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Enhanced Early Expansion and Maturation of Semi-Invariant NK T Cells Inhibited Autoimmune Pathogenesis in Congenic Nonobese Diabetic Mice

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Semi-invariant NK T cell (iNKT) deficiency has long been associated with the pathogenesis of type 1 diabetes (T1D), but the linkage between this the deficiency and T1D susceptibility gene(s) remains unclear. We analyzed NOD mice subcongenic for resistant alleles of Idd9 locus in search for protective mechanisms against T1D, and found that iNKT cell development was significantly enhanced with a more advanced mature phenotype and function in mice containing Idd9.1 sublocus of B10 origin. The enhanced iNKT cell development and function suppressed effector function of diabetogenic T cells. Elimination of iNKT cells by CD1d deficiency almost abolished T1D protection in these mice. Interestingly, although the iNKT cells were responsible for a Th2 orientated cytokine profile that is often regarded as a mechanism of T1D prevention, our data suggests that the Th2 bias played little if any role for the protection. In addition, dendritic cells from the congenic NOD mice showed increased abilities to engage and potentiate iNKT cells, suggesting that a mechanism mediated by dendritic cells or other APCs may be critical for the enhanced development and maturation of iNKT cells. The products of T1D susceptibility gene(s) in Idd9.1 locus may be a key factor for this mechanism. The Journal of Immunology, 2008, 181: 6789–6796.
NOD mice (20), Th2 deviation of autoreactive T cells has been considered as a regulatory mechanism in NOD.R28 mice. Id9 locus can be divided into three subloci: Id9.1, Id9.2, and Id9.3, each associated with different T1D prevalence (20), indicating the existence of numerous susceptibility genes within this region. Therefore, NOD.R28 mice are likely protected by multiple mechanisms conferred by different resistant alleles. Consistent with this notion, improved NKT cell development and function were not detected in NOD mice congenic for the resistant allele of the Id9.2/3 interval, suggesting that genes in the Id9.1 interval strongly affect the development and functions of NKT cells (18). In the present study, we investigated the role of NKT cells in T1D resistance in NOD mice congenic for different Id9 subloci, and found that restored development and function of NKT cells in NOD.R28 mice suppressed T1D pathogenesis, and elimination of NKT cells abolished T1D resistance in mice congenic for the resistant allele of Id9.1 sublocus. In addition, we found that NKT cells were responsible for the increased Th2 cytokines in these animals. However, Th2 cytokines were not required for T1D resistance.

Materials and Methods

Nodulation, NOD.scid, and IL-4-deficient NOD mice (NOD^delta/i^), as well as B6.g7 mice were purchased from The Jackson Laboratory. Stat6 and CD1d-deficient NOD mice were produced by backcrossing mutant genes from B6.g7 mice were purchased from The Jackson Laboratory. Stat6 and CD1d monomers and GalCer/CD1d tetramers were as previously described (23, 24).

Preparations of CD1d monomers were prepared by incubating detergent-free CD1d monomers at various concentrations of ligands. [3H]-thymidine incorporation was used to measure T cell proliferation and cytokine production was measured using ELISA (R&D Systems). For depletion of NKT cells, total splenocytes were incubated with PE labeled CD1d tetramers for 2 h, and washed three times. Cells were then incubated with anti-PE Ab conjugated microbeads. PE negative fraction was collected and stained with anti-TCR.

Cell preparation and T cell activation

Splenocytes from newly diabetic NOD mice were transferred into 7-wk-old NOD.scid or R28.scid mice (5 × 10^6/recipients). In some recipient mice, splenocytes (20 × 10^6/recipients) from 7- to 8-wk-old NOD or R28 mice were cotransferred with diabetic splenocytes. DX5^TcR^ thymocytes were isolated from 7- to 8-wk-old NOD.R28 mice using Ab conjugated microbeads and FACS sorting, and cotransferred (0.5 × 10^6/recipients) with the diabetic splenocytes (5 × 10^6/recipient). The onset of T1D was monitored twice a week by measuring glucose levels in both blood and urine. Mice were considered diabetic once blood glucose was higher than 12 mM.

Reconstitution of NOD.scid mice

NKT cells were isolated from the thymus of B6.g7 mice as described (23). In brief, thymocytes were first labeled with PE-conjugated anti-NK1.1 Ab, followed by anti-PE Ab conjugated microbeads (Milenyi Biotec). The purity of isolated NKT cells was then examined by FACS analysis using anti-TcR^FITC and allophycocyanin-labeled aGalCer/CD1d tetramer staining. For the activation of NKT cells, plate-bound aGalCer/CD1d monomers were prepared by incubating aGalCer and CD1d monomers at 4°C overnight, and then at 37°C for 6 h in 96-well plates. The coated plates were washed twice with PBS. NKT cells (2.5 × 10^6 cells/ml) were then added to the plates and incubated at 37°C for 48 h in the presence or absence of DCs from different donor mice.

Isolated DCs (10 × 10^6 cells/ml) were first stained with biotinylated anti-I-Ab Ab, followed by Streptavidin-PerCP. After incubating with thymic NKT cells (NK1.1-PE stained, 10^6 cells/ml) were then used for genotyping of Cd1d gene-deficient mice to ensure all these loci were NOD origin. Thus, NOD.R201 mice contained potential protection from a B10 allele of Idd9.1 region, we developed NOD.R201 mice by backcrossing NOD.B10-Idd9R11 (referred to NOD.R11 mice thereafter), and NOD mice congenic for Id9 locus (R450) mice were purchased from Tacomb Farms. NOD.R201 mice were developed by crossing R28 with NOD mice, followed by intercrossing the candidate heterogeneous offspring. A deficient Ii, Stat6, or Cd1d gene, as well as a scid mutation, was introduced into NOD.R28 or NOD.R201 mice from NOD background, and the congenic alleles of mutant gene carriers were fixed to homozygosity followed by intercrossing, to generate congenic NOD mice homozygous for the deficient genes. Because the Cd1d gene is located between Id9d3 and Id9/17/18 loci on chromosome 3, microsatellite markers D3Nds55, D3Nds40, D3mit230, D3mit40, and D3mit10 were used for genotyping of Cd1d gene-deficient mice to ensure all these loci were NOD origin. T1D incidence of NOD^delta/i^ mice was similar to that of NOD mice, indicating no potential flanking susceptibility or resistance gene was introduced during backcross.

All mice were maintained in a specific pathogen-free facility at University of California, according to the Institutional Animal Care and Use Committee guideline.

α-galactosylceramide (αGalCer) was provided by Kirin Brewery. All Abs were purchased from BD Pharmingen. αGalCer/CD1d tetramers were provided National Institutes of Health tetramer center. Preparations of CD1d monomers and αGalCer/CD1d tetramers were as previously described (23, 24).

Fig. 1. Restored iNKT cell development in NOD.R28 and NOD.R201 mice.


Reconstitution of NOD.scid mice

Statistical analysis

The results of the in vivo studies were analyzed with a Kaplan-Meier log-rank test and a Welch t test was used for in vitro data.

Results

Regulatory lymphocytes protected NOD.R28 mice

It has been demonstrated that a B10 allele of Idd9 interval strongly protected NOD.R28 mice from T1D. However, NOD.R11 that contain B10 allele of Idd9.2 and 9.3 regions were only partially protected, indicating that gene(s) in Idd9.1 region of NOD mice con flexibility for T1D susceptibility (20). To investigate the potential protection from a B10 allele of Idd9.1 region, we developed NOD.B10-Idd9R201 (NOD.R201 hereafter) mice by backcrossing NOD.R28 mice with NOD mice, followed by intercrossing their heterozygous candidate offspring. NOD.R201 mice contain a 10 cM interval of chromosome 4 of B10 origin, without overlapping with the B10 interval in NOD.R11 mice.

When TID incidence was examined, we found that none of 15 female NOD.R28 mice developed T1D within 30 wk after birth,
whereas 80% female NOD mice became diabetic. Consistent with the previous report (20), incidence of T1D of female NOD.R11 mice was ~30%. Among female NOD.R.201 mice, <15% mice developed T1D, suggesting a stronger protection by B10 Idd9.1 allele that might confer a protective mechanism different from Idd9.2/9.3 allele (Fig. 1b).

T1D susceptibility genes may be expressed in lymphocytes or in nonhematopoietic parenchyma cells. To distinguish these possibilities, we developed a line of NOD.R28.scid mice, and transferred splenocytes from newly diabetic NOD mice into R28.scid and NOD.scid mice. We found that most of R28.scid mice developed T1D as rapidly as did NOD.scid recipients, indicating that islets in NOD and NOD.B10-Idd9 mice were equally susceptible to autoimmune attacks (Fig. 1c). These results further suggest that the resistant allele of Idd9 locus confers regulatory mechanisms to suppress T1D pathogenesis, and that regulatory populations were of hematopoietic origin.

**NOD.R28 and NOD.R201 mice have improved development and function of iNKT cells**

To identify the regulatory populations, we first examined CD4+Foxp3 expressing regulatory T cells in spleen and pancreatic lymph nodes, and found no difference in the frequencies of these cells between NOD and NOD.R28 mice (data not shown). However, when we examined the other known regulatory T cell population, iNKT cells, using αGalCer loaded CD1d tetramers, we found significantly increased frequencies and numbers of iNKT cells in both the thymus and spleen of NOD.R28 and NOD.R201 mice, but not those of NOD.R11 mice in which the frequency of iNKT cells was similar to that in NOD mice (Fig. 2, a and b). A previous report suggested a genetic linkage between iNKT cell deficiency and Idd9.1 region in NOD mice (18). Our result provides clear evidence for this connection. Because the differences in the iNKT cell frequencies were more pronounced in the thymus, we analyzed thymic iNKT cells at different ages. The results revealed that at a very early stage (2 wk of age), there was no difference in frequency of iNKT cells in the thymus among different mice. However, iNKT population steadily expanded from 3 wk of age in NOD.R28 and NOD.R201, but not in NOD and NOD.R11 mice. And the differences in iNKT cells frequencies were reached to the peak in 8-10 wk old mice (Fig. 2c). Clearly, the defective ontogeny of thymic iNKT cells associated with a gene(s) in Idd9.1 region contributes to the thymic iNKT cell deficiency in NOD mice, and the gene product of B10 allele might promote the expansion of a predominant subset of immature iNKT cells in the thymus of 2- to 3-wk-old mice (27) in R28 and R201 mice. In addition, more thymic iNKT cells in NOD.R28 and NOD.R201 mice expressed NK cell markers, such as DX5, than those from NOD mice (Fig. 2d).

High proportion of DX5+ thymic iNKT cells were also detected in B10.g7 mice, but not in NOD mice congenic to B10 allele of Idd3 locus. Because the expression of NK cell marker is a late event in the iNKT cell development and is usually associated with maturation of iNKT cells, our observation suggested that the thymic iNKT cells in NOD.R28 and NOD.R201 mice differ from those in NOD mice in both expansion and maturation. Furthermore, we found that consistent with the increased frequency as well as potentially elevated iNKT cell functions, splenocytes from R28 and R201 mice produced significantly more IFN-γ and IL-4 than those from NOD mice (Fig. 2e), when stimulated with various concentrations of αGalCer, a specific ligand for iNKT cells. The increased cytokine production reflected the increased frequencies of iNKT cells in R28 and R201 mice. Depletion of iNKT cells using αGalCer/CD1d tetramer completely abolished cytokine responses by splenocytes from these mice (data not shown).

**iNKT cells protected NOD.R201 mice from T1D**

To investigate whether iNKT cells developed in NOD.R28 mice are capable of suppressing effector functions of autoimmune diabetic T cells, we purified thymic iNKT cells from NOD.R28 mice and cotransferred these cells with diabetic splenocytes into NOD.scid recipients. The transfer of diabetic splenocytes induced the onset of T1D in the recipient mice in 5 wk. However, a small number of iNKT cells from NOD.R28 mice delayed the pathogenic progression and reduced the incidence of T1D (Fig. 3a). Similarly, the cotransfer of splenocytes from NOD.R28 mice delayed and reduced T1D in NOD.scid recipients (Fig. 3a). To further define the role of iNKT cells in the T1D prevention in R201 mice, we transferred splenocytes from age-matched (8 wk) NOD and NOD.R201 mice into NOD.scid recipients. Splenocytes from NOD mice induced T1D in all recipient mice within 15 wk; in contrast, none of recipients of NOD.R201 splenocytes developed
FIGURE 3. Inhibition of T1D in NOD.R201 mice by iNKT cells. a, T1D incidence of NOD.scid recipients of diabetic splenocytes (5 x 10^6/recipient) in the presence or absence of thymic iNKT cells (0.5 x 10^6/recipient) from NOD.R28 mice. b, T1D incidence of NOD.scid recipients of splenocytes from newly diabetic NOD mice (5 x 10^6/recipient) cotransferred with splenocytes (10 x 10^6/recipient) from 7-wk-old NOD or NOD.R28 mice. c, FACS profile showing the efficiency of iNKT cell depletion from total splenocytes of NOD.R201 and NOD.R28 and NOD.R201 mice stimulated by GalCer (0, 20, 40, and 100 ng/ml). d, Proliferative responses of splenocytes from NOD, CD1d-deficient NOD, NOD.R28, and NOD.R201 mice stimulated by anti-CD3 Ab (0, 1, 2, and 5 µg/ml). e, Proliferative responses of splenocytes from NOD, CD1d-deficient NOD, NOD.R201, CD1d-deficient NOD.R201 mice, and NOD.R28 mice stimulated by GalCer (0, 20, 40, and 100 ng/ml). f, T1D incidence of female CD1d-deficient NOD, NOD.R28, and NOD.R201 mice. The difference between NOD.R201 \( ^{\text{CD1d}^{-/-}} \) and NOD.R201 \( ^{\text{CD1d}^{+/+}} \) was significant (p < 0.05).

T1D. However, when iNKT cells were depleted before the cell transfer (Fig. 3e), ~40% of the recipients of NOD.R201 splenocytes developed T1D (Fig. 3d).

To further investigate the role of iNKT cells in T1D prevention, we eliminated iNKT cells in NOD.R28 and NOD.R201 mice genetically by introducing a null \( \text{Cd}1d \) gene from CD1d-deficient NOD mice. As expected, iNKT cells were no longer detectable in the thymus and spleen of CD1d deficient R28 and R201 mice, indicating the abolished iNKT cell development (Fig. 3e and data not shown). Splenocytes from NOD, NOD\(^{\text{Cd1d}^{-/-}}\), NOD.R201, NOD.R201\(^{\text{Cd1d}^{-/-}}\), and NOD.R28 mice proliferated equally well in response to anti-CD3 stimulation (Fig. 3f). However, the splenocytes from NOD\(^{\text{Cd1d}^{-/-}}\) and NOD.R201\(^{\text{Cd1d}^{-/-}}\) mice no longer responded to the αGalCer stimulation, whereas splenocytes from R28 and NOD.R201 mice proliferated vigorously (Fig. 3g). These phenotypic and functional analyses further confirmed the lack of iNKT cells in both NOD.R28\(^{\text{Cd1d}^{-/-}}\) and NOD.R201\(^{\text{Cd1d}^{-/-}}\) mice. More than 50% of R201\(^{\text{Cd1d}^{-/-}}\) mice developed T1D, whereas the incidence of the disease in R201 mice that were heterogeneous in CD1d deficiency was much lower, similar to that of CD1d sufficient NOD.R201 mice (Fig. 3h). Therefore, iNKT cells conferred a significant protection to NOD.R201 mice from T1D.

However, CD1d-deficient NOD.R28 mice were still free of T1D, suggesting protective mechanisms additional to that mediated by iNKT cells in those mice.

Th2 deviation by iNKT cells plays a minor role in T1D prevention

iNKT cells are known for their capacity of cytokine burst, especially for Th2 cytokines. Th2 cytokines by iNKT cells were implicated as a regulatory mechanism for autoimmune disorders (15, 28). In contrast, a large number of IL-4-producing islet infiltrating cells were detected in NOD.R28 mice. As such, the Th2 deviation was considered as a potential protective mechanism in these animals (20). To identify the role of Th2 immune responses in NOD.R28 mice, we first investigated whether T cells from NOD.R28 mice produced increased Th2 cytokines. CD4\(^{+}\) T cells, including those CD1d tetramer-positive iNKT cells, purified from the spleens of NOD and NOD.R28 mice were activated using anti-CD3/CD28 stimulation. It is clear that these CD4\(^{+}\) T cells from both strains expressed similar levels of IFN-γ. However, CD4\(^{+}\) T cells from NOD.R28 mice produced much more IL-4 than those from NOD mice (Fig. 4a), as did the same population from NOD.R201 mice (Fig. 4c). These results suggested that the Th2 profile was an intrinsic property of CD4\(^{+}\) T cells of NOD.R28 and NOD.R201 mice. However, iNKT cells among the isolated T cells might contribute to the production of Th2 cytokines. Therefore, we activated CD4\(^{+}\) T cells from CD1d-deficient NOD and NOD.R28 mice, and found that both populations produced identical levels of IFN-γ, but very little IL-4 (Fig. 4, b and c). These results suggested a critical role of iNKT cells for the IL-4 production.

To test whether the Th2 profile is responsible for T1D prevention, we introduced IL-4 or Stat6 deficiency into NOD.R28 mice by crossing these mice with Il4 or Stat6 gene deficient NOD mice. IL-4 deficiency did not alter T1D pathogenesis in NOD mice (29), whereas T1D in Stat6 deficient NOD mice was slightly accelerated (Fig. 4d and Ref. 30). Stat6 is a signal transducer shared by IL-4 and IL-13, the different effects of deficient Il4 and Stat6 genes on T1D pathogenesis might be due to a
compensation of IL-4 deficiency by IL-13 in NOD\(^{+/−}\) mice, whereas in NOD\(^{\text{Stat} 6−/−}\) signals from both IL-4 and IL-13 were abolished. However, neither IL-4 nor Stat6 deficiency significantly increased T1D incidence in NOD,R28 mice, suggesting a dispensable role of Th2 cytokines for T1D resistance in these animals.

**DCs from NOD.R201 mice engage iNKT cells with high intensity**

Defective development and function may be intrinsic in iNKT cells in NOD mice (31). However, defects in other cell types that are critical for lipid Ag processing and CD1d recognition may also regulate development and function of iNKT cells. DCs play an important role in the development and activation of iNKT cells. In particular, we have found recently that DCs potentiate iNKT cells for their effective responses to suboptimal exogenous lipid- Ags through a CD1d dependent mechanism (23). Furthermore, iNKT cells may modulate function of DCs as a potential regulatory mechanism (32). To investigate the potential involvement of DCs in the improved development and function of iNKT cells and T1D prevention in NOD,R201 mice, we first compared the function of Ag presentation of DCs from NOD and NOD,R201 mice. DCs were isolated from NOD and NOD,R201 mice as APCs to activate CD8\(^+\) 8.3-T cells and CD4\(^+\) BDC2.5-T cells that expressed islet specific TCR transgenes. Naïve T cells from different TCR transgenic NOD donor mice were cultured with their peptide Ags NRP-A7 or 1040-51 for 72 h in the presence of different DCs, and the cytokine production was measured by ELISA. Identical levels of IFN-γ were produced by activated 8.3- and BDC2.5-T cells in the presence of DCs from different donor mice. BDC2.5 T cells produced no IL-4 but similar levels of both IFN-γ and IL-10 (Fig. 5, a and b and data not shown). These results indicated that DCs from NOD and NOD,R201 mice had similar abilities of Ag presentation and costimulation, and DCs from NOD,R201 mice did not promote Th2 polarization.

To test the ability of DCs for engaging and potentiating iNKT cells, we purified splenic DCs from NOD, NOD,R201 and B6.g7 mice. We also purified thymic iNKT cells from B6.g7 mice and cultured them with immobilized CD1d monomers loaded with a low concentration (1 ng/ml) of αGalCer in the presence or absence of various numbers of DCs derived from different donors. iNKT cells were not activated by αGalCer/CD1d complex alone until fresh DCs were added into the cultures, and the cytokine production by iNKT cells was increased proportionally to the numbers of DCs, as described previously (23). It was clear that DCs from NOD mice potentiated iNKT cells with a lower efficiency than those from NOD,R201 or B6.g7 mice, because

**FIGURE 4.** Enhanced Th2 responses and a dispensable role of Th2 cytokines in T1D prevention. a, IFN-γ and IL-4 production by CD4\(^+\) T cells purified from NOD and NOD,R28 mice. T cells were activated by immobilized anti-CD3 Ab (5 μg/ml) and anti-CD28 Ab (2 μg/ml) for 3 days, and cytokine production was measured by ELISA. Columns represent cytokine production by cells from individual mice. Similar results were obtained from three independent experiments. b and c, IFN-γ and IL-4 production by CD4\(^+\) T cells purified from CD1d sufficient and deficient NOD and NOD,R201 and mice. Representative data of three independent experiments with similar results are shown. d, T1D incidence by cells from individual mice. Similar results were obtained from three independent experiments. NOD,R201 and mice. Representative data of three independent experiments with similar results are shown.

**FIGURE 5.** Different engagement and potentiation of iNKT cells by DCs from NOD and NOD,R201 mice. a and b, IFN-γ and IL-10 productions by the activated islet-specific 8.3- and BDC2.5-T cells. T cells were activated by their peptide ligands presented by DCs from NOD or NOD,R201 mice. Representative data of two experiments with identical results are shown. c, IFN-γ and IL-4 productions by thymic iNKT cells from B6.g7 mice activated by immobilized αGalCer/CD1d complex in the presence or absence of splenic DCs from NOD, NOD,R201, or B10.g7 mice. Representative data of three independent experiments with similar results are shown. d, Frequencies of conjugates formed between iNKT cells with DCs from NOD, NOD,R201, or NOD\(^{\text{Cd1d−/−}}\) mice. Representative data of two similar experiments are shown. e, Profile of CD1d expression in splenic DCs purified from NOD and NOD,R201 mice.
iNKT cells produced much less IFN-γ and IL-4, in the presence NOD DCs compared with other DCs (Fig. 5c).

Potential of iNKT cells depends on Vα14 engagement with endogenous lipid Ags displayed on DCs (23). Therefore, we tested the engagement between iNKT cells and DCs directly by analyzing iNKT/DCs conjugate formation. Purified iNKT cells from B6 mice were incubated with DCs isolated from NOD and NOD.R201 mice, and samples were taken at various time points, fixed, and analyzed by FACS. DCs from CD1d-deficient NOD mice were also isolated and used as control. The frequencies of conjugates increased and reached the peak 40 min after the mixing of iNKT cells with DCs. However, iNKT cells conjugated with DCs from NOD.R201 mice more frequently and rapidly than DCs from NOD mice, whereas there was no conjugate was detectable with CD1d-deficient DCs (Fig. 5d). These results suggested that DCs from NOD and NOD.R201 mice had different ability to engage with iNKT cells. The conjugate formation between iNKT cells and DCs is CD1d dependent, however, we did not find different levels of CD1d expression in DCs from NOD and NOD.R201 mice (Fig. 5e).

Discussion

iNKT cell deficiency has long been associated with T1D pathogenesis, and this deficiency has been linked to multiple loci within NOD genome, including Idd6 on chromosome 6 (33), Nkt1 locus on chromosome 1, and Nkt2 locus on chromosome 2 (34). In addition, evidence suggested that development and function of iNKT cells might be under the control of multiple genes mapped to different Idd loci, including Idd3, Idd4 and Idd13, because introgression of these NOD-derived loci, individually, into B6 mice reduced either numbers or cytokine responses of iNKT cells (18). In contrast, B10 allele of Idd3, and I3 loci did not improve the development and function of iNKT cells in NOD mice. These studies indicated that iNKT cell deficiency is a result of complex interplay among different factors. However, these studies did not provide evidence for the connection between T1D susceptibility and iNKT cell deficiency (18, 33–35). For instance, T1D incidence was reduced in NOD mice congenic for the Idd6 locus of the B6 allele, but the development of iNKT cells was still defective in these mice (33). In contrast, development and cytokine production of iNKT cells were partially recovered in NOD.NKT1 mice (congenic for a B6-derived Nkt1 allele) that developed T1D as frequently as NOD mice (35), suggesting that Nkt1 locus does not encode T1D susceptibility and that incomplete restoration of iNKT cell functions could not provide T1D protection. Alternatively, it is possible that these observations argued against the linkage between T1D susceptibility and iNKT cell deficiency.

The current study presents direct evidence that impaired development of iNKT cells is a manifestation of a T1D susceptibility gene(s) in Idd9.1 sublocus. iNKT cells from NOD.R28 mice were capable of regulating diabetogenic T cells, and the T1D prevention in NOD.R201 mice depended on the development of iNKT cell population. Although resistant allele of Idd9.1 sublocus was intact in NOD.R201/Cd1d−/− mice, elimination of the development of iNKT cells almost abolished T1D protection for these mice. It is unlikely that Cd1d deficiency introduced additional T1D susceptibility, because the progression and incidence of T1D in parental NOD/Cd1d−/− mice were similar to WT NOD mice in our colony. Furthermore, we found that NOD.R28/Cd1d−/− mice were still highly resistant to T1D. These results were not surprising, and they suggest that although iNKT cell mediated regulation may be a component of protective mechanisms in NOD.R28 mice, the resistant allele of entire Idd9 locus confers multiple overlapping protective mechanisms that were still capable of protecting these animals. These observations emphasize that iNKT cell deficiency in NOD mice is a single factor that hold a delicate balance between the resistance and susceptibility to autoimmunity, but cannot determine the pathogenesis by itself.

Early expansion in the thymus indicates intensive and effective positive selection of iNKT cells in NOD.R28 and NOD.R201, but not in NOD and NOD.R11 mice. Because the positive selection for iNKT cells takes place among double positive thymocytes, a deficient positive selection in NOD mice is likely intrinsic for the T lineage. Therefore, the result of present study is consistent with early studies in which we and others found that development of iNKT cells in the NOD.scid reconstituted with pre-T cells depended on the donor origin (31, 36, 37). The positive selection of iNKT cells depends on endogenous lipid Ags displayed by CD1d molecules, and also requires homotypic interactions of Slam receptors and downstream recruitment of the adaptor SLAM-associated protein (SAP) (2, 6, 38). No difference was detected between NOD and other strains in the expression of CD1d. In contrast, it was reported that the Slamf1 expression in NOD thymocytes was low, suggesting an association of iNKT cell deficiency with Slamf1 locus on chromosome 1 (39). iNKT cell development was partially improved in NOD mice congenic for B6 allele of Slamf1 locus, however, the improvement did not alter T1D pathogenesis (35). It is possible that a high level of Slamf1 signal enhances positive selection of iNKT cells, but not their regulatory function. The gene encoding SAP resides in the X chromosome, therefore, SAP-mediated signal for positive selection would be identical in NOD, NOD.R28, and NOD.R201 mice. In contrast, analysis of DX5 expression revealed different maturation of thymic iNKT cells in NOD and NOD.R28 mice. DX5 is expressed on the semi-mature thymic iNKT cells that poise to migrate into the periphery (2). Together, our results suggest that both expansion and maturation as the results of positive selection are defective in NOD mice and these two events are responsible for iNKT cell deficiency mediated T1D susceptibility.

Lipid Ag processing and loading to CD1d molecules are critical for the development of iNKT cells (40–44). Multiple pathways may be involved in processing different glycolipids for the development of iNKT cells (5). Whether there is difference in lipid processing pathways between NOD and other strains of mice remains unknown. However, distinct abilities of DCs from NOD and NOD.R201 mice to engage and potentiate iNKT cells are implausible. These abilities depend on the levels and patterns of endogenous lipid Ags displayed by CD1d. We have found that B cells poorly engaged and potentiate iNKT cells, although they expressed CD1d at levels similar to that of DCs. Forced high expression of CD1d that was able to traffic through later lysosomes enhanced their interaction with iNKT cells. In contrast, disrupting various lipid-processing pathways in DCs abolished their ability to potentiate iNKT cells (23). Although DCs play a role in negative but not positive selection of iNKT cells (4, 45), the different interactions of iNKT cells with DCs from NOD and NOD.R201 mice may reflect a lower capacity of lipid processing in NOD mice than that in NOD.R201 mice, leading to different development of iNKT cells.

The regulatory mechanism mediated by iNKT cells remains to be identified. Th2-orientated responses have long been associated with iNKT cells and implicated as candidate (1, 15). Our results showed that the correction of iNKT cell deficiency enhanced Th2 cytokine production in both NOD.R28 and NOD.R201 mice. It might be resulted from selective expansion or differentiation of subsets of iNKT cells that preferentially produce Th2 cytokines (1, 46, 47). Different properties of DCs may also enhance the production of Th2 cytokines (48). Although the mechanism remains to be
identified, it was clear that Th2 cytokines were not required for TID prevention in NOD.R28 mice. DCs may be an important player in nNKT cell mediated immune regulation through a cytokine-independent mechanism, because the engagement with nNKT cells modulates functional status (23, 32, 49). Although DCs from NOD.R28 mice did not alter the priming of islet specific T cells in cultures, it is possible that DCs’ ability to support autoimmune responses and effector differentiation is altered in vivo by continuing interactions with nNKT cells.

Our study further defines the location of TID susceptibility gene in Idd9 locus responsible for nNKT cell deficiency. NOD.B6-Idd1Id mice harboring a B6-derived chromosome 4 interval overlapping 8 cM distal, but not proximal region of Idd9.1 locus, still suffered nNKT cell deficiency (50). The results from R201 and NOD.B6-Idd1Id mice indicate that the Idd9.1 sublocus contains different susceptibility T1D genes, and T1D-associated functional nNKT deficiency may be encoded by one such susceptibility gene at the proximal end. The identities of numerous susceptibility genes, such as Cd30, Cd137, and Tnfr2 in Idd9.2 and 9.3 loci have been proposed in the early study (20), although the candidacy of the genes still remains to be confirmed. The identity of candidate gene in Idd9.1 locus remains elusive, although the products of some genes, including Lmb1 and Csf1, are potentially associated with development and differentiation of immune cells. It is also noteworthy that this region has also been associated with multiple autoimmune syndromes in other strains (51).

Disclosures

The authors have no financial conflict of interest.

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