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*ImmuunoHorizons* 2020, 4 (12) 797-808
doi: https://doi.org/10.4049/immunohorizons.2000100
http://www.immunohorizons.org/content/4/12/797

This information is current as of January 7, 2021.

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**Supplementary Material**  
http://www.immunohorizons.org/content/suppl/2020/12/15/4.12.797.DCSupplemental

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Altered Innate-like T Cell Development in Vα14-Jα18 TCRα Transgenic Mice

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ABSTRACT

CD1d-restricted invariant NKT (iNKT) cells are innate-like T cells that respond to glycolipids, a class of Ags that are invisible to conventional T cells. iNKT cells develop in the thymus where they receive strong “agonist” TCR signals. During their ontogeny, iNKT cells differentiate into discrete INKT1, INKT2, and INKT17 effector subsets akin to helper CD4 T cells. In this study, we found that transgenic (Tg) expression of the canonical Vα14-Jα18 TCRα-chain at the double-positive thymocyte stage led to premature iNKT cell development and a cell-intrinsic bias toward INKT2 cells, due to increased TCR signaling upon selection. Consistent with the strong INKT2 bias, innate memory CD8+ T cells were found in greater numbers in Vα14 Tg mice, whereas the prevalence of mucosa-associated invariant T cells was reduced. INKT cells from Vα14 Tg mice were hyporesponsive to stimulation by their cognate Ag α-galactosylceramide. Finally, Vα14 Tg mice displayed increased B16F10 melanoma tumor growth compared with wild-type mice. This study reveals some of the limitations of Vα14 Tg mice and warrants the cautious interpretation of past and future findings using this mouse model. Immunohorizons, 2020, 4: 797–808.

INTRODUCTION

Type I or invariant NK T (iNKT) cells are thymus-derived innate-like αβT lymphocytes that play a key role in the regulation of tissue homeostasis as well as in a wide range of immune responses, including antitumoral activity (1–4). iNKT cells respond to Ags that are invisible to conventional T cells, namely endogenous and exogenous lipid-based Ags (e.g., α-galactosylceramide [αGC]) presented by the quasi-monomorphic MHC class Ib molecule CD1d (1–3). They recognize CD1d:glycolipid complexes through semi-invariant TCRs composed of a canonical and invariant Vα14-Jα18 (Vα24-Jα18 in humans) TCRα-chain paired with a limited number of Vβ segments (Vβ8, 7, and 2 in mice; Vβ11 in humans). Like classical CD4+ TH cells and innate lymphoid cells, iNKT cells are composed of discrete preprogrammed Th1-related cytokine-secreting subsets, namely INKT1 (which produce...
IFN-γ), iNKT2 (IL-4), and iNKT17 (IL-17A/F) (2, 5, 6). Effector differentiation and functional programming are believed to be initiated in the thymus during their development. This complex process involves many intrinsic and environmental cues that instigate an intense genomic reprogramming controlling differentiation and effector fate commitment. Mapping the development and functional differentiation of iNKT cells within a thymus has been extensively investigated but is still controversial.

Pioneer studies proposed a linear model of differentiation/maturation in which newly selected iNKT cells (iNKT0 or stage 0) sequentially matured through stages 1–3, characterized by the expression of CD44 and then NK1.1 (7, 8). Functionally, maturing iNKT cells progressively acquired the capacity to produce either IFN-γ or IL-17A while they concomitantly repressed/lost their ability to produce IL-4 (7, 8). More recently, the Hogquist laboratory suggested that discrete functional iNKT subsets (iNKT1, iNKT2, and iNKT17) derived from a common multipotent CCR7+ precursor (5, 9). However, these two models may not be mutually exclusive, and recent comprehensive transcriptomic-based data proposed a mixed model in which iNKT1 and iNKT17 subsets derive from iNKT2 cells (10–12). In addition, these studies suggested an additional level of heterogeneity in thymic iNKT subsets, including both intermediate precursor cells and discrete iNKT1 subsets that may exert diverse functions (10, 11).

Emerging evidence indicates that iNKT cells can influence the thymic microenvironment in several ways (2, 4). For instance, IL-4 produced by thymic iNKT2 cells conditions naive CD8+ T cells to express the transcription factor Eomesoderm (Eomes), which confers them innate memory hallmarks in the absence of cognate Ag encounter (5, 13). iNKT2-derived IL-4 also increases the number and functions of regulatory T cells (14) and together with IL-13 regulates mature T cell thymic egress (15). Interestingly, iNKT2-derived IL-4 appears to regulate the functions of immune cells beyond the lymphocyte lineage, such as CD1d2-expressing thymic dendritic cells, to produce the CCR4 ligands CCL-17 and CCL22 (5). At least some iNKT1 cells are long-term thymus residents (16), but the role these cells (as well as iNKT17 cells) may play in thymus homeostasis is unknown.

Because of the relative low iNKT cell abundance in certain tissues, mice expressing a rearranged V µ 14-Jα 14 canonical TCRα chain transgene (hereafter called Va14 transgenic [Tg] or Tg mice) have been generated (17–19) and widely used to evaluate the ontogeny of these cells, their functional properties, and their role in proof-of-concept preclinical models (19–24). To avoid untimely expression of the canonical TCRα transgene, the Bendelac group recently generated a novel Va14 Tg strain whereby the canonical TCRα transgene is expressed at the double-positive (DP) stage under the control of the minimal CD4 promoter, enhancer, and intronic silencer (19). However, whether this convenient mouse model faithfully recapitulates iNKT cell development and effector functions remains an open question.

In this study, we found that iNKT cell differentiation in Va14 Tg mice is heavily skewed toward the iNKT2 subset. This bias is cell intrinsic, imprinting early in life, and largely maintained as mice age. Of importance, newly selected iNKT0 in these mice expressed higher levels of TCRβ and the transcription factors Nur77, Egr2, PLZF, and GATA-3, reminiscent of increased TCR signaling (25–29). The development/homeostasis of some lineages of innate T cells, such as innate memory CD8+ T cells and mucosa-associated invariant T (MAIT) cells, were affected. Functionally, iNKT cells from Va14 Tg mice produced less IFN-γ and IL-4 than their wild-type (WT) counterpart in response to αGC. Finally, in line with the strong iNKT2 bias, Va14 Tg mice displayed increased B16F10 melanoma tumor growth compared with WT mice. This was associated with a T cell exhaustion phenotype in the tumor microenvironment of Va14 Tg mice. This study reveals some of the inherent limitations of Va14 Tg mice and should encourage future users to cautiously interpret their findings.

**MATERIALS AND METHODS**

**Mice and reagents**

Va14-Jµ18 Tg mice (Va14 Tg) were a generous gift from Dr. Albert Bendelac (University of Chicago, Chicago, IL). CD1D1−/−CD1D2−/− mice were generated and generously provided by Dr. Chyung-Ru Wang (Northwestern University) (30). NUR77-GFP (C57BL/6-Tg [Nρ4a1-EFP/cre]280Khog/J) (25) and CD45.1 mice (B6.SJL-PtprcPepcb/BoyJ) mice were purchased from The Jackson Laboratory. All transgenes were maintained as hemizygous. All strains were bred in-house and maintained at the Division of Comparative Medicine, University of Toronto animal facility, under specific-pathogen-free conditions. All animal procedures were approved by the Faculty of Medicine and Pharmacy Animal Care Committee at the University of Toronto (animal use protocols 20012454 and 20012439). Mice were analyzed at 6–8 wk of age, unless specified otherwise. Only littermate mice were used, except for bone marrow chimeras. αGC (KR7N7000) was purchased from Diagnomice. Abs used were purchased from eBioscience, BioLegend, or BD Biosciences. PBS57-loaded and -unloaded biotinylated CD1d monomers and 5-OP-RU- and 6-FP-loaded MRI tetraviruses were obtained from the National Institutes of Health Tetramer Core Facility. CD1d monomers were tetramerized by addition of fluorochrome-conjugated streptavidin.

**Bone marrow chimeras**

Bone marrow was collected from femur and tibia of C57BL/6 CD45.1 congenic mice and Va14 Tg (CD45.2) mice and injected i.v. (2 × 10⁶ cells per recipient) either separately or in a 3:1 (WT/Va14 Tg) ratio into lethally irradiated mice (2 × 550 cGy). Mice were given neomycin-supplemented water (2 g/l neomycin sulfate) for 2 wk postirradiation. Mice were bled 6 wk postinjection, and blood cells were stained for CD45.1 and CD45.2 to assess chimerism by flow cytometry. Mice were analyzed 9 wk posttransfer and analyzed by flow cytometry.

https://doi.org/10.4049/immunohorizons.2000100
Flow cytometry
Cells were stained as previously described (31). After staining with LIVE/DEAD Fixable Aqua and Abs for surface markers, cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and stained with Abs against selected cytokines for 30 min at room temperature. For the detection of transcription factors, cells were fixed and permeabilized using the Intracellular Fixation and Permeabilization Kit (eBioscience) and stained with Abs against transcription factors for 30 min. In some experiments, iNKT cells were first stained with PE-labeled CD1d tetramers and then magnetically enriched using the EasySep PE Positive Selection Kit (STEMCELL Technologies). Samples were acquired on the LSR II, LSR Fortessa, and LSR Fortessa X-20 cytometers in the Faculty of Medicine Flow Cytometry facility at the University of Toronto.

Stimulation assays
For in vitro stimulations, 5–10 × 10^6 cells were cultured in 48-well plates (500 ml per well) and stimulated with PMA (100 ng/ml) and ionomycin (1 μg/ml) or the respective amount of vehicle DMSO in complete RPMI 1640 for 4 h at 37°C in the presence of a Protein Transport Inhibitor Cocktail (Thermo Fisher Scientific). For in vivo stimulations, mice were injected with 0.5 μg of αGC, and spleen and liver cells were collected after 90 min. Cells were then analyzed by flow cytometry.

B16F10 tumors
C57BL/6 WT and Vo14 Tg mice were s.c. injected in the left flank with 5 × 10^5 B16F10 cells in 100 μl of PBS. Tumor growth was measured every day, and tumor volume was calculated as follows: tumor volume = length × width^2 × 0.52. Mice were euthanized when tumors reached 2000 mm^3. Tumors and inguinal lymph nodes (iLNs) (draining and contralateral) were harvested and processed into single-cell suspensions. Tumors were minced to 1-mm pieces and digested with 1 mg/ml type IV collagenase and 0.1 mg/ml DNase in a shaker at 150 rpm at 37°C for 30 min. After filtration, cells were separated using a 33% Percoll gradient. Cells were then analyzed by flow cytometry.

H&E staining and imaging
Whole thymi were fixed in formalin (Sigma-Aldrich) for several days at 4°C and submitted to The Centre for Phenogenomics (Mount Sinai Hospital and The Hospital for Sick Children) for embedding, sectioning, and H&E staining. H&E-stained slides were viewed and imaged using the Zeiss Axiocam 506 Mono microscope and ZenBlue software, respectively.

Data analysis
GraphPad Prism was used to calculate the normality of data sets and apply the appropriate statistical tests, typically Mann-Whitney or Student t test, as indicated. For comparisons of cell origins within chimeras, the nonparametric Wilcoxon signed-rank paired t test was used. Tumor growth curves were longitudinally analyzed with type II ANOVA and pairwise comparisons using the Tum-Growth software tool (32). The p values are as follows: *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

RESULTS

Cell-intrinsic iNKT2 bias in Vo14 Tg mice
Vo14 Tg mice have been widely used to study the development and functions of iNKT cells. Mice expressing the rearranged Vo14-Jo18 canonical TCRα-chain under the control of the minimal CD4 promoter and enhancer (19) (hereafter named Vo14 Tg or Tg) have increased frequency of up to 50-fold iNKT cells in all tissues tested, relative to WT littermate control mice (Fig. 1A). In addition, thymic cellularity was reduced by up to 75% in adult Vo14 Tg mice compared with WT mice (Fig. 1B), which is in agreement with findings in other TCRα Tg mice (18, 33, 34). We found a significant increase of stage 1 (CD44^− NK1.1^−) and stage 2 (CD44^+ NK1.1^−) iNKT cells paralleled with a decrease in stage 3 (CD44^+ NK1.1^+) iNKT cell frequency in the thymus of Tg mice as compared with WT mice (Fig. 1C), in agreement with a previous study (34). The absolute number of stage 1 and 2 iNKT cells was increased in Tg mice, whereas stage 3 iNKT cell numbers were not affected (Fig. 1C). Similar results were also found in peripheral tissues, except the liver, in which the differences were minimal (Supplemental Fig. 1A). We also found an increased frequency and absolute number of iNKT2 cells in the thymus of Tg mice, whereas the proportion, but not absolute number, of iNKT1 and 17 was decreased (Fig. 1D). This suggests that iNKT2 cells account for the higher number of stage 1 and 2 iNKT cells. Similar findings were observed in peripheral tissues, although the results were somewhat more heterogeneous (Supplemental Fig. 1B). Therefore, iNKT cell effector differentiation is skewed toward iNKT2 in Vo14 Tg mice.

Growth software tool (32). The endogenous rearrangement of the Vo14 and Jo18 gene segments occurs 1–2 d before birth (35), and only very few immature iNKT cells can be detected in newborn mice following enrichment procedures (36). We found that Vo14 Tg and WT 3-d-old littermate mice had similar thymic cellularity (Fig. 2A). However, whereas iNKT cells were barely detectable in WT pups, these cells were already found at high frequency in Vo14 Tg mice (Fig. 2B), which indicates precocious expression of the TCRα transgene and therefore premature iNKT cell development. When
compared with adult WT thymi, 3-d-old Tg thymi showed increased proportions of stage 1 and 2 iNKT cells and a strong decrease in stage 3 iNKT cells (Fig. 2C). The frequency of iNKT2 and 17 was increased in the thymus of 3-d-old Tg mice, compared with WT adult mice, whereas the frequency of iNKT1 was reduced (Fig. 2D). Therefore, iNKT cell development and biased effector differentiation in Vα14 Tg mice occurs very early in life.

Previous studies have shown that the population of iNKT2 cells tends to decrease with age (5, 31). Therefore, we investigated whether the increased prevalence of iNKT2 cells would be normalized in older Vα14 Tg mice. We found that 20-wk-old Tg mice maintained the reduced thymic cellularity (Fig. 2E) and increased thymic iNKT cell prevalence (Fig. 2F) observed in adult mice. Similarly, increased frequency of stage 1 and 2 iNKT cells as well as an increase in iNKT2 cell proportion and decrease in iNKT1 prevalence were maintained in the thymus and peripheral tissues of 20-wk-old Tg mice, compared with littermate WT mice (Fig. 2G, 2H, Supplemental Fig. 2A, 2B). These results indicate that the iNKT2 cell subset bias is maintained in Vα14 Tg mice as they age.

Increased TCR expression and signaling in Tg iNKT cells
The sharp iNKT2 bias observed in Vα14 Tg led us to investigate TCR signaling in these mice (37, 38). For this, we crossed Vα14 Tg mice with Nur77GFP reporter mice and measured Nur77GFP expression levels in iNKT cells as a proxy for TCR signal strength (25). Total thymic iNKT cells from Vα14 Tg Nur77GFP mice expressed higher levels of Nur77GFP compared with littermate Nur77GFP controls (data not shown). Next, we assessed Nur77GFP expression in different iNKT cell subsets following magnetic enrichment. We found that iNKT0 (defined as CD69+ CD24hi), iNKT1 (CD24+ Sdc1− NK1.1+), and iNKT2 (CD24+ Sdc1− NK1.1−) all expressed higher levels of Nur77GFP in Vα14 Tg Nur77GFP mice compared with littermate Nur77GFP control mice (Fig. 3A). The highest increase in Nur77GFP expression was seen within iNKT0, suggesting that iNKT cells in Vα14 Tg mice receive stronger TCR signaling from the earliest stage of development. In addition, the expression of the transcription factors Egr2, PLZF, and GATA-3, which are induced by TCR signaling (27, 29, 39), was also increased in iNKT0 cells from Vα14 Tg mice compared with WT mice.

FIGURE 1. Cell-intrinsic biased iNKT cell effector differentiation in Vα14 Tg mice.
(A) Thymus, spleen, liver, iLNs, and mLN were obtained from 6- to 8-wk-old WT and Vα14 Tg (Tg) mice. Representative FACS plots (left panel) and frequency of iNKT cells (right panel) out of live CD45+ CD19− cells are shown. (B) Absolute number of thymocytes in WT and Tg mice. (C) Representative FACS plots (left), frequency (middle), and absolute numbers (right) of stage 1, 2, and 3 iNKT cells in the thymus. (D) Representative FACS plots (left), frequency (middle), and absolute numbers (right) of iNKT1, 2, and 17 cell subsets in the thymus. (A–D) Data show individual mice and the mean values ± SEM. *p < 0.05, **p < 0.01, ****p < 0.0001, Mann-Whitney. (E) Bone marrow chimera schematic (top panel). Frequency of iNKT1, 2, and 17 cell subsets and strain of origin (CD45.1 or CD45.2) in thymus, spleen, and iLNs from chimeras (bottom panels) out of live CD19− CD24− cells (six mice per group). *p < 0.05, **p < 0.01, Wilcoxon signed-rank paired t test.

https://doi.org/10.4049/immunohorizons.2000100
mice (Fig. 3B–D). iNKT2 cells from Vα14 Tg mice expressed lower levels of Egr2 and GATA-3 compared with WT iNKT2 cells, whereas their expression in iNKT1 and iNKT17 subsets was similar between strains (Fig. 3B, 3C). All iNKT subsets expressed higher TCRβ levels in the thymus and peripheral tissues of Vα14 Tg mice (Fig. 3E, Supplemental Fig. 3). However, CD1d tetramer binding avidity was unaffected, except for iNKT2 cells, which showed reduced CD1d tetramer binding avidity in Vα14 Tg compared with WT mice (Fig. 3F). These findings are consistent with a previous study that showed lower CD1d tetramer binding in stage 1 iNKT cells from Vα14 Tg thymi as well as reduced expression of Egr2 and GATA-3 in iNKT cells at stages 1 and 2 (34). Nur77 expression was reduced in TCRβ+ thymocytes from Vα14 Tg mice, which was limited to CD8+ TCRβ+ thymocytes (Fig. 3G).

Next, we attempted to correct the iNKT2 bias by reducing the expression of CD1d in these mice. For this, we crossed Vα14 Tg mice with CD1d+/− mice and evaluated the iNKT effector subset distribution. All CD1d+/− mice, regardless of Vα14 transgene expression, showed reduced CD1d levels in thymocytes compared with CD1d+/− littermates (Fig. 3H). Despite this, the phenotype of iNKT cells from Vα14 Tg CD1d+/− mice and Vα14 Tg CD1d+/− littermates was identical (Fig. 3I). In summary, these data show that iNKT cells in Vα14 Tg mice express higher levels of TCR and sustain greater TCR signaling than in WT mice at the early stage of development and that the iNKT2 bias cannot be reversed by reducing CD1d expression in thymocytes.

T cell development in Vα14 Tg mice
Given the reduced thymic cellularity, and the roles iNKT cell subsets may play in thymic homeostasis (2), we sought to investigate whether T cell development was affected in Vα14 Tg mice. First, H&E staining revealed that the overall thymic architecture was preserved and that defined cortex and medulla could be found in both genotypes, with perhaps more diffused medullary regions in Tg mice (Fig. 4A). Next, we analyzed thymocyte development, excluding PBS57-CD1d tetramer-positive thymocytes from the analysis because of their abundance in Tg mice (Fig. 4B). The frequency and absolute numbers of DP thymocytes were reduced in Vα14 Tg mice compared with WT controls. The frequency and absolute numbers of DN thymocytes were increased, and the absolute numbers were unaffected. The frequency of CD4 SP thymocytes was unchanged, but their absolute number was slightly reduced. Finally, the distribution and absolute numbers of the different DN stages (DN1–4) were similar between Vα14 Tg and WT mice. Consistent with the drastic reduction of DP cells, we also detected a higher frequency of cell death in Vα14 Tg thymi (Fig. 4C), suggesting that thymocytes may be dying at the DP stage. In mixed bone marrow chimeras, cell death was cell intrinsic, and significantly increased in CD45.2 (Tg) thymocytes, compared with CD45.1 (WT) thymocytes (Supplemental Fig. 4A). In addition, although CD1d expression by thymocytes is reduced in CD1d+/− Vα14 Tg mice (Supplemental Fig. 4B), this suggests that iNKT cells do not directly kill thymocytes. Moreover,
immature thymocytes (CD24+) expressed higher TCRβ levels in Vα14 Tg mice (Fig. 4D), suggesting that more immature TCR-expressing DPs accumulate in Vα14 Tg thymi. Therefore, we assessed the expression of CD5 and CD24, which are upregulated and downregulated upon positive selection, respectively (40–42). The frequency of TCRβhi CD5+ and TCRβhi CD24hi thymocytes was increased in Vα14 Tg mice, although their absolute numbers were reduced (Fig. 4E). Among DP thymocytes, the slight increase in the frequency of positively selected and mature thymocytes was not significant, but their absolute numbers were reduced in Vα14 Tg mice (Fig. 4F). Taken together, these results suggest that many of the immature DP thymocytes fail to be positively selected and die by neglect, which likely contributes to reduced thymus size.

**Altered cytokine production in Vα14 Tg mice**

In the thymus, iNKT cells produce IL-4 at steady state, which can impact the development and function of other cells (2, 5). Upon in vitro stimulation with PMA and ionomycin, IL-4+ iNKT cells were found at higher frequencies in Tg mice, but this was only significant in the thymus (Fig. 5A, 5B). IFN-γ+ iNKT cells were significantly reduced in the spleen of Tg mice (Fig. 5B). IL-17A+ iNKT cells were reduced in the thymus and spleen from Tg mice (Fig. 5A, 5B). Similar frequencies of TNF-α+ iNKT cells were found in the two groups of mice in all tissues tested (Fig. 5B). However, i.v. injections of the cognate iNKT cell ligand αGC led to severely blunted IFN-γ and IL-4 production in the spleen and liver of Vα14 Tg mice, compared with WT mice (Fig. 5C). The proportion of IL-17A+ iNKT cells was also reduced in livers from Vα14 Tg mice (Fig. 5C). These data show that peripheral iNKT cells found in Vα14 Tg mice are hyporesponsive to TCR-mediated stimulation.

**Homeostasis of Eomes+ CD8 T cells and MAIT cells is affected in Vα14 Tg mice**

IL-4 released by iNKT cells at steady state is required for the development of innate memory Eomes+ CD8 T cells (5, 13) and Cxcl10+ MAIT cells (49). Consistent with the higher frequency of IL-4+ iNKT cells in the thymus, Vα14 Tg mice exhibited increased frequencies and absolute numbers of Eomes+ CD8 T cells in the thymus and peripheral tissues.
Innate-Like T Cell Development in Va14 Tg Mice

Increased numbers of Eomes+ CD8 T cells persisted in 20-wk-old mice (Supplemental Fig. 2C, 2D). A recent study suggested that iNKT and MAIT cells compete for similar resources and/or niche (43). In agreement with this, we found that both the frequency and absolute numbers of MAIT cells were reduced in most tissues from Va14 Tg mice respective to WT mice, except for the mesenteric lymph node (mLN) and thymus, where they were unchanged and increased, respectively (Fig. 6B). Moreover, the phenotype of these MAIT cells was also affected in peripheral tissue of Va14 Tg mice, with a consistent reduction of RORγt+ MAIT17 cell frequency and concomitant increase in the proportion of T-bet+ MAIT1 cells (Fig. 6C). We found no significant difference in the numbers and subset distribution of γδ T cells between WT and Va14 Tg mice (data not shown). Altogether, these results show that numerical and/or functional alterations of iNKT cells in Va14 Tg mice are associated with changes in the homeostasis of other innate-like T cells.

FIGURE 4. T cell development in Va14 Tg mice. (A) H&E histological analysis of 6- to 8-wk-old WT and Tg mice (three mice per group). (B) CD4 and CD8 expression on CD1d tetramer-negative thymocytes from WT and Tg mice (top panels). CD25 and CD44 expression on CD1d tetramer-negative CD4/8+ thymocytes (bottom panels). Data show representative plots (left panels) and mean values ± SEM (right panels). (C) Frequency of LIVE/DEAD Fixable Aqua-positive thymocytes from WT and Tg mice. (D) Representative histogram of TCRβ expression by immature CD24+ CD1d tetramer-negative thymocytes from WT and Tg mice. (E) Frequency and absolute numbers of TCRβ+ CD5+ (top panels) and TCRβ+ CD24+ (bottom panels) in CD1d tetramer-negative thymocytes. Data show mean values ± SEM (left panel) and representative FACS plots (left panels). (F) Frequency and absolute numbers of TCRβ+ CD5+ (top panels) and TCRβ+ CD24+ (bottom panels) in CD1d tetramer-negative CD4/8 DP thymocytes. Data show mean values ± SEM (left panel) and representative FACS plots (right panel). Data combined from two to three independent experiments. *p < 0.05, **p < 0.001, ****p < 0.0001, Mann-Whitney.

Va14 Tg mice have higher susceptibility to B16F10 tumor development

Given the well-documented antitumor role of iNKT cells, we decided to investigate whether tumor development was affected in Va14 Tg mice using the B16F10 melanoma model. We injected B16F10 melanoma cells s.c. in one of the flanks of Va14 Tg and WT mice, monitored tumor growth, and analyzed tumor-infiltrating lymphocytes (TILs). Tumor growth was accelerated in Va14 Tg mice (Fig. 7A), which could not be prevented by i.p. αGC injections (data not shown). This suggested that despite having increased numbers of iNKT cells, these mice were not able to efficiently control tumor growth. iNKT cells could be readily identified among TILs in both groups of mice, albeit at a higher frequency in tumors recovered from Va14 Tg mice (Fig. 7B). Interestingly, most of the iNKT cells within WT TILs were T-bet+ iNKT1 cells, whereas iNKT17 cells were rare and iNKT2 cells were barely detectable (Fig. 7C). TILs recovered from Va14 Tg mice contained a significant increase in iNKT2 cells and a concomitant decrease in iNKT1 and absence of iNKT17 cells (Fig. 7C). In addition, we observed a reduced frequency of CD69+ iNKT cells (Fig. 7D) and higher proportion of PD-1+ T cells (Fig. 7E) in TILs from Va14 Tg mice compared with WT mice, suggesting reduced iNKT cell activation and increased T cell exhaustion. Altogether, these results indicate that Va14 Tg mice have an altered tumor immune environment and higher susceptibility to B16F10 tumor development.

https://doi.org/10.4049/immunohorizons.2000100
DISCUSSION

Vo14 Tg mice have been widely used for the study of iNKT cells because of the increased number of these otherwise rare cells (17–19). In this article, we show that iNKT cells develop prematurely in these mice, with a cell-intrinsic bias toward iNKT2, as well as functional alterations, which ultimately leads to a reduced capacity to promote tumor rejection.

iNKT cell selection requires stronger TCR signals than conventional T cells. Within this range, higher TCR signaling favors iNKT2 differentiation, whereas intermediate and low TCR signaling are associated with iNKT17 and iNKT1 differentiation, respectively (5, 37, 38, 44, 45). In Vo14 Tg mice, iNKT cells express higher TCR levels, starting with iNKT0. However, only iNKT2 have lower CD1d tetramer binding avidity. This is in agreement with a recent study that reported lower CD1d tetramer mean fluorescence intensity for stage 1 iNKT cells in these mice (34). In our study, lower Nur77, Egr2, and GATA-3 expression at stage 1/2 led the authors to conclude that the altered phenotype of iNKT cells in Vo14 Tg mice was due to reduced TCR signaling and a block at stage 1. Although TCR signaling was not assessed in iNKT0 cells, these authors found lower TCR expression in this population from Vo14 Tg mice. In our study we found that iNKT0 from Tg mice had higher expression of TCRβ, Nur77, Egr2, PLZF, and GATA-3 compared with WT iNKT0, which is reminiscent of increased TCR signaling during selection and may favor subsequent differentiation toward iNKT2 (25–29, 39). In such a scenario, excessive TCR signals may trigger negative selection, which could explain the lower TCR avidity found in iNKT2 as well as some of the phenotypic and functional alterations we reported (46). The Joseph et al. (34) study used magnetic depletion of CD8+ cells, which could exclude some newly selected iNKT0 that have not yet downregulated CD4 and CD8. In addition, we detected a high proportion of CD24+ CD69* cells among thymic Vo14 Tg iNKT cells (data not shown), which we excluded from our analysis to focus on positively selected iNKT0 cells. Increased TCR signaling and GATA-3 expression may also lead to aberrant expression of Th-POK, which has been shown to regulate the effector differentiation and functional response of iNKT cells (47–50).

Some thymic iNKT2 cells produce IL-4 at steady state, which influences the development and function of other cells in the thymic microenvironment (5), which includes the differentiation of CD8* SP thymocytes into memory-like Eomes* cells (5, 13). In line with this, Vo14 Tg thymi showed increased frequency of IL-4* iNKT cells after in vitro PMA/ionomycin stimulation and higher...
abundance of Eomes$^+$ CD8 T cells. Thymic IL-4 can also induce the egress from mature CD4$^+$ SP (15), and this possibly accounts for the reduced number of CD4$^+$ SP thymocytes in V$\alpha_{14}$ Tg mice.

MAIT cell numbers are found in larger numbers in CD1d-decient mice (43) and are reduced in peripheral tissues of V$\alpha_{14}$ Tg mice. This suggests that these two populations of innate-like T cells may compete for limited resources and/or a shared niche within tissues. We found that ROR$\gamma^+$ MAIT17 are particularly reduced in peripheral tissues but not in the thymus. It was previously shown that thymic IL-4 can induce the production of the chemokines CCL17 and CCL22 by dendritic cells (5), whereas in vitro treatment of thymic epithelial cells with IL-4 induces the expression of ccl21 and cxcl10 (15). Additionally, IL-4 can inhibit the expression of CCR6, a chemokine receptor usually expressed by ROR$\gamma^+$ cells (51, 52). As MAIT17 cells express CCR6 (53, 54), it is possible that NKT2-derived IL-4 controls their egress through the regulation of chemokines and/or chemokine receptors, including CCR6.

Decreased thymic cellularity is a common feature of TCR Tg mice, including V$\alpha_{14}$ Tg mice (18, 33, 34). The TCR$\alpha$ locus rearranges at the DP stage of thymocyte development expression. In the V$\alpha_{14}$-Ja18 Tg mice we have used in this study, expression of the rearranged TCR$\alpha$-chain is controlled by the CD4 promoter; thus, thymocytes can express a mature TCR as soon as they enter the DP stage. This early TCR expression may cut off pre-TCR/TCR$\beta$ signals, which promote proliferation and survival, whereas thymocytes may lack expression of other molecules that may normally be expressed later during the DP stage. In addition, our results suggest that many TCR-expressing DP thymocytes may not be positively selected. It is known that the V$\alpha_{14}$-Ja18 canonical TCR$\alpha$-chain dominates the interaction between the TCR and CD1d-lipid complexes (55–58). This canonical TCR$\alpha$-chain limits the combinatorial diversity of the TCR repertoire and likely imposes constraints on the positive selection of conventional T cells by self-peptide:MHC complexes. This may explain the accumulation of TCR$\beta^+$ DPs and increased thymocyte death by neglect in V$\alpha_{14}$ Tg thymi. Rearrangement of the canonical TCR$\alpha$-chain normally starts late at the DP stage in the fetal thymus slightly before birth (35). Because of its premature expression in V$\alpha_{14}$ Tg mice, iNKT cells develop earlier than in WT mice, at a suboptimal time and/or location within the thymus, when and where other critical factors or resources may be missing.

iNKT cells generally promote antitumor immunity (59–63). For instance, in the B16F10 mouse metastatic melanoma model, it was demonstrated that IFN-$\gamma$ produced by iNKT cells following aGC treatment was essential for NK cell activation and the antitumor response (60). Despite a higher prevalence of iNKT cells within and outside B16F10 tumors, these grew faster in V$\alpha_{14}$ Tg mice.
mice. Altered iNKT cell phenotype and function, altered T cell selection and homeostasis, and enhanced T cell exhaustion could all contribute to enhanced tumor growth in Vα14 Tg mice. Specifically, the higher prevalence of iNKT2 may contribute to a higher amount of IL-4 in the tumor microenvironment, which can impair the antitumor response (64). Although the frequency and number of iNKT1 cells within tumors from Vα14 Tg remain arguably high, these cells may be functionally impaired. In agreement with this, iNKT cells from Vα14 Tg mice displayed reduced cytokine production after in vivo stimulation with aGC. Additionally, a previous study reported that thymic iNKT-derived IL-4 promotes the generation of CD103+ activated/memory-like regulatory T cells, which have greater immunosuppressive capacity (14). Further studies could assess whether such a phenomenon occurs in Vα14 Tg mice, especially during antitumor responses.

Altogether, our findings indicate that the development and function of iNKT cells are severely impacted in Vα14 Tg mice. In addition, these mice show alteration of other innate-like T cell populations, such as innate memory CD8 T cells and MAIT cells. In conclusion, our findings call to caution in using these mice for developmental and functional studies of iNKT cells.

DISCLOSURES

The authors have no financial conflicts of interest.

REFERENCES


