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3 **NOD mice contain an elevated frequency of iNKT17 cells: implications in type 1**
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6 **diabetes**

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32 **Short running title:** iNKT17 cells exacerbate type 1 diabetes

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51 **Non standard abbreviations:** α -GalCer, α -galactosylceramide, CIA, collagen induced
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54 arthritis, ROR γ t, Retinoic acid receptor-related orphan receptor γ t.

Abstract

Invariant natural killer T (iNKT) cells are a distinct lineage of innate-like T lymphocytes and converging studies in mouse models have demonstrated the protective role of iNKT cells in the development of type 1 diabetes. Recently, a new subset of iNKT cells, producing high levels of the pro-inflammatory cytokine IL-17, has been identified (iNKT17 cells). Since this cytokine has been implicated in several autoimmune diseases, we have analyzed iNKT17 cell frequency, absolute number and phenotypes in the pancreas and lymphoid organs in non-obese diabetic (NOD) mice. The role of iNKT17 cells in the development of diabetes was investigated using transfer experiments. NOD mice exhibit a higher frequency and absolute number of iNKT17 cells in the lymphoid organs as compared to C57BL/6 mice. iNKT17 cells infiltrate the pancreas of NOD mice where they express IL-17 mRNA. Contrary to the protective role of CD4⁺ iNKT cells, CD4⁻ iNKT cells containing iNKT17 cells enhance the incidence of diabetes. Treatment with blocking a anti-IL-17 antibody prevents the exacerbation of the disease. This study reveals that different iNKT cell subsets play distinct roles in the regulation of type 1 diabetes. iNKT17 cells that are abundant in NOD mice, exacerbate diabetes development.

Introduction

Invariant natural killer T (iNKT) cells represent a distinct lineage of T cells that co-express a highly conserved $\alpha\beta$ T cell receptor (TCR) along with typical surface receptors for natural killer cells. The invariant TCR α chain of iNKT cells is encoded by V α 24-J α 18 gene-segments in humans and V α 14-J α 18 gene-segments in mice. The TCR β chain is also strongly biased, encoded by V β 11 gene-segment in humans and V β 8.2, V β 7 and V β 2 gene-segments in mice. These lymphocytes recognize both self and microbial glycolipid antigens presented by the non-classical class I molecule CD1d. iNKT cells are characterized by their capacity to produce rapidly large amounts of both Th1 (IFN- γ , TNF- α) and Th2 (IL-4, IL-13) cytokines, which enables them to exert beneficial, as well as deleterious, effects in a variety of inflammatory or autoimmune diseases [1, 2].

Converging studies in mouse models suggest that iNKT cells can prevent the development of type 1 diabetes [3]. iNKT cells are reduced in number in diabetes-prone NOD mice [4, 5], and increasing the number of iNKT cells by adoptive transfer [6, 7] or via the introduction of a V α 14-J α 18 transgene, reduces significantly the progression of the disease [6]. A similar protection was observed after specific iNKT cell stimulation with exogenous ligands, α -galactosylceramide (α -GalCer) and its analogues [8-11]. Early reports suggested that iNKT cell protection was associated with the induction of a Th2 response to islet auto-antigens [8, 10-12]. However, following studies using the transfer of anti-islet T cells showed that iNKT cells inhibit the differentiation of these auto-reactive T cells into effector cells during their priming in pancreatic lymph nodes (PLN) [13, 14]. This regulatory role of iNKT cells could be explained by their ability to promote the recruitment of tolerogenic dendritic cells [14, 15].

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3 It is now well established that iNKT cells can be divided into several subpopulations using
4 various cell surface markers, these subsets exhibiting diverse functions. According to the
5 expression of the CD4 molecule, human iNKT cells have been shown to express a Th1 or Th0
6 cytokine profile [16, 17]. In the mouse, CD4⁻ iNKT cells are more potent to promote tumor
7 rejection [18]. Recently, a new population of CD4⁻ NK1.1⁻ iNKT cells producing high levels
8 of the pro-inflammatory cytokine IL-17 together with low IL-4 and IFN- γ levels in response
9 to several iNKT cell ligands, has been identified and named iNKT17 cells [19]. Consistent
10 with their ability to produce IL-17 rapidly and independently of IL-6, iNKT17 cells, unlike
11 naive T cells, were found to express constitutively IL-23R and ROR γ t [20-22].

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Much of the focus on IL-17 secreting cells has been on their role in promoting organ-specific
autoimmunity and chronic inflammatory conditions [23]. In the past few years, results have
suggested that it was not IL-12 and Th1 cells that are required for the induction of
experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) but
rather IL-23 and Th17. EAE can be induced by the transfer of IL-17 producing autoreactive T
cells and IL-17 deficient mice had reduced susceptibility to CIA and EAE. Unregulated Th17
responses or overwhelming IL-17 production from T cells and other sources is also associated
with chronic inflammation in rheumatoid arthritis patients [23].

Recent studies suggest that IL-17 might also be involved in the development of type 1
diabetes. Transfer of in vitro polarized BDC2.5 Th17 cells into NOD SCID mice induced
diabetes in recipient mice with similar rates of onset as transfer of Th1 cells [24-26].
However, the exact role of IL-17 in the pathogenesis of type 1 diabetes remains unclear as the
neutralization of IL-17 inhibited the disease transfer in one of the studies but not in two
others. Treatment with an anti-IL-17 monoclonal antibody (mAb) protected NOD mice
against diabetes only when performed at late stage of disease development [27]. Although it is
clear that Th17 cells play an important role in some autoimmune disease models, their precise

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3 role in diabetes remains to be elucidated. All these observations on the role of IL-17 and
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5 iNKT cells in autoimmune diseases led us to characterize iNKT17 cells in the NOD mouse
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8 and to investigate whether these cells play a pathogenic role in diabetes.
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16 Results

19 **Enhanced iNKT17 cell population in NOD mice compared to C57BL/6 mice**

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21 To investigate the role of iNKT17 cells in type 1 diabetes, we have compared the frequency
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23 and absolute number of these cells in NOD and C57BL/6 mice. C57BL/6 mice were used as
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25 control mice, since they develop neither diabetes nor other autoimmune pathologies. iNKT17
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27 cells were analyzed in the thymus, spleen, inguinal LN (ILN) and PLN. ILN were used as
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29 control tissue since they are enriched in iNKT17 cells [28]. IL-17 production by iNKT cells
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31 was detected after CD1d- α GalCer tetramer staining and stimulation with phorbolmyristyl
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33 acetate (PMA) and ionomycin (Fig. 1A). As previously shown in C57BL/6 mice, iNKT17
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35 cells do not express the NK1.1 marker. These cells are also NK1.1⁺ in NK1.1 congenic NOD
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37 mice used for this analysis (Fig. 1B). Interestingly, iNKT17 cell frequency was four to six
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39 fold increased in NOD mice as compared to C57BL/6 mice (Fig. 1B and C). This difference
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41 was also observed in terms of absolute number (Fig. 1D). Of note, in PLN of NOD mice,
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43 iNKT17 cells represent 13% of total iNKT cells compared to only 2% in C57BL/6 mice. The
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45 high frequency and absolute number in PLN of NOD mice suggest that iNKT17 cells could
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47 play a role in the development of type 1 diabetes.
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57 **Enhanced expression of IL-17 associated genes by thymic iNKT cells from NOD mice**

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3 Previous studies have shown that unlike Th17 cells, iNKT17 cells are generated during
4 thymic differentiation [19]. iNKT cell maturation can be divided in three differentiation
5 stages; stage 1 (CD44⁻ NK1.1⁻), stage 2 (CD44⁺ NK1.1⁻ CD4⁻ or CD4⁺) and stage 3 (CD44⁺
6 NK1.1⁺). We have analyzed the expression of genes usually associated with the iNKT17
7 lineage in thymic iNKT cells. Quantitative-PCR data show that *il-17a* gene is mainly
8 transcribed in stage 2 CD4⁻ iNKT cells and to a lesser extent in stage 1 and stage 2 CD4⁺
9 iNKT cells (Fig. 1D). In agreement with our results obtained by intracellular IL-17 staining,
10 IL-17A mRNA level is increased (10-fold) in stage 2 CD4⁻ iNKT cells from NOD as
11 compared to C57BL/6 mice. Analysis of mRNA encoding ROR γ t, which is required for
12 iNKT17 cell differentiation [21], revealed its high expression in the stage 2 CD4⁻ iNKT cells
13 and 3-fold increased in NOD mice. IL-23R is constitutively expressed by iNKT17 cells [20],
14 and its expression is high in stage 2 CD4⁻ iNKT cells however there is not significant
15 difference between NOD and C57BL/6 mice. Interestingly, stage 2 CD4⁻ iNKT cells also
16 expressed IL-22 mRNA and this expression is 4-fold higher in cells from NOD mice. These
17 data showing a higher transcription of *il-17a*, *roryt* and *il-22* genes in iNKT cells from NOD
18 mice strengthen the differences in iNKT cells between this autoimmune strain and C57BL/6
19 mice.
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46 **iNKT17 cells infiltrate the pancreas of NOD mice**

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48 To determine whether iNKT17 cells infiltrate the pancreas of NOD mice, we have analyzed
49 pancreatic infiltrates from NOD and V α 14 NOD transgenic mice that express iNKT cell
50 characteristic TCR α chain and exhibit a 10 fold increased frequency and number of iNKT
51 cells in lymphoid tissues [6] as well as in the pancreas [29]. iNKT17 cells represent 6% of all
52 iNKT cells infiltrating the pancreas in NOD and V α 14 NOD mice (Fig. 2A). We next
53 assessed whether this frequency varies at different stages of insulinitis. At 6 weeks of age NOD
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3 mice have a small infiltrate of hematopoietic cells, at 12 weeks peri-insulinitis is more abundant
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5 and at 20 weeks many pancreatic islets are characterized by a destructive insulinitis leading to
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7 diabetes onset [30]. Indeed, we observed an increased frequency of pancreatic infiltrating
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9 hematopoietic (CD45⁺) cells with aging (Fig. 2B). Even though, iNKT17 cell frequency
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11 among iNKT cells as well as iNKT cell frequency among CD45⁺ cells infiltrating pancreas
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13 remained stable (Fig. 2B), the number of iNKT17 cells increased with the enhanced
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15 infiltration of pancreas, meaning that they could participate in the destruction of islet cells.
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17 CCR6 and CD103 integrin expression has been described on iNKT17 cells [28] and CCR6
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19 has been involved in the recruitment of pathogenic Th17 cells in CIA [23]. All iNKT17 cells
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21 from ILN are CD103⁺ and the level of CD103 expression is higher in iNKT17 cells of NOD
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23 mice as compared to C57BL/6 mice (Fig. S1). iNKT17 cells from ILN are mainly CCR6⁺,
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25 whereas in PLN and spleen only a fraction of iNKT17 cells express CCR6 and CD103 (Fig.
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27 S1). The analysis of CCR6 and CD103 expression on pancreatic iNKT17 cells showed that,
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29 while 60% of iNKT17 cells expressed CD103 integrin, most of them were negative for CCR6
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31 (Fig. 3C). These data suggest that iNKT17 cell recruitment in the pancreas is independent of
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33 CCR6, whereas CD103 could play a role in the retention of these cells.
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43 **Pancreatic iNKT17 cells express IL-17A mRNA in the absence of exogenous stimulation.**

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45 To determine whether iNKT17 cells express IL-17A mRNA in the absence of exogenous
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47 stimulation such as PMA and ionomycin, iNKT cells were purified from the pancreas, PLN
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49 and ILN from V α 14 NOD mice. Expression of other genes usually associated with iNKT17
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51 cells were also assessed by quantitative-PCR (Fig. 3D). IL-21 and IL-22 mRNA were barely
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53 detectable in the three organs analyzed. Interestingly, *il-17a* gene was expressed at much
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55 higher level in pancreatic iNKT cells than in iNKT cells from PLN and ILN (6 and 13-fold
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57 increased respectively). A similar trend was observed for *il-17f* gene. In contrast, *roryt* and *il-*
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3 *23r* gene expression was not significantly different in iNKT cells from pancreas and ILN.
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5 These data show that even though iNKT17 cells are present in these three tissues, they are
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7 expressing IL-17A mRNA only in the pancreas.
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10 Since previous studies have shown that iNKT17 cells can secrete IL-17 through TCR
11 engagement [20], we investigated whether CD1d was required for IL-17A mRNA expression
12 by iNKT17 cells in the pancreas (Fig. 3E). To address this question, we used V α 14 NOD
13 mice expressing CD1d solely in the thymus (CD1d^{pLck} V α 14 NOD mice) [31]. ROR γ t, IL-23R
14 and IFN- γ mRNA expression was similar in pancreatic iNKT cells from both types of mice.
15 However, IL-17A mRNA expression was significantly decreased (3-fold) in iNKT cells from
16 mice lacking peripheral CD1d expression. Altogether, our data suggest that iNKT17 cells are
17 activated locally in the pancreas in a CD1d-dependent manner.
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32 **CD4⁻ iNKT cells containing iNKT17 cells enhance the incidence of diabetes.**

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34 To evaluate the role of iNKT17 cells in type 1 diabetes, we reconstituted immunodeficient
35 NOD mice with different iNKT cell subsets and analyzed the induction of diabetes after
36 transfer of anti-islet BDC2.5 T cells [32]. Since there is no specific antibody available to
37 purify iNKT17 cells, we first determine the frequency of iNKT17 cells in different iNKT cell
38 subpopulations divided according to CD4 and NK1.1 expression of donor cells. As shown in
39 Fig. 3A and Fig. S2, iNKT17 cells are mainly present in the CD4⁻ iNKT cell population and at
40 a higher frequency among NK1.1⁻ CD4⁻ iNKT cells. Therefore, we enriched iNKT17 cells
41 based on their lack of CD4 expression and they represent around 23% of the injected CD4⁻
42 iNKT cell population (Fig. 3B). Recipient NOD mice were reconstituted with CD4⁻ or CD4⁺
43 iNKT cells, which were detected in pancreas before BDC2.5 T cell transfer (Fig. 3B). In order
44 to detect an eventual pathogenic role of iNKT17 cells, all recipient mice were injected with a
45 low number of BDC2.5 T cells, which induces around 30% of diabetes in control mice devoid
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3 of iNKT cells (Fig. 3C). Interestingly, in the group of mice reconstituted with CD4⁻ iNKT
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5 cells, the incidence of diabetes was significantly (P=0.036) increased and reached 70%. In
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7 contrast, reconstitution with CD4⁺ iNKT cells significantly (p=0.033) prevented the
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9 development of diabetes. Moreover, when CD4⁻ iNKT cells were further divided according to
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11 NK1.1 expression, only NK1.1⁻ CD4⁻ iNKT cells containing the higher frequency of iNKT17
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13 cells exacerbated diabetes (Fig. 3D).
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17 Since diabetes induced by diabetogenic BDC2.5 T cells is associated with their production of
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19 IFN- γ [13], we have analyzed whether the presence of iNKT cell subsets have influenced their
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21 production of IFN- γ and IL-17. As previously described [13], in diabetic control mice devoid
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23 of iNKT cells, BDC2.5 T cells produced large amount of IFN- γ in both PLN and pancreas
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25 (Fig. 4A). In diabetic mice reconstituted with CD4⁻ iNKT cells, production of IFN- γ by
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27 BDC2.5 T cells was similar as in diabetic control mice and production of IL-17 remained low,
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29 less than 1%. While cytokine production by BDC2.5 T cells was similar in both groups of
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31 mice, the frequency of BDC2.5 T cells in the pancreas was increased in mice reconstituted
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33 with CD4⁻ iNKT cells (44 \pm 3.1%) compared to control mice (32 \pm 1.4%). These data suggest
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35 that the enhanced incidence of diabetes in mice reconstituted with CD4⁻ iNKT cells is due to
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37 the increased frequency of diabetogenic BDC2.5 T cells. Indeed, the frequency of pathogenic
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39 BDC2.5 T cells is probably a key parameter controlling the development of diabetes, since
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41 non-diabetic mice reconstituted with CD4⁺ iNKT cells contained only 0.9 \pm 0.2% and 12 \pm 6.4%
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43 of BDC2.5 T cells in their PLN and pancreas, respectively. Our results highlight the
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45 pathogenic role of CD4⁻ iNKT cells. To demonstrate the key role of IL-17, produced by
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47 iNKT17 cells, we treated mice with an anti-IL-17 antibody. Importantly, this treatment
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49 abolished the deleterious role of CD4⁻ iNKT cells whereas it does not alter the incidence of
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51 diabetes induced by BDC2.5 T cells alone (Fig. 4B). Altogether, our results show that CD4⁻
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3 iNKT cells containing iNKT17 cells exacerbate the development of diabetes in an IL-17-
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5 dependent manner.
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8 9 10 **α GalCer treatment abolishes IL-17 production by iNKT cells.**

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12 It has been well established that activation of iNKT cells by repeated α GalCer injections
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14 prevent the development of diabetes in NOD mice [8, 10, 15]. Autoimmunity prevention
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16 correlated with the ability of α GalCer to induce iNKT cell anergy and to strongly suppress
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18 their IFN- γ production while IL-4 production was less inhibited [33]. Interestingly, we have
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20 observed that α GalCer treatment suppressed not only IFN- γ by iNKT cells but also their IL-
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22 17 production whereas it does not inhibit IL-10 production (Fig. 5). This inhibition of IL-17
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24 production could be critical in the protective role of α GalCer treatment.
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35 **Discussion**

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39 Our study reveals that NOD mice exhibit a high frequency of iNKT17 cells, which produce
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41 IL-17 in the pancreas and can exacerbate diabetes development upon cell transfer. This study
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43 suggests that IL-17 can participate to the pathology of type 1 diabetes. The role of IL-17 in
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45 autoimmune diabetes was first suggested by the low IL-17 production observed in NOD mice
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47 protected against the disease after treatment with a modified self-peptide [25]. More recent
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49 studies showed that IL-17 neutralization with specific antibodies prevents the development of
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51 diabetes in NOD mice [27]. Different immune cell populations can secrete IL-17 [34]. The
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53 role of Th17 cells in diabetes remains unclear. Indeed the induction of the disease in NOD
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55 SCID mice after transfer of in vitro polarized Th17 anti-islet T cells was abolished by anti-IL-
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3 17 treatment in one study but not in two others [25, 26]. It has been reported that IL-17-
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5 producing $\gamma\delta$ T cells do not exacerbate diabetes upon co-transfer into NOD/SCID mice [35].
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8 iNKT17 cells represent a new subset of IL-17-producing cells [19] and we observed an
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10 increased frequency of this cell population in NOD mice as compared to non-autoimmune
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12 C57BL/6 mice. iNKT17 cells from NOD and C57BL/6 mice exhibit a similar phenotype,
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14 mainly CD4⁻ and NK1.1⁻. iNKT17 cells are generated in the thymus where they constitutively
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16 express IL-17 mRNA [21, 22]. The analysis of thymic iNKT cells showed higher frequency
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18 and absolute number of iNKT17 cells in NOD mice compared to C57BL/6 mice. Furthermore
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20 the analysis of the thymic stage 2 CD4⁻ iNKT cell subset (containing iNKT17 cells) showed
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22 an enhanced expression of ROR γ t and IL-23R mRNAs, two key molecules controlling IL-17
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24 lineage [21]. Thus, our data suggest that the high frequency of iNKT17 cells in the peripheral
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26 tissues is subsequent to an elevated frequency of iNKT17 cells in the thymus of NOD mice,
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28 which could be due to an elevated expression of ROR γ t in thymic iNKT cells upon their IL-
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30 17 lineage commitment.
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36 Not only are iNKT17 cells present at high frequency in NOD mice but more importantly, they
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38 infiltrate pancreatic islets of NOD mice. NOD pancreatic islets express the adhesion molecule
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40 E-cadherin, which interacts with the integrin CD103 [36]. Interestingly, 60% of pancreatic
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42 iNKT17 cells expressed CD103 integrin and retention of iNKT17 cells in the pancreas could
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44 be due to CD103/E-cadherin interactions as previously described for diabetogenic CD8 T
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46 cells in the context of islet allografts [37]. Moreover, CD103 can act as a co-activation
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48 molecule in human T lymphocytes [38] and could play a similar role in the activation of
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50 iNKT17 cells in the pancreas. While CCR6 is involved in the recruitment of Th17 cells in the
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52 target tissue in autoimmune CIA [39], the recruitment of iNKT17 cells in the pancreas is
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54 probably independent of CCR6 since most of them do not express this molecule.
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58 Alternatively, lack of expression of CCR6 might be due to downregulation upon entry into
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3 inflamed pancreas. Even though it has been suggested that iNKT17 cells are characterized by
4 CCR6 and CD103 expression, the expression of these molecules by iNKT17 cells varies
5 depending on tissues.
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10 Since IL-17 protein is not detectable in absence of exogenous activation [19, 20], we analyzed
11 IL-17 mRNA and other mRNA associated with the IL-17 response. Importantly, IL-17
12 mRNA level was much higher in iNKT cells from the pancreatic islets than from PLN and
13 ILN. Not such difference in the mRNA level was observed for ROR γ t and IL-23R between
14 these three tissues. Flow cytometry data showed that iNKT17 cells represent respectively 40%
15 of iNKT cells in ILN, 12% in PLN and 6% in pancreas. The discrepancy between the
16 frequency of iNKT17 cells in these three tissues and the spontaneous level of IL-17 mRNA
17 suggest that pancreatic iNKT17 cells are locally activated in this tissue. Interestingly, IL-17,
18 but not IFN- γ , mRNA expression by pancreatic iNKT cells was strongly decreased in mice
19 lacking peripheral CD1d expression, demonstrating that local iNKT17 cell activation involves
20 CD1d recognition. The residual expression of IL-17 mRNA in the absence of peripheral
21 CD1d expression suggests that other local factors, such as IL-23 or IL-1 β , could participate in
22 the activation of iNKT17 cells [40]. IL-1 β is an interesting candidate since it is present in
23 inflamed pancreatic islets [41].
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43 Transfer experiments of iNKT cell subsets reveal the pathogenic role of CD4⁻ iNKT cells
44 containing iNKT17 cell population in the development of diabetes. Reconstitution of
45 immunodeficient NOD mice with CD4⁻ iNKT cells enhanced the incidence of diabetes after
46 injection of a low dose of BDC2.5 T cells. Similar exacerbation of diabetes incidence was
47 observed after reconstitution with NK1.1⁻ CD4⁻ iNKT cell population, which exhibits a high
48 frequency of iNKT17 cells. However, due to cell number limitation most of our experiments
49 were performed with the whole CD4⁻ iNKT cell population. Treatment with anti-IL-17
50 antibodies abolished the pathogenic role of CD4⁻ iNKT cells suggesting that iNKT17 cells are
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3 the critical players in the exacerbation of diabetes, however we cannot rule out that other cell
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5 types producing IL-17 are also participating. Unfortunately, we could not directly demonstrate
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7 that only iNKT17 cells were involved in the deleterious effect of CD4⁻ iNKT cells since there
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9 is presently no specific surface marker to purify this cell population. IFN- γ is also produced
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11 by CD4⁻ iNKT cells and this cytokine could also participate to the exacerbation of diabetes,
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13 however no exacerbation was observed after reconstitution with NK1.1⁺ CD4⁻ iNKT cells
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15 producing high amount of IFN- γ but low level of IL-17. Of note, CD4⁻ iNKT cells alone do
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17 not induce diabetes after transfer into immunodeficient NOD mice (data not shown).
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19 Therefore, we can propose that iNKT17 cells enhanced diabetes incidence through different
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21 mechanisms. *In vitro* data have shown that IL-17 synergizes with other cytokines such as
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23 IFN- γ and IL-1 α/β to induce iNOS expression and subsequent NO production in insulinoma
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25 cells or in pancreatic islets of NOD mice [42]. Similarly in the pancreas, IL-17 produced by
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27 iNKT cells could synergize with IFN- γ secreted by BDC2.5 T cells to induce high expression
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29 of NO in β -cells resulting in their destruction. A deleterious loop could take place since β -cell
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31 death induced by NO would promote self-antigen presentation by dendritic cells to BDC2.5 T
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33 cells. This mechanism could explain the higher frequency of BDC2.5 T cells observed in the
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35 PLN and the pancreas of mice transferred with CD4⁻ iNKT cells as compared to mice devoid
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37 of iNKT cells. Furthermore, it has been shown that IL-17A and IL-17F can induce CXCL10
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39 chemokine expression in lung epithelial cells [43, 44]. Production of CXCL10 by pancreatic
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41 β -cells could contribute to the recruitment of auto-reactive T cells expressing the CXCR3
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43 chemokine receptor as previously shown in several mouse models of type 1 diabetes [45, 46].
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45 Thus, iNKT17 cells might not be involved in the initiation of the insulinitis but rather could
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47 participate in the exacerbation of β -cell death and diabetes onset.
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49 Our data reveal a functional dichotomy between CD4⁺ and CD4⁻ iNKT cell subsets in the
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51 control of diabetes development. While CD4⁻ iNKT cells exacerbate the incidence of diabetes,
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3 CD4⁺ iNKT cells strongly protect mice against diabetes induced by BDC2.5 T cells. Our
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5 transfer experiments demonstrate the protective role of CD4⁺ iNKT cells as it was previously
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7 suggested in NOD mice deficient for CD38 [47]. iNKT cells represent a heterogeneous
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9 population, each subset of iNKT cells exhibiting different functions, either deleterious or
10
11 beneficial toward diabetes development. Protection by iNKT cells is probably not only due to
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13 their total frequency but also to the ratio between the different iNKT cell subsets. This
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15 hypothesis is a possible explanation for the controversial role of iNKT cells in diabetic
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17 patients. In contrast to NOD mice, some authors failed to detect differences in iNKT cell
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19 frequencies and IL-4 production between diabetic patients and healthy subjects [48].
20
21 Autoimmune diabetes is generally considered a Th1-type pathology, but recent reports
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23 suggested that IL-17-producing cells are enhanced in diabetic patients and allegedly
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25 contribute to disease severity [49]. We have recently reported that human iNKT cells produce
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27 IL-17 under pro-inflammatory conditions [50]. IL-17-producing cells in T1D patients [49]
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29 express CCR6 similarly to IL-17-producing human iNKT cells [50]. Therefore, our data
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31 prompt further analysis of iNKT cell subpopulations in patients with a peculiar emphasis on
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33 determining the cytokine profile not only of circulating iNKT cells, but more relevantly of
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35 iNKT cells from tissues such as PLN and pancreas.
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Materials and methods

Mice

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52 C57BL/6J, NOD, C α ^{-/-} NK1.1 NOD, BDC2.5 C α ^{-/-} NOD, V α 14 NOD, CD1d^{pLck} V α 14 NOD,
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54 V α 14 C α ^{-/-} NOD mice have already been described [6, 13, 31]. NK1.1 V α 14 C α ^{-/-} NOD were
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56 generated for iNKT cell subset transfer experiments. NK1.1 NOD females were used for flow
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58 cytometry analysis of Fig.1 [51]. Females were used between 6 and 20 weeks of age. All
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3 experimental protocols were approved by the local ethic committee on animal
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5 experimentation.
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10 **Flow cytometry**

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12 CD1d- α GalCer tetramer staining was performed as previously described [52]. Then cells
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14 were stained at 4°C in PBS containing 5% FCS and 0.1% NaN₃. Fc γ R were blocked with
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16 2.4G2 mAb. Surface staining was performed with anti-CD44 (clone IM7), anti-NK1.1 (clone
17
18 PK136), anti-TCR β (clone H57-597), anti-CD4 (clone RM4-5), anti-CD45 (clone 30F11),
19
20 anti-CD90.2 (clone 30H12), anti-CD45.2 (clone 104), anti-CD103 (clone 2E7) (BD
21
22 Pharmingen) and anti-CCR6 (clone 140706 - R&D). For intracellular staining, cells were
23
24 stimulated for 4h at 37°C with 10 ng/mL of PMA, 1 μ g/mL of ionomycin in the presence of
25
26 10 μ g/mL of brefeldin A (all from Sigma). Then cells were surface stained, fixed,
27
28 permeabilized using a commercial kit (BD Pharmingen) and stained with anti-IL-17 (clone
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30 TC11-10H10), anti-IFN γ (clone XMG1.2), anti-IL-4 (clone 11B11) and anti-IL-10 (clone
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32 JES5-16E3) (BD Pharmingen). Cells were analyzed on a FACS Aria (BD).
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41 **Preparation of iNKT cells and quantitative PCR**

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43 Thymic cells were expanded 5 days in the presence of 20 ng/mL of IL-7 (R&D). iNKT cells
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45 were sorted as TCR β ⁺ CD1d- α GalCer tetramer⁺ cells and according to various markers CD44,
46
47 NK1.1 and CD4 expression, using FACS Aria. Ten thousand iNKT cells were collected in
48
49 RLT buffer with 1% of β -mercaptoethanol. mRNA was isolated using RNeasy Mini Kit
50
51 (Qiagen) and reverse transcribed with Superscript III (Invitrogen). Quantitative-PCR was
52
53 realized with SYBR Green (Roche) and analyzed with LightCycler 480 (Roche).
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Preparation of pancreatic islet cells

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3 Pancreatic islet cells were prepared as previously described [53]. Pancreata were perfused
4 with a solution containing collagenase P (Roche), dissected free from surrounding tissues and
5
6 with a solution containing collagenase P (Roche), dissected free from surrounding tissues and
7
8 digested at 37°C for 10 min. Islets were then purified on a Ficoll gradient and disrupted by
9
10 adding cell dissociation buffer (GIBCO) for 10 min at 37°C.
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12 13 14 15 **Cell purification for transfer experiments**

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17 iNKT cells from spleen and mesenteric LN of CD45.1^{+/+} CD90.1^{+/+} V α 14 C α ^{-/-} NOD mice
18 were enriched by negative selection and then sorted as CD4⁻ or CD4⁺ CD1d- α GalCer
19 tetramer⁺ cells. Sorted cell purity was >96%. CD62L⁺ BDC2.5 T cells were isolated from
20 CD45.2^{+/+} CD90.1^{+/+} BDC2.5 C α ^{-/-} NOD mice. Splenocytes were enriched in T cells by
21 negative selection and CD62L⁺ cells were positively selected using biotinylated anti-CD62L
22 mAb and Streptavidin microbeads (Miltenyi Biotec). CD62L⁺ BDC2.5 T cell purity was
23 >92%. Similar procedures were used for the reconstitution with NK1.1⁻ or NK1.1⁺ CD4⁻
24 iNKT cells. Donor cells were obtained from NK1.1 V α 14 C α ^{-/-} NOD mice.
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39 **Adoptive transfer experiments and diabetes diagnosis**

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41 At 2 weeks of age, CD45.1^{+/+} CD90.1^{+/+} C α ^{-/-} NOD mice were reconstituted i.v with 1.5 x 10⁶
42 CD4⁻ or CD4⁺ iNKT cells from CD45.1^{+/+} CD90.2^{+/+} V α 14 C α ^{-/-} mice. Mice were injected i.p
43 with PK136 mAb (50 μ g/mouse of on days 15, 17, 26 and with 100 μ g/mouse on day 32). At
44
45 with PK136 mAb (50 μ g/mouse of on days 15, 17, 26 and with 100 μ g/mouse on day 32). At
46
47 6 weeks of age, recipient mice were injected i.v with 10⁴ naïve CD62L⁺ BDC2.5 T cells from
48 CD45.2^{+/+} CD90.1^{+/+} BDC2.5 C α ^{-/-} mice. Diabetes analysis was also performed in mice
49 reconstituted with NK1.1⁻ or NK1.1⁺ CD4⁻ iNKT cells. In some experiments mice were
50 injected i.p with 200 μ g of blocking anti-mouse IL-17 antibody (CA028_00511) or isotype
51 control (101.4) on days 0, 2, 4, 6 and 8 after BDC2.5 T cell transfer (day 0). Reagents were
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3 provided by UCB Celltech. Overt diabetes was defined by two consecutive positive
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6 glucosuria tests and glycemia >200 mg/dL.
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10 **Statistical Analysis**

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12 Statistical analyses were performed with the nonparametric Mann-Whitney U test. The log-
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15 rank test was used for the comparison of diabetes incidence.
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For Peer Review

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Figures

Figure 1. Enhanced iNKT17 cell population in NOD mice as compared to C57BL/6 mice.

Organs were harvested from 6 to 10 week-old female NOD (white bars) and C57BL/6 (black bars) mice and cell suspensions were prepared. (A-C) Intracellular IL-17 staining of iNKT cells was performed after stimulation with PMA/ionomycin in the presence of brefeldin A for 4h. iNKT cells were detected using CD1d- α GalCer tetramers in combination with anti-TCR β and their subsets using anti-IL-17 and anti-NK1.1 mAbs. (A) Representative FACS profiles of IL-17 production by iNKT cells from thymus, spleen, ILN and PLN. (B) Frequency of IL-17 producing cells among iNKT cells and (C) absolute number of IL-17 producing iNKT cells in these lymphoid organs. (D) Enhanced expression of IL-17 lineage associated genes by thymic iNKT subsets in NOD mice. Thymic stage 1 (CD44⁻ NK1.1⁻), stage 2 (CD44⁺ NK1.1⁻ CD4⁻ or CD4⁺) and stage 3 (CD44⁺ NK1.1⁺) iNKT cells were sorted from C57BL/6 (black bars) and NOD mice (white bars). The levels of IL-22, IL-17A, ROR γ t and IL-23R mRNAs were evaluated by quantitative PCR. Data were normalized to *gapdh* housekeeping gene. Cumulative data from four independent experiments, each performed with 4 to 10 pooled mice, are shown. Data correspond to the mean \pm SD; * $p \leq 0.05$ between NOD and C57BL/6 mice.

Figure 2. iNKT17 cells infiltrate the pancreas of NOD mice and express IL-17 mRNA in the absence of exogenous stimulation.

(A-C) Cell suspensions from pancreatic islets of females were prepared and intracellular IL-17 staining of iNKT cells was performed as described in Figure 1. iNKT cells were detected

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3 using CD1d- α GalCer tetramers in combination with anti-CD45, anti-TCR β and their subsets
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5 using anti-IL-17, anti-CD4, anti-CD103 and anti-CCR6 mAbs. (A) Representative FACS
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7 profiles of IL-17 and CD4 expression by pancreatic iNKT cells from 12 week-old mice NOD
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9 and V α 14 NOD. (B) Pancreatic islet cells of NOD (white bars) and V α 14 NOD (black bars)
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11 females were prepared. Frequency of pancreatic infiltrating CD45⁺ cells (top panel), iNKT
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13 cells (medium panel) and iNKT17 cells (low panel) at different ages and at diabetes onset
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15 (n.d.= not done). (C) Representative FACS profiles of IL-17 and CD103 or CCR6 expression
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17 by pancreatic iNKT cells. Each experiment was performed with 3-8 pooled mice. (D-E) The
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19 levels of IL-17A, IL-17F, IL-21, IL-22, ROR γ t, IL-23R and IFN- γ mRNA were evaluated by
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21 quantitative PCR and the data were normalized to *gapdh* housekeeping gene. (D) iNKT cells
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23 were purified from the pancreas (black bars), PLN (grey bars) and ILN (white bars) from 12
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25 week-old V α 14 NOD mice. (E) Pancreatic iNKT cells were obtained from 12 week-old V α 14
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27 NOD (white bars) and CD1d^{pLck} V α 14 NOD mice (black bars). Cumulative data from four
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29 independent experiments are shown, each performed with 3-6 pooled mice. Data correspond
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31 to the mean \pm SD; * $p \leq 0.05$ between the different tissues (D) or between different mice (E).
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41 **Figure 3. CD4⁻ iNKT cells containing iNKT17 cells enhanced the incidence of diabetes.**

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43 (A) Cytokine analysis by intracytoplasmic staining of iNKT cell subsets. Pooled splenocytes
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45 and mesenteric LN cells from V α 14 C α ^{-/-} NOD donor mice were stimulated and stained as
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47 described in Figure 1. iNKT cells were detected using CD1d- α GalCer tetramers in
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49 combination with anti-TCR β , anti-CD4 and anti-NK1.1 mAbs. (B-C) iNKT cell subset
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51 purification and cell transfer into recipient mice. (B) Intracellular staining of iNKT cells from
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53 pooled spleen and mesenteric LN (MLN) of V α 14 C α ^{-/-} NOD donor mice (left panel). CD4⁻ or
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55 CD4⁺ iNKT cells were purified by cell-sorting and 1.5×10^6 cells were injected into 2 week-
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57 old C α ^{-/-} NOD mice (middle panel). Four weeks later, frequency of CD90.2⁺ CD4⁻ or CD4⁺
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3 iNKT cells were analyzed in the pancreas of Ca^{2+} NOD recipient mice (right panel). (C)
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5 Incidence of diabetes in recipient mice reconstituted with $CD4^{+}$ iNKT cells (triangles), $CD4^{+}$
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7 iNKT cells (asterisks) or PBS (circles), then injected with 10^4 BDC2.5 T cells four weeks later
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9 (day 0). (D) Similar transfer experiments were performed with $NK1.1^{-}$ $CD4^{-}$ (inverted
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11 triangles) and $NK1.1^{+}$ $CD4^{-}$ (stars) iNKT cell subsets.
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17 **Figure 4. Analysis of diabetogenic T cells and role of IL-17 in the exacerbation of**
18 **diabetes by $CD4^{+}$ iNKT cells.**
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21 (A) Frequency of $CD45.2^{+}$ $CD4^{+}$ BDC2.5 T cells (left panel) and their production of IFN- γ
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23 and IL-17 (right panel) in PLN and pancreas of each group of recipient mice, twelve days
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25 after BDC2.5 T cell transfer. Data represent the mean \pm SD of two independent experiments.
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29 (B) Incidence of diabetes in recipient mice reconstituted with $CD4^{+}$ iNKT cells and injected
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31 with 10^4 BDC2.5 T cells four weeks later. Mice were treated with anti-IL-17 antibodies (full
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33 triangle) or isotype control (empty triangle) on days 0, 2, 4 and 6 after BDC2.5 T cell transfer.
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38 **Figure 5. Repeated α GalCer injections inhibit IL-17 production by iNKT cells.**
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41 Five week-old NOD females were treated twice a week for three weeks with α GalCer (5 μ g,
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43 i.p.). One week after the last injection mice were sacrificed, the spleen harvested and the
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45 splenocytes were cultured with or without 100 ng/ml of α GalCer for three days. IL-4, IL-10,
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47 IL-17 and IFN- γ levels were measured in supernatants by ELISA as previously described [6,
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49 19]. Data correspond to the mean \pm SD of four replicates; * $p \leq 0.05$ between mice pretreated,
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51 or not, by α GalCer.
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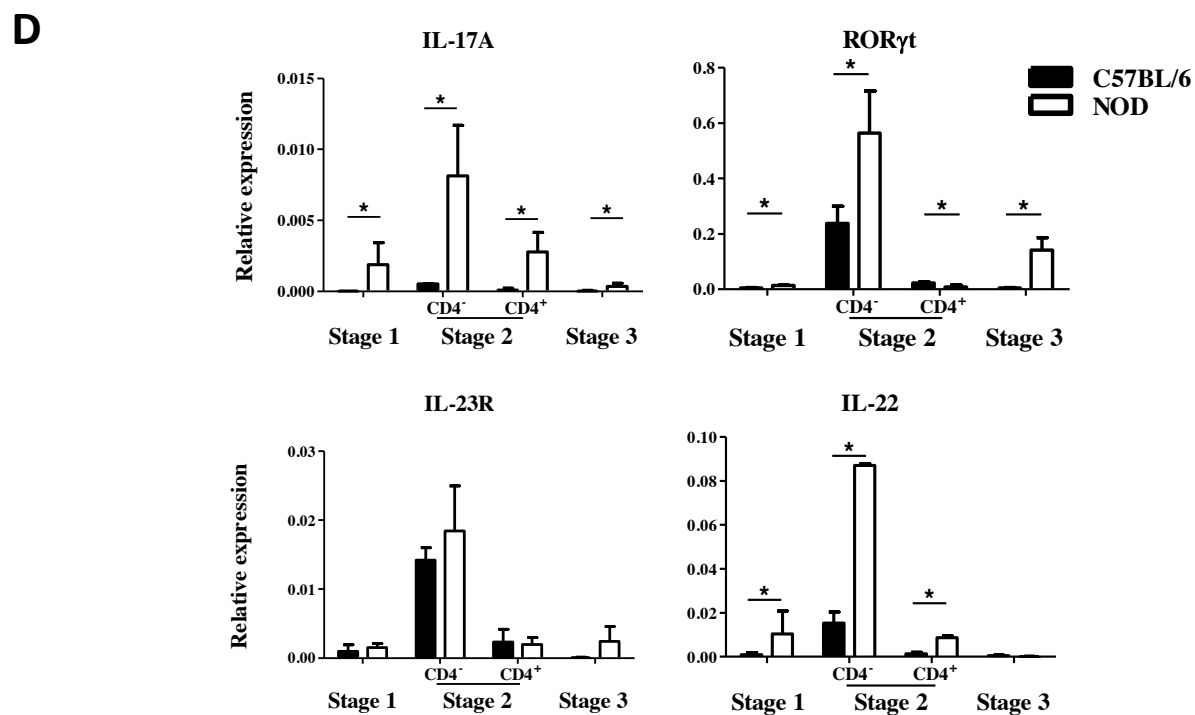
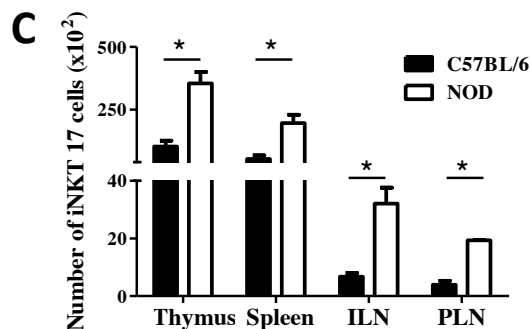
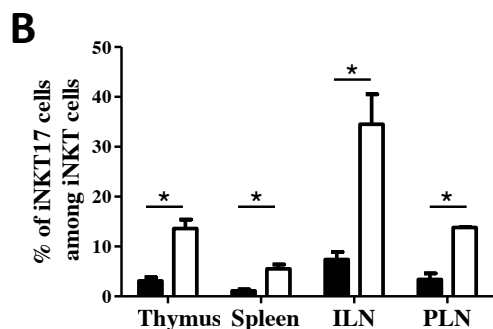
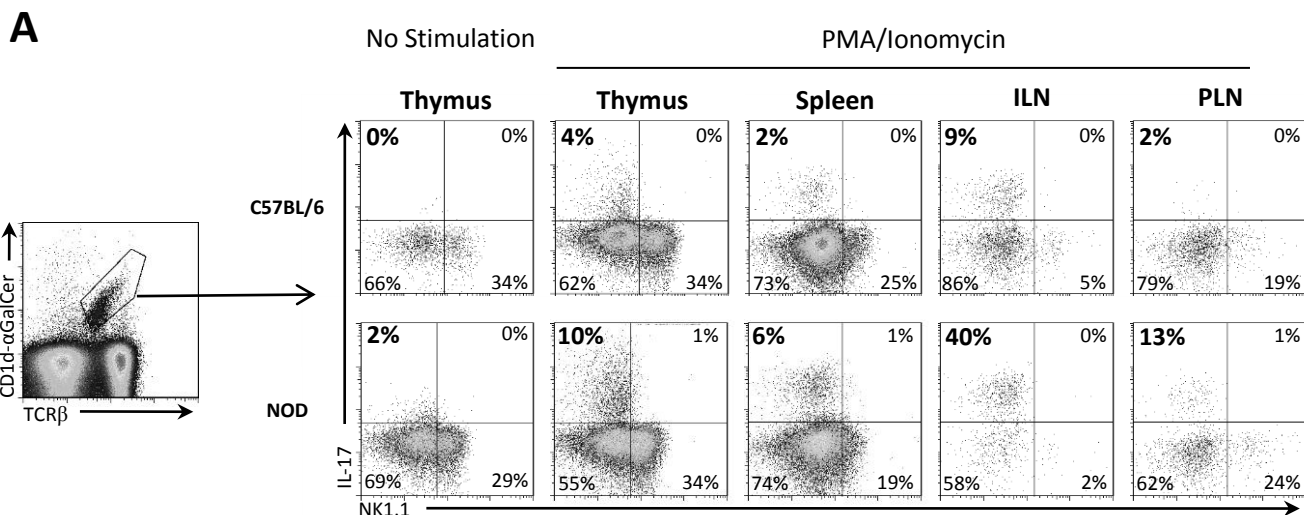
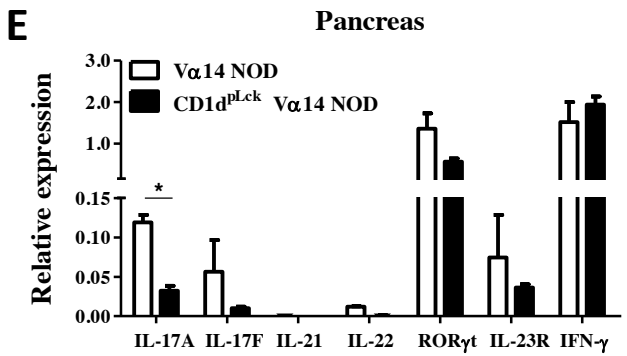
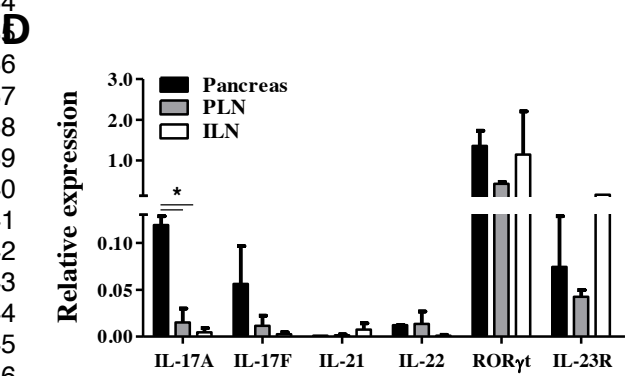
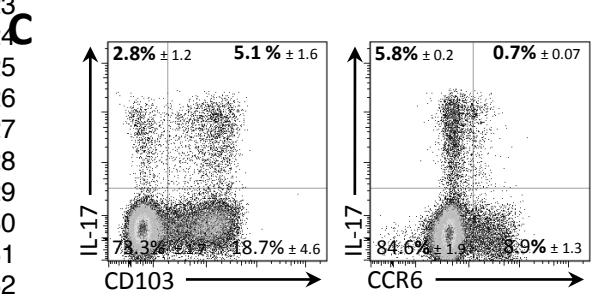
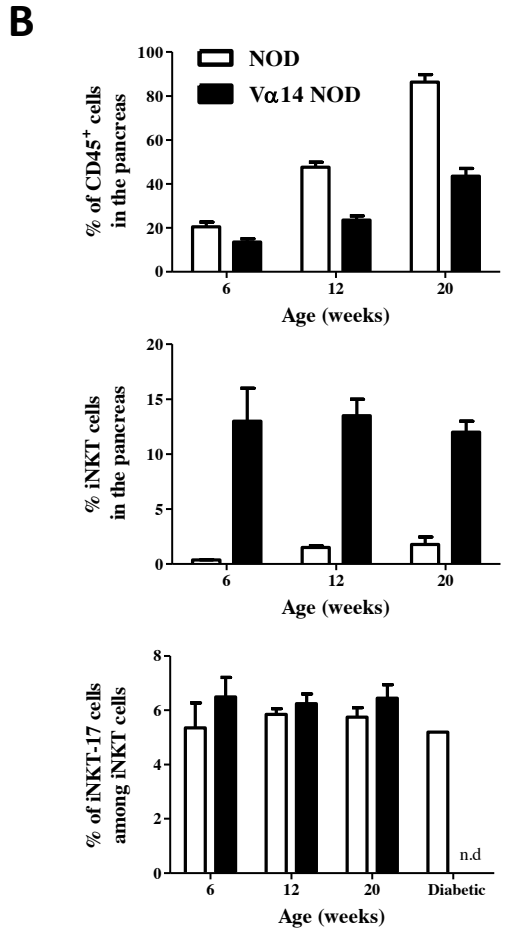
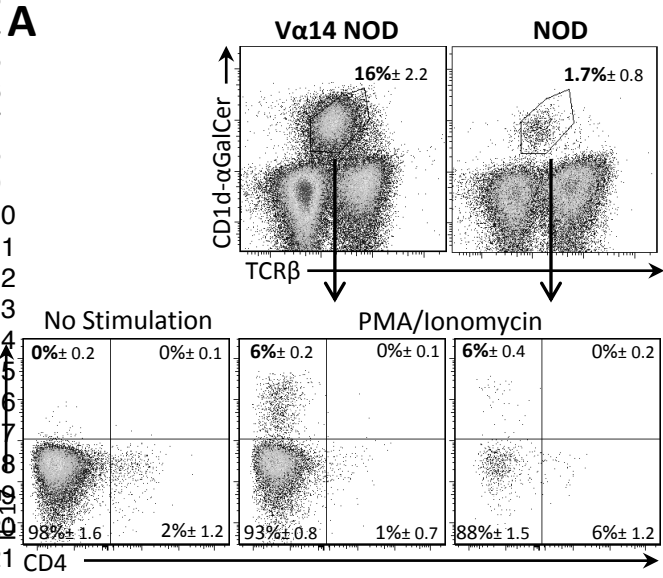
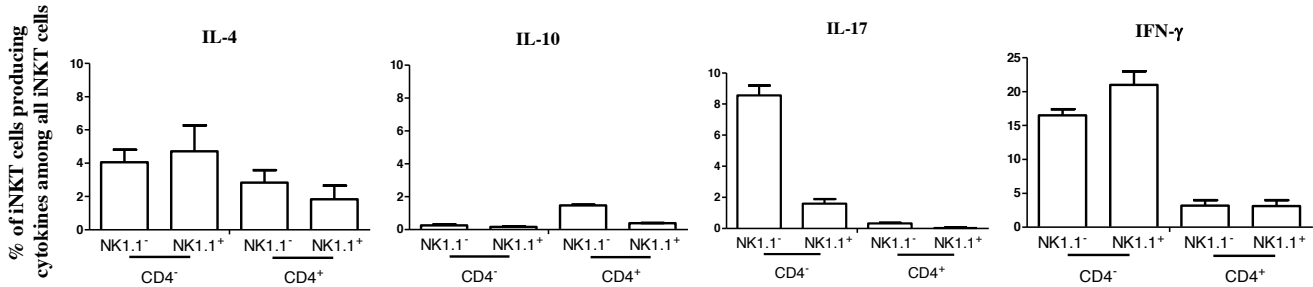


Figure 2

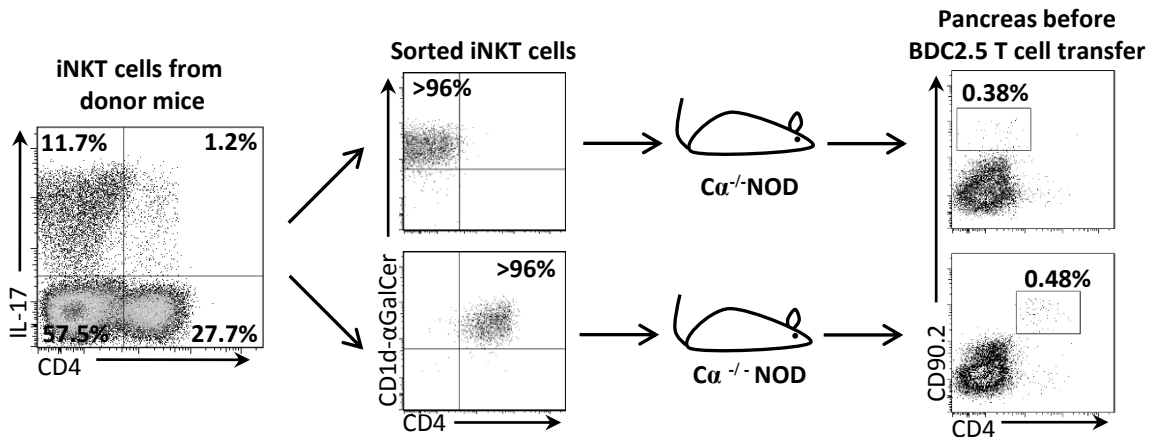
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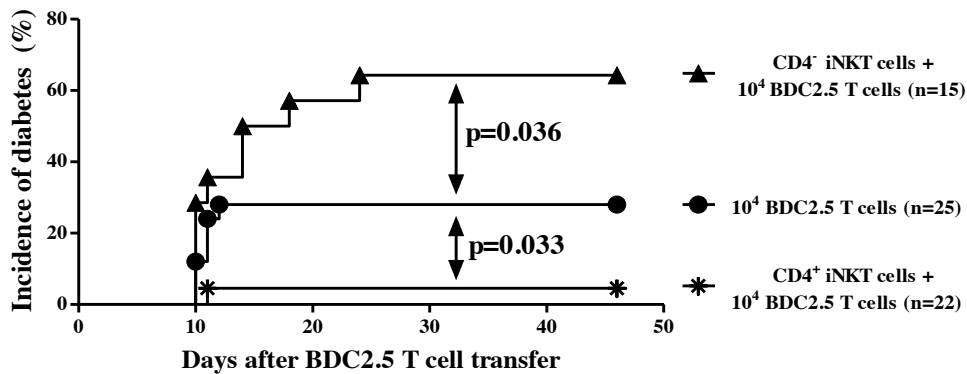
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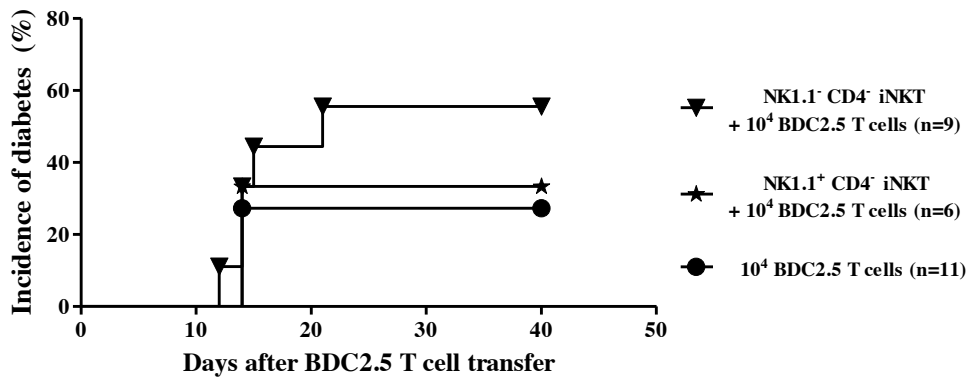
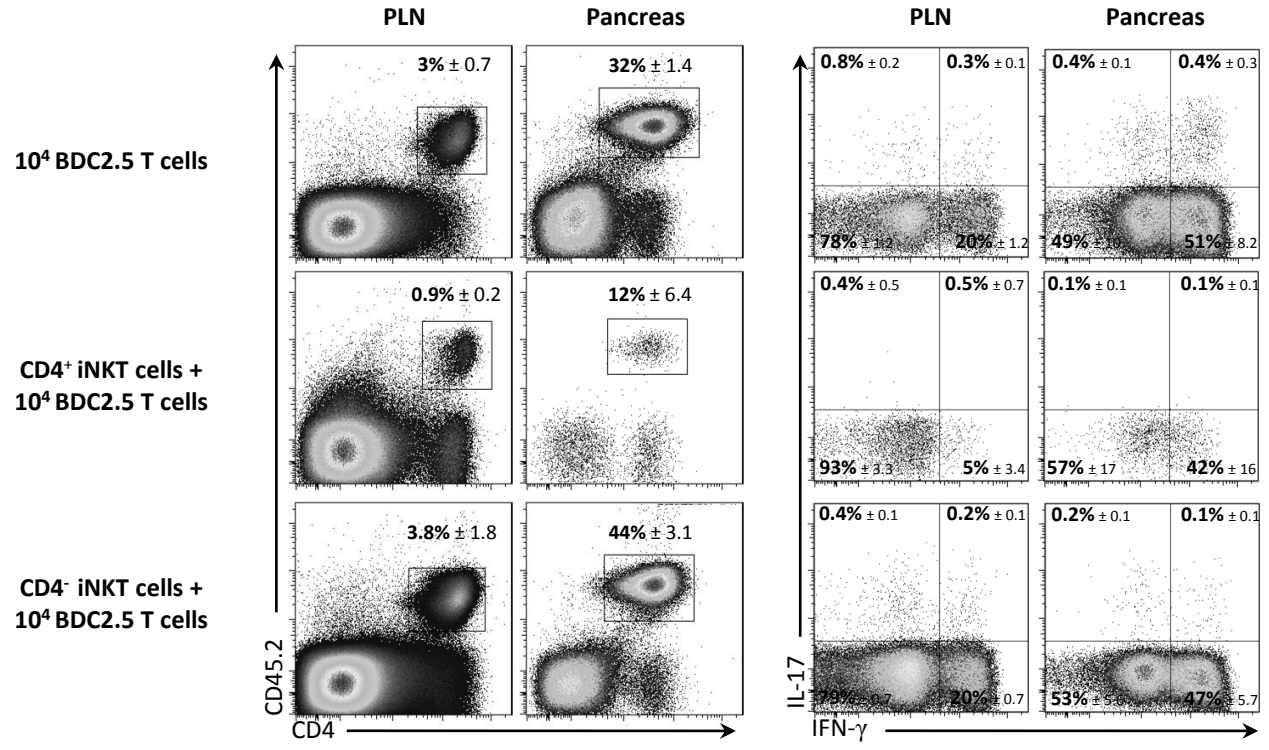


Figure 4

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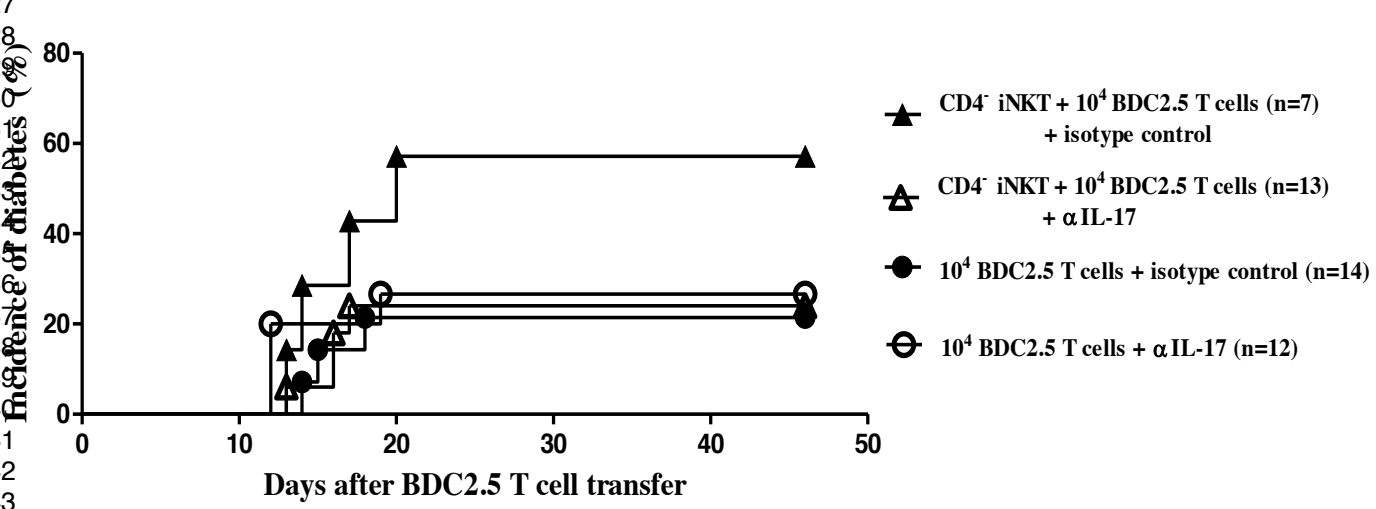


Figure 5

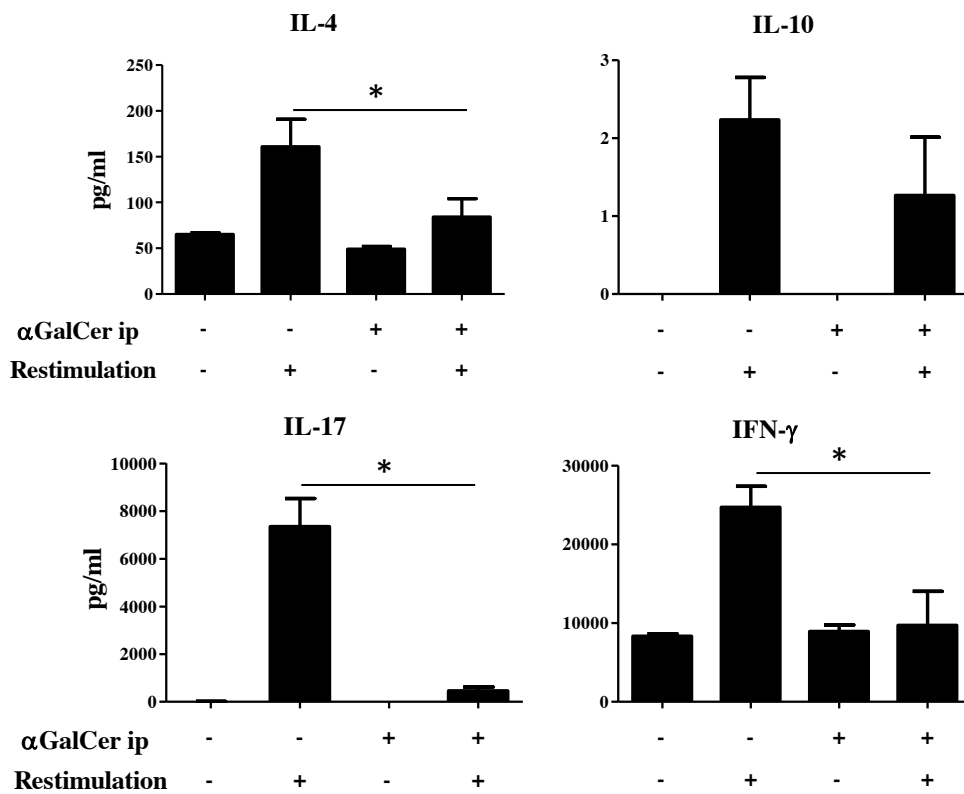


Figure S1

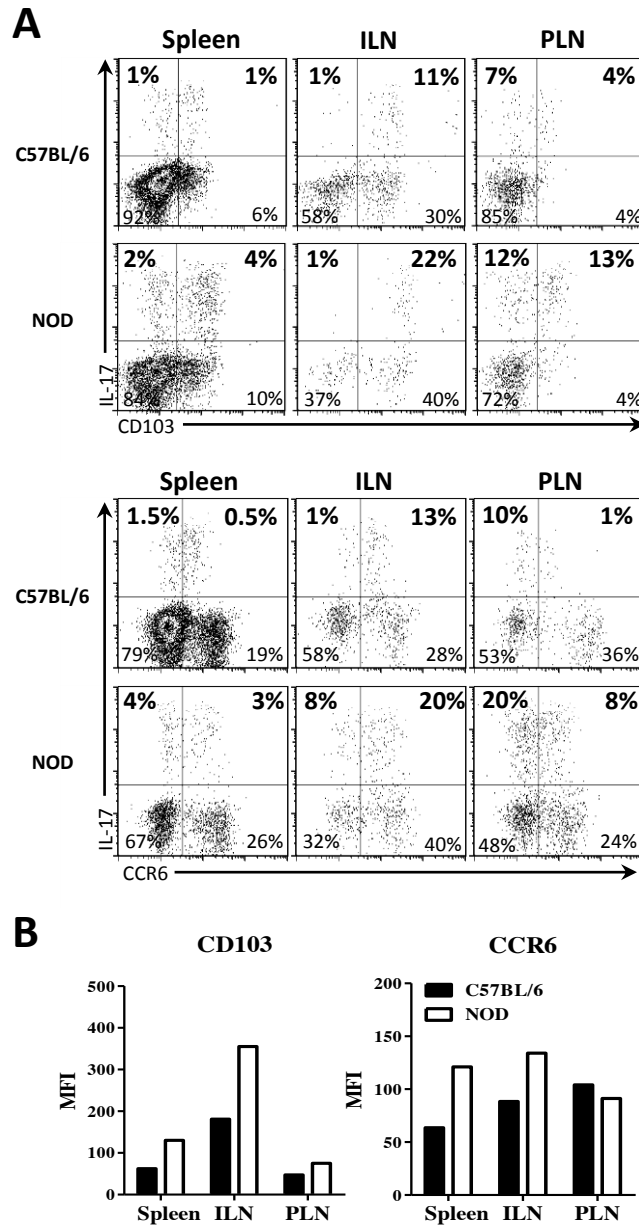


Figure S1. CCR6 and CD103 expression of iNKT17 cells from NOD and C57BL/6 mice. Organs were harvested from 6 to 10 week-old female C57BL/6 (black bars) and NOD (white bars) mice and cell suspensions were prepared. Intracellular IL-17 staining of iNKT cells was performed as described in Figure 1. iNKT cells were detected by flow cytometry using CD1d- α GalCer tetramers in combination with anti-TCR β and their subsets using anti-IL-17, anti-CD103 and anti-CCR6 mAbs. (A) Representative FACS profiles of iNKT cells from spleen, ILN and PLN showing the expression of IL-17 and CD103 (top panel) or CCR6 (bottom panel). (B) CD103 and CCR6 MFI values of IL-17 producing iNKT cells. The data are representative of two independent experiments, each performed with 5-6 pooled mice.

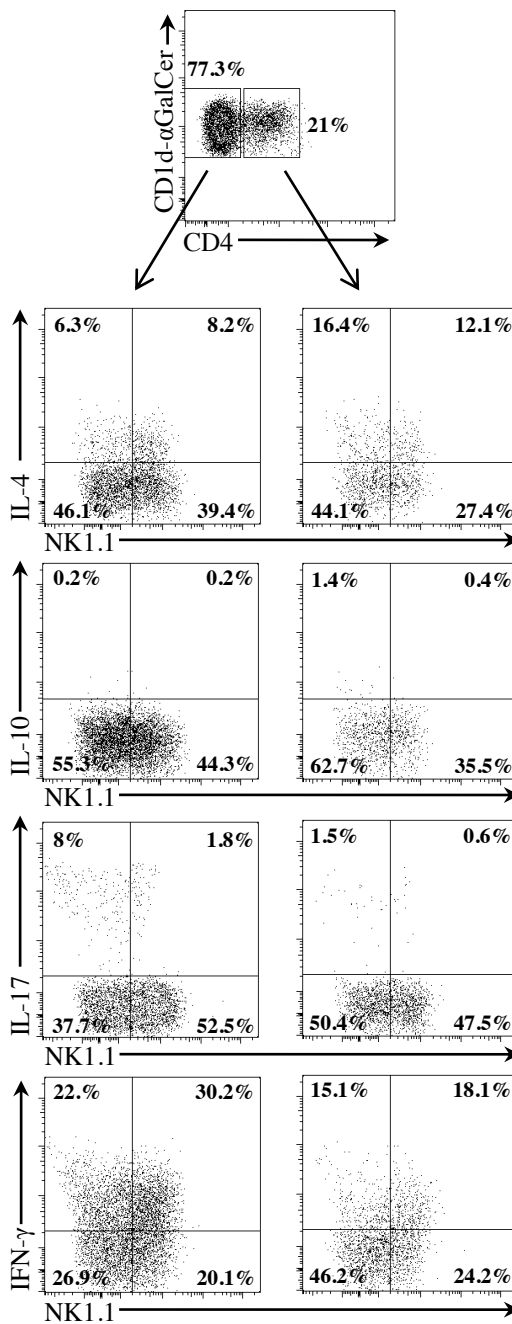


Figure S2. Characterization of iNKT cell subsets from donor mice. Cytokine analysis by intracytoplasmic staining of iNKT cell subsets. Pooled splenocytes and mesenteric LN cells from *Vα14 Cα^{-/-}* NOD donor mice were stimulated and stained as described in Figure 1. iNKT cells were detected using CD1d-αGalCer tetramers in combination with anti-TCRβ, anti-CD4 and anti-NK1.1 mAbs. Cytokines were detected using anti-IL-4, anti-IL-10, anti-IL-17 and anti-IFN-γ mAbs