



New Genetically Manipulated Mice Provide Insights Into the Development and Physiological Functions of Invariant Natural Killer T Cells

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Invariant natural killer T (iNKT) cells are a unique T cell subset that exhibits characteristics of both innate immune cells and T cells. They express V α 14-J α 18 (*Trav11-Traj18*) as an invariant chain of the T cell receptor (TCR) and are restricted to the MHC class I-like monomorphic antigen presenting molecule CD1d. iNKT cells are known as immune regulators that bridge the innate and acquired immune systems by rapid and massive production of a wide range of cytokines, which could enable them to participate in immune responses during various disease states. Thus, *Traj18*-deficient mice, *Cd1d*-deficient mice, or iNKT cell-overexpressing mice such as iNKT TCR α transgenic mice and iNKT cell cloned mice which contain a V α 14-J α 18 rearrangement in the TCR α locus are useful experimental models for the analysis of iNKT cells *in vivo* and *in vitro*. In this review, we describe the pros and cons of the various available genetically manipulated mice and summarize the insights gained from their study, including the possible roles of iNKT cells in obesity and diabetes.

Keywords: invariant natural killer T cells, CD1d, *Traj18*, iPSC, obesity, adipose tissue, cloned mice, thymic differentiation

INTRODUCTION

Invariant natural killer T (iNKT) cells (also called type I NKT cells) are characterized by the expression of an invariant T cell receptor (TCR), V α 14-J α 18 (*Trav11-Traj18*) paired with V β 8.2 (*Trbv13-2*), V β 8.1 (*Trbv13-3*), V β 7 (*Trbv29*), or V β 2 (*Trbv1*) in mice and the V α 24-J α 18/V β 11-D β 2-J β 2.7 (*TRAV10-TRAJ18/TRBV25-1-TRBD2-TRBJ2-7*) pair in humans. The iNKT cells differ from classical $\alpha\beta$ T cells in recognizing (glyco)lipid antigens (Ags) in conjunction with the monomorphic MHC class I-like CD1d molecule (1, 2). The prototypic Ag recognized by iNKT cells is the glycosphingolipid α -galactosylceramide (α -GalCer), originally isolated from the marine sponge *Agelas mauritianus*. It was identified from structure-activity relationship studies around the glycosphingolipid Agelashpin 9b by the pharmaceutical division of Kirin Brewery Co. Ltd. in a screen for naturally occurring molecules that prevented tumor metastases in mice *in vivo* (3). The synthetic derivative compound, also known as KRN7000 (α -GalCer C26:0),

retains the activity of Agelasphin 9b while being much easier to synthesize (4). α -GalCer and its derivatives have been used in many different studies and are highly potent iNKT cell modulators both in humans and in mice (5, 6). Recent studies have demonstrated that iNKT cells, even though they all express the same invariant Ag receptor, can be classified into different functional subtypes, interferon (IFN)- γ -producing iNKT1, interleukin (IL)-13/IL-4-producing iNKT2, and IL-17A-producing iNKT17 (7). When activated by α -GalCer, iNKT cells rapidly produce these various types of cytokines, resulting in bystander immune modulating functions leading to activation and inhibition of various immune effector cells, including NK cells, macrophages, granulocytes, dendritic cells (DCs), basophils, and eosinophils in the innate system as well as CD4⁺ T and CD8⁺ T cells in the acquired system. Therefore, iNKT cells participate in broad spectrum regulation of immune homeostasis and in various disease states including infection, autoimmunity, allergy, antitumor responses, metabolic disorders, allograft rejection, and graft-vs-host disease (8, 9).

Numerous studies investigating the role of iNKT cells have utilized mouse models of iNKT cell deficiency. One such model directly targets J α 18 (*Trajl18*^{-/-}) (10), which is required for iNKT-TCR formation. However, the overall TCR repertoire diversity is impaired in *Trajl18*^{-/-} mice, in which *Trajl18* was replaced with a PGK-Neo^r cassette, which had inadvertent but substantial effects on transcription and TCR α gene rearrangements (11). Another model makes use of mice deficient in CD1d (*Cd1d1*^{-/-}) (12), which prevents the development of any CD1d-restricted T cells including iNKT cells. However, in mice, although not in humans, there has been a gene duplication event and so two homologous genes, *Cd1d1* and *Cd1d2*, encode CD1d proteins. Even though *Cd1d1*^{-/-}*Cd1d2*^{-/-} mice have also been developed (13, 14), the role of CD1d2 in iNKT cell development and function is still unclear. Consequently, any changes in immunological activity attributed to iNKT cells that are based on studies of *Trajl18*^{-/-} or *Cd1d*^{-/-} mice need to be reassessed.

On the other hand, mice that have been genetically manipulated to have elevated numbers of iNKT cells are also useful tools for iNKT cell study. Therefore, rearranged V α 14-J α 18 and V β 8 genes were introduced into recombination-activating gene-deficient mice, and there was preferential generation of iNKT cells but no mature B and T lymphocytes (15). iNKT-TCR α transgenic mice that overexpressed iNKT-TCR α (mV α 14-J α 18) were firstly generated by Bendelac et al. (16) resulting in preponderance of iNKT cells, while abnormal development of other immune cells were also observed. A human iNKT-TCR α (hV α 24-J α 18) transgenic mouse has also been developed by similar approach (17). Moreover, iPS cell lines obtained by reprogramming of mature iNKT cells (iNKT-iPSC) from C57BL/6 (B6) mice preferentially generate iNKT cells but no conventional $\alpha\beta$ T or $\gamma\delta$ T cells, NK cells, DCs or B cells *in vitro* (18). Furthermore, mice generated from the iNKT-iPSC had a much larger number of iNKT-like cells (19) compared to mice with a rearranged V α 14-J α 18 transgene (16). It is therefore important to compare the development and function of iNKT cells and their subtypes that differentiate *in vivo* in these iNKT cell overexpressed mice.

Trajl18-DEFICIENT AND Cd1d-DEFICIENT MICE

Because iNKT cells are highly conserved among species including mice and humans, mouse models of iNKT cell deficiency represent useful tools for the analysis of iNKT cell biology. However, as described above, the originally generated *Trajl18*^{-/-} mice (10) lack transcripts not only of *Trajl18* but also of genes encoding J α regions upstream of *Trajl18*, resulting in an almost 60% reduction in the diversity of the TCR α repertoire (11). It is possible that the lower overall $\alpha\beta$ TCR diversity of the original *Trajl18*^{-/-} mice contributed to the divergent results that have been reported by some of the studies that used the mice. Recently, four new *Trajl18*^{-/-} mouse lines have been established by different research groups including ours. Two lines were generated by classical P1 bacteriophage cyclization recombination/locus of crossover in P1 (*Cre/loxP*) technology (20, 21), a third was generated by transcription activator-like effector nuclease (TALEN) methodology (TALEN-*Trajl18*^{-/-}) (22), and a fourth by using the clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 technology (CRISPR-*Trajl18*^{-/-}) (23). All four groups analyzed TCR α diversity in CD4⁺CD8⁺ double-positive (DP) thymocytes by next-generation sequencing and found that the usage frequency of J α gene segments in these new *Trajl18*^{-/-} mouse lines was comparable with WT B6 mice (20–23).

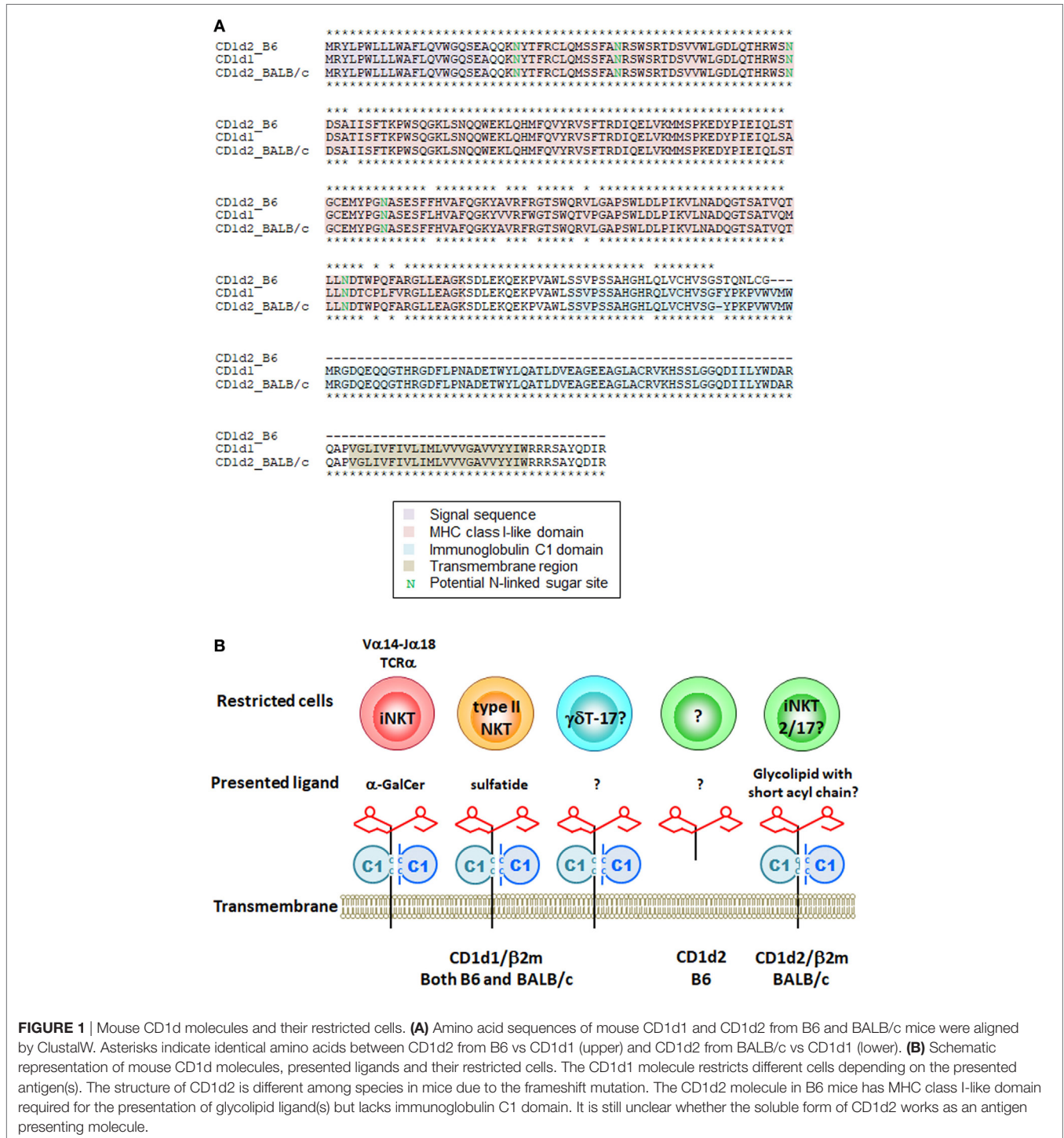
In addition to canonical V α 14-J α 18 iNKT cells, another minor α -GalCer/CD1d reactive subset of T cells harboring *Trav10*-*Trajl50* was recently described as type Ib NKT cells (24). However, type Ib NKT cells were discovered in mice that lack expression of *Trajl* gene segments upstream of *Trajl18* (10). We (23) and Chandra et al. (20) could not detect any type Ib NKT cells in the new mouse strains lacking iNKT cells. By contrast, Zhang et al. (22) did find type Ib NKT cells in their TALEN-*Trajl18*^{-/-} mice. However, these mice express *Trav11*-*Trajl18* mRNA with a partial deletion, indicating that a mutant iNKT-TCR α has the unexpected ability to recognize α -GalCer/CD1d. Based on these results, we should rethink the existence of type Ib NKT cells.

It is known that iNKT cells are restricted by CD1d molecules, but that two CD1d isoforms, CD1d1 and CD1d2, are present in mice. Two gene manipulated lines has been developed, *Cd1d1*^{-/-} (12) and *Cd1d1*^{-/-}*Cd1d2*^{-/-} (13, 14). iNKT cells are severely impaired in both lines, indicating that CD1d2 cannot substitute for CD1d1 to support iNKT cell development. CD1d1 and CD1d2 in 129/Sv mice share 93% amino acid identity. Although CD1d2 on thymocytes cannot substitute for the development of iNKT cells (25), we cannot eliminate the potential role of CD1d2 in the development or function of iNKT cells. Sundararaj et al. (26) recently reported that the structure of the CD1d2 A'-pocket was restricted in size compared with CD1d1 in complex with endogenous lipids or a truncated acyl-chain analog of α -GalCer. They found that the majority of iNKT cells in the *Cd1d1*^{-/-} mice showed an increase in the iNKT2 and iNKT17 populations and a concomitant decrease in iNKT1 compared with WT mice (26). A small but consistent increase in the proportion of cells using the V β 8 gene segment, concomitant to a reduction in V β 7 gene usage, was also observed for CD1d2-selected iNKT cells

compared with CD1d1-selected iNKT cells (26). B6 mice, but not BALB/c or 129/Sv mice, harbor a two-nucleotide insertion in exon 4 of *Cd1d2*, which encodes the $\alpha 3$ domain (27). This frameshift mutation introduces a stop codon, abolishing surface expression but possibly still allowing expression of a soluble CD1d2 molecule (Figure 1A). These results indicate that the CD1d2 molecule can present different sets of self-antigen(s) in the thymus of different mouse strains, thereby potentially impacting

the development of iNKT cells. Even though *Cd1d2*^{-/-} mice have not yet been established, they should provide an answer to this controversy.

CD1d is also an Ag presenting molecule for cells other than iNKT cells. Another type of CD1d-restricted cell is the type II or variant NKT cell, which has a more diverse TCR repertoire and appears to recognize various lipid Ags including sulfatides, also known as 3-O-sulfogalactosylceramide, which are a class



of glycolipids that contain a sulfate group (28). Unfortunately, no tools are yet available that can be used to analyze the entire population of type II NKT cells. Thus, when we discuss type II NKT cells, it is important to understand the advantages and limitations of each experimental tool, as well as the precise definition of type II NKT cells being analyzed (29). There is also a report that the homeostasis of liver-resident IL-17A-producing $\gamma\delta T$ ($\gamma\delta T$ -17) cell depends on hepatocyte-expressed CD1d that presents lipid Ag (30); however, it is unclear whether CD1d is required for the development of $\gamma\delta T$ -17 cells in the thymus (Figure 1B).

Despite a high degree of conservation, subtle but important differences exist between the CD1d Ag presentation pathways of humans and mice. Wen et al. (31) have generated a human CD1d knock-in mouse (hCD1d-KI) which substitute mouse *Cd1d1* to human *CD1D* locus. Reduced numbers of iNKT cells were observed, but at an abundance comparable to that in most humans. They further generated human iNKT-TCR α chain knock-into the hCD1d-KI (32). Similar to humans, the mice developed a subset of CD8 $\alpha\beta^+$ iNKT cells among other human-like iNKT subsets. The results support human *CD1D* is functionally and phenotypically ortholog of mouse *Cd1d1*. These hCD1d-KI mice will allow more accurate *in vivo* modeling of human iNKT cell responses as some human pathogens specifically target human CD1D for pathogenicity and will facilitate the preclinical optimization of iNKT cell-targeted immunotherapies.

INKT CELLS AND OBESITY

Obesity research is an illustrative example of how the different genetically engineered animals have been employed to study the role of iNKT cells in a complex disease. Both the original *Tra18*^{-/-} (10) and the *Cd1d*^{-/-} (12–14) mice have been used by many different research groups to study the role of iNKT cells and/or type II NKT cells in obesity-related pathologies in high fat diet (HFD)-induced obesity models. However, these studies have reported very conflicting results, with some groups finding no effect (33, 34), some protection (35–37) and others finding promotion (38, 39) of obesity-associated disease.

Among these studies, only one paper, that published by Lynch et al. (33), has focused on the protective role of iNKT cells in obesity by studying HFD-induced obese *Tra18*^{-/-} mice on a B6 background. In this study, they found that when mice lacking iNKT cells were placed on an HFD they showed enhanced weight gain, larger adipocytes, fatty livers, and insulin resistance. By contrast, many other research groups suggested a pathogenic role of iNKT cells in obesity by showing an ameliorated metabolic phenotype in HFD-induced obese *Tra18*^{-/-} or *Cd1d*^{-/-} mice. In the studies that used B6 background *Tra18*^{-/-} mice, Wu et al. (39) reported ameliorated hepatic steatosis, glucose tolerance, and insulin sensitivity, as well as reduced tissue inflammation in *Tra18*^{-/-} mice on an HFD. Pathological roles of NKT cells in obesity were also reported by Satoh et al. (38) in *Cd1d*^{-/-} mice; however, no difference in the metabolic parameters between *Tra18*^{-/-} and WT B6 mice on an HFD was observed in their study, arguing for a pathogenic role of type II NKT rather than iNKT cells in these pathologies. Similarly, Kotas et al. (40) and Lee et al. (41) have also reported a minor role of iNKT cells in the

development of obesity, by comparing *Tra18*^{-/-} and *Cd1d*^{-/-}*Cd1d2*^{-/-} mice with WT B6 mice on an HFD.

Many reasons for these divergent results have been proposed and discussed, including the age, gender, and background of the mice, HFD type and duration, and the gut flora or environmental microbial distribution among the animals employed by the different research groups. Nevertheless, if we focus only on the results obtained from B6 background *Tra18*^{-/-} mice, it is interesting to note that most of the studies have used an HFD of 60% fat calories, and none of them have reported a decreased level of weight gain in *Tra18*^{-/-} mice as compared with WT B6 mice (36–40). It seems that a consensus has been reached that iNKT cells do not participate in promoting the development of obesity, at least as measured by gain in bodyweight.

To exclude the possible effect of impaired TCR repertoire diversity on diet-induced obesity observed in the original *Tra18*^{-/-} mice (10, 11), we re-investigated the contribution of iNKT cells to the development of obesity using our CRISPR-*Tra18*^{-/-} mice (23) with an unbiased TCR repertoire. The results were clear cut, obese CRISPR-*Tra18*^{-/-} mice gained less body weight and had smaller visceral fat-pads and adipocytes, less fat deposits in the liver, and ameliorated glucose tolerance and insulin resistance (23). The ameliorated levels were almost equivalent to those seen in obese *Cd1d*^{-/-} mice, indicating that iNKT cells play a pathogenic role in diet-induced obesity and that the impact of CD1d deficiency on metabolism is iNKT cell dependent.

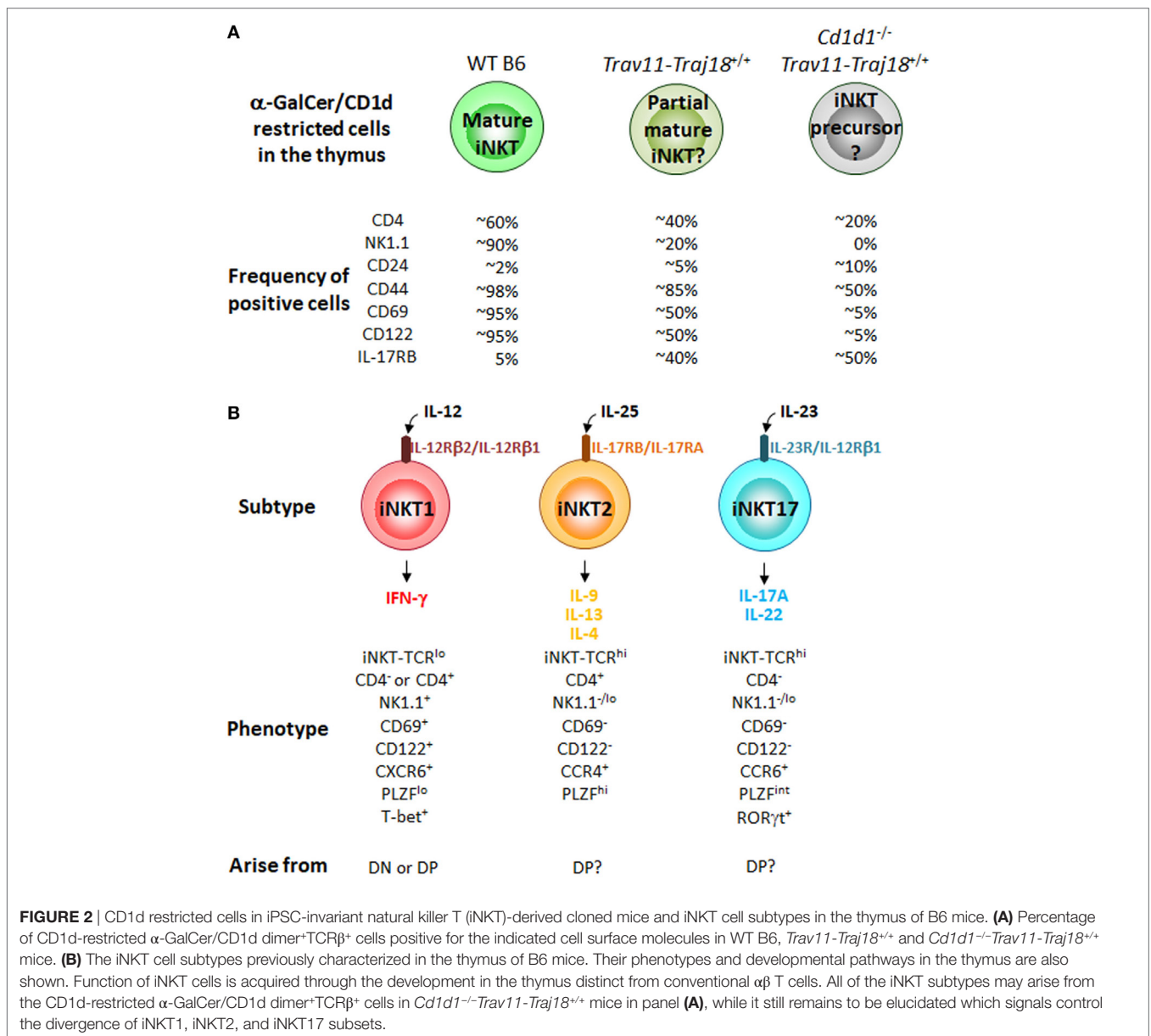
It is notable that one of the T cell populations affected by impaired TCR repertoire diversity in the original *Tra18*^{-/-} mice, the mucosal-associated invariant T (MAIT) cells that use the invariant $V\alpha 19$ -J $\alpha 33$ (*Trav1*-*Traj33*) chain in mice (42), were recently reported to have an altered distribution and cytokine productions in obese patients, and were found to be positively associated with insulin resistance (43, 44). Considering the potential role of MAIT cells and other T cell subsets in obesity, results obtained with the original *Tra18*^{-/-} mouse model should be interpreted with caution.

INKT-TCR α TRANSGENIC AND INKT CELL CLONED MICE

Mice that have been genetically manipulated to have elevated numbers of iNKT cells were first attempted to be generated by over-expressing $V\alpha 14$ -J $\alpha 18$ iNKT TCR α (m $V\alpha 14$ -J $\alpha 18$) as a transgene (16). Consistent with the results from *Cd1d*-deficient mice (12–14), the m $V\alpha 14$ -J $\alpha 18$ transgenic mice exhibited increased IL-4 and immunoglobulin (Ig) E in serum, indicating that mouse iNKT cells are one of the important sources of IL-4 and IgE. Because both human and mouse iNKT cells are restricted to α -GalCer/CD1d, a human iNKT TCR α (h $V\alpha 24$ -J $\alpha 18$) transgenic mouse has also been developed (17). Interestingly, analysis of the mice and derived h $V\alpha 24$ -J $\alpha 18^+$ T cells revealed that type 1 diabetes [insulin-dependent diabetes mellitus (IDDM)] is associated with an extreme T helper (Th) 1 phenotype of h $V\alpha 24$ -J $\alpha 18^+$ T cells, suggesting that there is a strong link between h $V\alpha 24$ -J $\alpha 18^+$ T cells and human type 1 diabetes. On the other hand, there is evidence that IL-4 exerts a dominant-negative effect on the progression to IDDM in non-obese diabetic (NOD)

mice (45–47), and NOD mice with the mV α 14-J α 18 transgene were protected from diabetes (48), indicating that not only the number but also the phenotype of iNKT cells influences the incidence of diabetes both in humans and mice. The fact that the gut microbiota can impact iNKT cell development and functions (49–51) and is associated with diabetes onset, regulatory imbalance, and IFN- γ levels in NOD mice should be also considered (52). Another iNKT cell-overexpressing mouse line was derived from iPSCs. iPSCs hold tremendous potential for applications not only in drug discovery, regenerative medicine, and cell replacement therapy (53–55), but also in basic biology. We have succeeded in reprogramming splenic iNKT cells from WT B6 mice (18). These iPSC-iNKT cells could be differentiated into iNKT cells *in vitro* and secreted large amounts of IFN- γ . Importantly, iPSC-iNKT cells recapitulated the known adjuvant

effects of natural iNKT cells and suppressed tumor growth *in vivo*. These studies demonstrate the feasibility of expanding functionally competent iNKT cells *via* an iPSC phase, an approach that may be adapted for iNKT cell-targeted therapy in humans (56, 57). We further succeeded in generating iNKT cell cloned *Trav11-Traj18*^{+/+} mice from iPSC-iNKT cells through germline transmission and breeding with WT B6 mice (19). The absolute numbers and percentages of α -GalCer/CD1d dimer⁺ TCR β ⁺ cells in the thymus and periphery of *Trav11-Traj18*^{+/+} mice were elevated by 10–20-fold compared to B6 mice and 10–20-fold compared to B6 mice and by 3–10-fold compared to iNKT-TCR α transgenic mice due to the bypass of TCR α rearrangement at the double-negative (DN) stage. They lacked $\gamma\delta$ T cells due to the deletion of the δ locus and had reduced numbers of $\alpha\beta$ T cells while NK, B, and DC numbers were normal. However, the surface phenotype of α -GalCer/CD1d



dimer⁺ TCRβ⁺ cells in *Trav11-Traj18^{+/+}* mice was different from that in WT B6 mice; there was a partial reduction of CD44⁺ cells and changes in the CD4⁺:NK1.1⁺ ratio (19). We think this is due to the shortage of CD1d molecules in the face of an excess number of α-GalCer/CD1d dimer⁺TCRβ⁺ cells because the surface phenotype of the iNKT cells changed into the mature phenotype as seen in WT B6 when these cells were sorted and transferred into *Traj18^{-/-}* mice (58). Generation of *Trav11-Traj18^{+/+}* mice carrying a *Cd1d1* transgene should clarify this point.

Trav11-Traj18^{+/+} mice on a *Cd1d1^{-/-}* background have also been generated (59). Interestingly, these mice have thymic CD1d-restricted α-GalCer/CD1d dimer⁺TCRβ⁺ cells, which are considered to be iNKT cells before CD1d selection. The frequency of positive cells of CD44, CD4, and NK1.1 by thymic α-GalCer/CD1d dimer⁺TCRβ⁺ cells from *Cd1d1^{-/-}Trav11-Traj18^{+/+}* mice is further lower than those from *Trav11-Traj18^{+/+}* mice (Figure 2A), suggesting that CD1d plays a role in the induction of these surface molecules on iNKT cells.

INKT CELL DEVELOPMENT IN THE THYMUS

Until recently, the iNKT cell field had embraced a sequential lineage developmental model (60) in which “developmental intermediates” produce Th2-type cytokines and “mature” NK1.1⁺ iNKT cells produce Th1 cytokines. However, based on the finding of the expression of distinct transcription factors, T-bet (*Tbx21*), PLZF (*Zbtb16*), and RORγt (*Rorc*) (61) and surface markers, CD4, NK1.1, and IL-17RB (7) in iNKT cell subsets, we considered an alternative “lineage diversification” model for iNKT cells (62), analogous to the differentiation of effector Th cells (63) and innate lymphoid cells (64, 65). Three major subsets of iNKT cells (iNKT1, iNKT2, and iNKT17) that produce distinct cytokines have been defined (7, 61, 66, 67) (Figure 2B), and these represent diverse lineages and not developmental stages, as previously thought. In fact, it was recently reported that some iNKT1 cells developed through an alternative DN pathway that bypasses the DP pathway (68), supporting the above findings that iNKT subtypes possibly arise from different precursors in the thymus. Of note, thymic α-GalCer/CD1d dimer⁺TCRβ⁺ cells from *Cd1d1^{-/-}Trav11-Traj18^{+/+}* mice described above exhibit the precursors of all iNKT cell subtypes. The poised effector state is

acquired during development in the thymus, where iNKT precursors differentiate into one of three distinct subsets, while it has still been unclear which signals control the divergence of iNKT1, iNKT2, and iNKT17. The precise molecular mechanisms should be clarified that are important for iNKT lineage diversification but are dispensable for conventional αβ T cell development. Taken collectively, it can be proposed that the acquisition of diverse functional characteristics by iNKT subtypes might be dependent on the environment providing an appropriate cytokine milieu, as well as on the cytokine receptor signaling in precursor cells undergoing CD1d selection.

CONCLUSION

The gene manipulated mice described here will reveal more insights into mouse iNKT cell development and function, and these insights should also be applicable to human iNKT cell studies. Overall, some reported differences between *Cd1d^{-/-}* and *Traj18^{-/-}* mice are likely due to the loss of some T cell populations including MAIT cells in the original *Traj18^{-/-}* mice. *Traj18^{-/-}* mice with unbiased TCR diversity will inform us of the actual role of iNKT cells and type II NKT cells. *Cd1d1^{-/-}Trav11-Traj18^{+/+}* mice may further reveal differences in iNKT cell thymic development and may account for the observed mouse strain specific differences. iNKT cells hold great promise for treatment of a myriad of diseases, and these gene manipulated mice will be invaluable in deciphering the role of iNKT cells in health and disease.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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