cells producing IFN-γ. All these parameters of CL4-TCR T cell activation were also increased in pancreatic lymph nodes of Vα14 transgenic mice. This enhanced activation of CL4-TCR T cells eventually led to accelerated and more severe insulitis and was reflected by an accelerated development of diabetes and a higher incidence of diabetes.

The prodiabetogenic effect of iNKT cells in this model of type 1 diabetes is in contrast to previous results showing that iNKT cells can protect NOD mice from the development of diabetes (13–16). Several parameters could explain the different role of iNKT cells on the various models of type 1 diabetes. In the present study, diabetes is induced by cell transfer and immunization with a specific peptide in presence of CFA, whereas the other models were spontaneous, in NOD mice, or induced by cell transfer of diabetogenic BDC2.5 CD4 T cells without further exogenous stimulation (13–16, 18). However, one should note that the development of another autoimmune disease, experimental autoimmune encephalomyelitis (EAE), is prevented in Vα14 transgenic NOD mice, even though this disease is induced by immunization in presence of CFA that promotes a strong local response in popliteal lymph nodes (34). Moreover, Vα14 NOD mice transferred with BDC2.5 CD4 T cells are still protected against diabetes despite additional immunization with a specific peptide in the presence of CFA (A. Lehuen, unpublished observations). It is important to note that s.c. injection of CFA had a similar effect on iNKT cells in both genetic backgrounds, NOD and (BALB/c × NOD)F1, in particular in regards to IFN-γ production by iNKT cells (data not shown). Interestingly, iNKT cells also promoted CL4-TCR T cell response in vivo in nonimmunized Ins-HA mice as well as in vitro culture. Taken together, these data suggest that immunization in the presence of CFA cannot by itself explain the discrepancy on the pro-

Not only the lineage but also the characteristic of each diabe-
togenic T cell clone might influence its susceptibility to be regu-
lated by iNKT cells. The HA-specific CD8 T cells studied in this work exhibit a high avidity for the HA peptide, as illustrated by the requirement of only low peptide concentration (≤1 nM) to induce half-maximal proliferation of naïve CL4-TCR T cells (32). In addition, early activation and full differentiation of naïve CL4-TCR T cells do not require the engagement of costimulatory molecules (37). This strong avidity of CL4-TCR T cells for HA Ags is possibly the consequence of a high-affinity TCR because monomeric K^d-HA_{512-520} complexes were able to bind and to trigger the CL4-

FIGURE 5. iNKT cells help the differentiation of diabetogenic CL4-TCR T cells into IFN-γ-producing cells. Ly5.1^-CL4-TCR T cells (3 × 10^6) were injected i.v. into control and Vα14 Ins-HA Ly5.1^-mice. Six days after immunization, popliteal and pancreatic lymph node cells from both types of recipient mice were stimulated with PMA plus ionomycin, and then surface and intracytoplasmic stainings were performed. Cells were stained with anti-CD8, anti-Ly5.1, and anti-IFN-γ mAbs. A. Dot plots were gated on donor Ly5.1^-CD8^- CL4-TCR T cells. Values in the dot plots correspond to the percentage of CL4-TCR T cells producing IFN-γ. B. Mean ± SD values obtained from three individual mice. Similar results were obtained in two independent experiments.
injection and myelin immunization were performed simulta-
neously in B10.PL mice (39). Interestingly, exacerbation of EAE
by iNKT cells was associated with an increased Th1 response
against self-Ags (39). This is reminiscent to what is observed in the
present study in which iNKT cells favor CL4-TCR T cell differ-
entiation into pathogenic Tc1 effectors.
The regulation by iNKT cells of CD8 T cell responses in autoimmunity had not been investigated before; however, several reports have already described the influence of iNKT cells on other immune responses mediated by CD8 T cells. In several tumor models, such as 15-12RM fibrosarcoma, 4T1 breast carcinoma, and CT26 colon carcinoma, iNKT cells inhibit immunosurveillance mediated by antitumor CD8 T cells (41, 42). In contrast, iNKT cells promote CD8 T cell responses against MBL-2 T lymphoma (43). Similarly, iNKT cells strongly enhance malaria-specific CD8 T cell responses induced by malaria vaccines (44). In another study, priming of CD8 T cells in vivo with peptide-loaded dendritic cells was more efficient when iNKT cells were simultaneously stimulated by α-GalCer (45). In these previous studies, the enhancement of CD8 responses by iNKT cells was associated with stronger Tc1 responses, whereas the inhibition of antitumor CD8 response was associated with Th2 cytokine production by iNKT cells. The present study reveals a Tc1-promoting effect of iNKT cells in type 1 diabetes similarly as already described in these other immune responses in which iNKT cells promote CD8 T cell responses.

Increased Tc1 differentiation of diabetogenic CL4-TCR T cells, after immunization, might be due to the accumulation of iNKT cells into popliteal lymph nodes, with these iNKT cells producing very large amounts of IFN-γ. This proinflammatory effect of iNKT cells is comparable to what has been described in several infectious models such as injection of Salmonella typhimurium and mycobacterial glycolipids (46, 47). Activated iNKT cells probably provide important T cell help to CL4-TCR T cells during their priming in popliteal lymph nodes. This iNKT cell help might potentiate secondary CD8 T cell stimulation in pancreatic lymph nodes and inside pancreatic islets in a fashion similar to what has been demonstrated for the help provided by conventional CD4 T cells (48–50). iNKT cells present in pancreatic lymph nodes might not be able to control these strongly stimulated diabetogenic CD8 T cells. This is reminiscent of the observation that iNKT cells were not able to prevent type 1 diabetes development induced by already primed Th1 BDC2.5 CD4 T cells, in contrast to the protective role of iNKT cells when naive BDC2.5 T cells were injected (18).

The present study using the Ins-HA model might be relevant to situations in which type 1 diabetes would be induced by epitope mimicry. Indeed, it has been suggested that type 1 diabetes could be triggered by viral infection, such as coxsackie virus containing an epitope shared by the glutamic acid decarboxylase islet autoantigen (51). It would be interesting to determine the influence of iNKT cells in other models of type 1 diabetes induced by β cell islet-specific CD8 T cells. Two models could be analyzed, either

![FIGURE 7. iNKT cells promote CL4-TCR T cell response in absence of immunization. Ly5.1<sup>−</sup> CL4-TCR T cells (3 × 10<sup>6</sup>) were injected i.v. into control and V<sup>α14</sup> Ins-HA Ly5.1<sup>−</sup> mice. Cells from popliteal and pancreatic lymph nodes were stained with anti-CD8 and anti-Ly5.1 mAbs. A, Expansion of CL4-TCR T cells between days 4 and 6. Values in the dot plots correspond to the percentages of Ly5.1<sup>−</sup> CD8<sup>+</sup> CL4-TCR T cells. B, IFN-γ production at day 6 after transfer. Popliteal and pancreatic lymph node cells were stimulated with PMA plus ionomycin, and then surface and intracytoplasmic stainings were performed. Dot plots were gated on donor Ly5.1<sup>−</sup> CD8<sup>+</sup> CL4-TCR T cells. Values in the dot plots correspond to the percentage of CL4-TCR T cells producing IFN-γ. Similar results were obtained with three individual mice.

![FIGURE 8. iNKT cells promote CL4-TCR T cell response in vitro. Naïve Ly5.1<sup>−</sup> CL4-TCR T cells were cultured in the presence of APC, rIL-2, and the HA<sub>512-520</sub> peptide, and iNKT cells were added to some wells as indicated. IFN-γ and TNF-α production was measured by intracytoplasmic staining after 4 days of culture.](http://www.jimmunol.org/)

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diabetes induced by LCMV infection of mice expressing the nucleoprotein of lymphocytic choriomeningitis virus in pancreatic β cells (52) or diabetes induced by the transfer of 8.3 diabetogenic CD8 T cells (53). According to the strength of diabetes induction, iNKT cells could mediate opposite effects, protective or exacerbating. Therefore, further studies should be performed before considering iNKT cell-based therapy to prevent type 1 diabetes.

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Disclosures

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References