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Increased Peripheral IL-4 Leads to an Expanded Virtual Memory CD8\(^+\) Population

Vanessa Kurzweil,* Ami LaRoche,† and Paula M. Oliver*†

Memory-phenotype CD8\(^+\) T cells can arise even in the absence of overt Ag stimulation. Virtual memory (VM) CD8\(^+\) T cells are CD8\(^+\) T cells that develop a memory phenotype in the periphery of wild-type mice in an IL-15–dependent manner. Innate CD8\(^+\) T cells, in contrast, are memory-phenotype CD8\(^+\) T cells that develop in the thymus in response to elevated thymic IL-4. It is not clear whether VM cells and innate CD8\(^+\) T cells represent two independent T cell lineages or whether they arise through similar processes. In this study, we use mice deficient in Nedd4-family interacting protein 1 to show that overproduction of IL-4 in the periphery leads to an expanded VM population. Nedd4-family interacting protein 1\(^{-/-}\) CD4\(^+\) T cells produce large amounts of IL-4 due to a defect in JunB degradation. This IL-4 induces a memory-like phenotype in peripheral CD8\(^+\) T cells that includes elevated expression of CD44, CD122, and Eomesodermin and decreased expression of CD49d. Thus, our data show that excess peripheral IL-4 is sufficient to cause an increase in the VM population. Our results suggest that VM and innate CD8\(^+\) T cells may be more similar than previously appreciated.

Abbreviations used in this article: cKO, conditional knockout; Eomes, Eomesodermin; HP, homeostatic proliferation; KO, knockout; Ndfip1, Nedd4-family interacting protein 1; PLZF, promyelocytic leukemia zinc finger protein; VM, virtual memory; WT, wild-type.

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Fetal-liver chimeras

Livers were isolated from embryonic WT (CD45.1+) or Ndfip1−/− (CD45.2+) mice, and single-cell suspensions were prepared by mashing through a 70-µm filter. Cells were resuspended in freezing media (90% FCS, 10% DMSO) and kept at −80°C until used. Thawed cells were resuspended in sterile PBS and injected i.v. into sublethally irradiated Rag1−/− recipients, ~5 × 10⁵ cells/mouse. The chimeras were used in experiments 6 wk later to allow reconstitution of the T cell compartment.

Abs and reagents

The following fluorochrome-conjugated Abs were purchased from Bio-Legend, eBioscience, or BD Pharmingen: anti-mouse CD3 (17A2), CD4

![Diagram](http://www.jimmunol.org/)

**FIGURE 1.** Cell-extrinsic memory phenotype of Ndfip1−/− CD8+ T cells. (A) Representative histograms of CD44, CD122, CD124, Eomes, and CD49d expression on splenic WT and Ndfip1KO CD8+ T cells. (B) CD44, CD122, CD124, Eomes, and CD49d expression on splenic WT and Ndfip1KO CD8+ T cells based on flow cytometric analysis. (C–F) WT (CD45.1+) or Ndfip1KO (CD45.2+) fetal liver was transferred singly or as a 50:50 mix into irradiated Rag1−/− recipients. Six weeks later, splenic CD8+ T cells were isolated and analyzed by flow cytometry. (C) Representative histograms of CD44 and CD122 expression on CD8+ T cells from straight WT or Ndfip1KO chimeras (top) or from within one mixed chimera (bottom). (D) CD44 and CD122 expression in straight and mixed chimeras based on flow cytometric analysis. (E and F) Spleen cells were stimulated with anti-CD3 for 4 h. (E) Representative histograms of IL-4 expression on CD4+ T cells from straight WT or Ndfip1KO chimeras (left) or from within one mixed chimera (right) after stimulation with anti-CD3 for 4 h. (F) IL-4+ cells as a percentage of total CD4+ T cells in straight and mixed chimeras after stimulation with anti-CD3 for 4 h. The p values were determined by unpaired Student t test, except for comparisons within individual mixed chimeras in (D) and (F), which used paired Student t test. Data are representative of three to five (A and B) or two (C–F) independent experiments.
Flow cytometry

Single-cell suspensions of lymphocytes isolated from spleen or thymus were stained for 10 min on ice with live/dead stain, blocked for 5 min with Fc block, then stained an additional 25 min with Abs directed against surface Ags. Cells were then washed twice with PBS plus sodium azide plus FCS (FACS buffer). For NKT cell identification, CD1d tetramer was included in the surface Ag stain. For H2Kb tetramer staining, single-cell suspensions of combined spleen and lymph nodes were enriched for CD8+ T cells through negative selection using rat anti-mouse I-A/E (M5/114.15.2) and goat anti-rat IgG magnetic beads (New England Bioslabs), according to the manufacturer’s protocol. Tetramer was added after blocking with Fc block. Cells were then incubated on ice for 1 h, washed twice with FACS buffer, and stained 25 min for surface Ags. For intracellular cytokine staining, after surface staining cells were fixed for 20 min on ice with BD CytoFix/CytoPerm and washed twice with 1× BD perm/wash buffer. Cells were then resuspended in perm/wash buffer plus Abs directed against cytokines and stained on ice for 1 h. For intracellular transcription factor staining, after surface staining cells were fixed 1–16 h with eBioscience Foxp3 fix/perm solution and washed twice with 1× permeabilization buffer. Cells were then resuspended in FACS buffer plus Abs directed against transcription factors and stained on ice for 1 h. For PLZF staining, cells were then washed and stained an additional 30 min with anti-mouse IgG1-FITC. Flow cytometry was performed on a LSRi or LSRFortessa (BD Biosciences), and results were analyzed using FlowJo software (Tree Star). Dead cells were excluded from analysis.

ELISA

Spleen cultures were set up at 1 × 10^6 or 2 × 10^6 cells/ml in complete DMEM. T cells were activated with 5 ng/ml soluble anti-CD3, and supernatants were collected 22 h after stimulation. NKT cells were activated with 200 ng/ml PBS44, and supernatants were collected 4 d after stimulation. All supernatants were kept at −80°C until used. ELISA was performed using the eBioscience Ready-Set-Go IL-4 kit, according to the manufacturer’s instructions.

Results

Innate-like CD8+ T cells develop in Ndfip1-deficient mice

Although Ndfip1 has a well-characterized role in activating ubiquitin complexes that prevent IL-4 production in CD4+ T cells, its function, if any, in CD8+ T cells is unknown. We therefore examined the phenotype of Ndfip1−/− CD8+ T cells ex vivo by staining splenocytes from WT or Ndfip1−/− mice for markers of activation and then analyzed expression by flow cytometry. We found that Ndfip1−/− mice have an increased percentage of memory-phenotype CD8+ T cells (Fig. 1). Ndfip1−/− CD8+ T cells are significantly more likely than WT CD8+ T cells to express high levels of CD44, CD122 (IL-2Rα), and the transcription factor Eomes (Fig. 1A, 1B). Notably, Ndfip1−/− CD8+ T cells express less CD49d (α4 integrin) than WT CD8+ T cells (Fig. 1A, 1B). Low CD49d expression on CD44high cells has been described in HP memory and VM populations, and CD49d has thus been suggested as a potential marker to distinguish true Ag-experienced memory cells from other memory-phenotype cells (8, 9, 11).

Interestingly, Ndfip1−/− CD8+ T cells also express increased levels of CD124 (IL-4Rα) (Fig. 1A, 1B). CD124 is upregulated on T cells in response to IL-4 exposure (22), and high CD124 levels have been observed on innate-like CD8+ T cells that develop in an IL-4-rich environment (12). This suggested that exposure to cytokine may drive Ndfip1−/− CD8+ T cells to acquire a memory-like phenotype. To test this, we generated chimeras by injecting WT (CD45.1+) or Ndfip1−/− (CD45.2+) fetal liver alone (straight chimeras) or mixed in equal measure (mixed chimeras) into lethally irradiated Rag1−/− recipients. After reconstitution, we analyzed splenocytes from the chimeras by flow cytometry. As expected, CD8+ T cells from the straight Ndfip1−/− chimeras expressed higher levels of both CD44 and CD88
T cells from the straight WT chimeras (Fig. 1C, 1D). However, there was no significant difference between WT and Ndfip1−/− cells that developed in the mixed chimeras, and this was largely due to increased expression of CD44 and CD122 on WT cells that developed in the presence of Ndfip1−/− cells (Fig. 1C, 1D). Thus, the memory-like phenotype of Ndfip1−/− CD8+ T cells is cell extrinsic. In contrast, upon stimulation with anti-CD3, Ndfip1−/− CD4+ cells but not WT CD4+ cells produced IL-4 even in the mixed chimeras, suggesting that IL-4 production from Ndfip1−/− cells drives acquisition of a memory phenotype in bystander CD8+ T cells (Fig. 1E, 1F). CD8+ T cells, in contrast, did not produce any IL-4 (data not shown).

Loss of Ndfip1 could potentially affect many cell types. To confirm that the memory phenotype of Ndfip1−/− CD8+ T cells does not require loss of Ndfip1 outside the T cell compartment, we crossed Ndfip1flo/flo mice to mice expressing the Cre recombinase under the control of the CD4 promoter (CD4-cre+). The resulting mice (conditional KOs [cKOs]) lack Ndfip1 only in T cells. CD8+ T cells from these cKOs have a memory-like phenotype similar to that of CD8+ T cells from Ndfip1−/− mice (Supplemental Fig. 1A, 1B, Fig. 1A, 1B). As expected, the CD4+ T cells from cKO mice are more likely than control cells to produce IL-4 in response to stimulation (Supplemental Fig. 1C). These data indicate that loss of Ndfip1 in T cells only is sufficient to induce increased frequency of memory-like CD8+ T cells. To determine whether Ndfip1−/− mice have an increased frequency of NKT or TCRγδ T cells, as has been observed in models of innate CD8+ T cells (15), we stained Ndfip1−/− and WT spleen and thymus using CD1d tetramer loaded with PBS-57 (an α-galactosylceramide analog) and anti-TCRγδ Ab. As shown in Supplemental Fig. 2A and 2B, Ndfip1−/− mice contain normal frequencies of TCRγδ T cells and a slightly lower frequency of NKT cells than WT mice. We also analyzed PLZF expression by intracellular staining and flow cytometry and determined that neither NKT cells nor TCRγδ T cells in Ndfip1−/− mice express elevated PLZF levels compared with WT (Supplemental Fig. 2C). Finally, to compare IL-4 production by WT and Ndfip1−/− NKT cells, we stimulated splenocyte cultures in vitro for 4 d with α-galactosylceramide analog PBS44, and then collected supernatants and analyzed by ELISA. We observed increased IL-4 production from Ndfip1−/− NKT cells, although this was not statistically significant (Supplemental Fig. 2D).

The memory phenotype of Ndfip1KO CD8+ T cells is largely IL-4 dependent

The previous experiments suggested that increased IL-4 in Ndfip1−/− mice causes CD8+ T cells to develop a memory-like phenotype. To test whether the CD8+ T cell phenotype is really dependent on IL-4, we compared splenocytes from Ndfip1−/−IL-4−/− mice and control Ndfip1+/+IL-4−/− mice. Loss of IL-4 almost completely ablated the increase in memory-phenotype cells observed in Ndfip1−/− mice (Fig. 2). Expression of CD122, CD49d, and CD124 was not statistically different in Ndfip1−/− IL-4−/− CD8+ T cells compared with IL-4−/− controls (Fig. 2). Furthermore, the percentage of cells expressing high levels of CD44 or Eomes was drastically reduced in Ndfip1−/− IL-4−/− CD8+ T cells compared with Ndfip1−/− cells from IL-4−/− sufficient mice, although a small but statistically significant increase compared with IL-4−/− controls remained (Figs. 1A, 1B, 2). Therefore, the overwhelming majority of memory-phenotype CD8+ T cells in Ndfip1−/− mice require IL-4 for their development or maintenance.

Because CD4+ T cells are major producers of IL-4 in Ndfip1−/− mice, we attempted to generate mice lacking CD4+ T cells by Figure 3. Memory-like phenotype in Ndfip1−/− CD8+ T cells first arises in the periphery. (A) Representative histograms of CD44 expression on CD8+ T cells from spleen and thymus in Ndfip1KO and control Ndfip1+/+ mice at 5, 8, and 28 d old. (B) CD44, CD122, and CD124 expression on CD8+ T cells from spleen and thymus based on flow cytometric analysis. (C) Spleen and thymus cells were stimulated 4 h with PMA and ionomycin, and culture supernatants were analyzed for IL-4 by ELISA. The p values were determined by unpaired Student t test. Error bars = SEM. n = 4–8 per time point. *p < 0.05, **p < 0.01, ***p < 0.001.
FIGURE 4. Memory-like phenotype in Ndfip1−/− CD8+ T cells is independent of nominal Ag exposure. (A) Representative contour plots of WT sample stained for surface markers and SIINFEKL-loaded tetramer, as described in Materials and Methods (left); WT sample stained (Figure legend continues).
crossing Ndfip1−/− mice to mice lacking all conventional MHC class II genes (MHCII−/− mice). Surprisingly, we observed that Ndfip1−/− MHCII−/− CD8+ T cells had a phenotype similar to Ndfip1−/− CD8+ T cells (Supplemental Figs. 1A, 1B, 3A, 3B). MHCII−/− mice have previously been described as containing a small percentage of mature peripheral CD4+ T cells that may be selected on nonconventional MHC molecules (23). We analyzed splenocytes from Ndfip1−/− MHCII−/− mice and confirmed that ~2–3% of lymphocytes were CD4+ T cells (Supplemental Fig. 3C). Nearly 100% of these cells were CD44high (data not shown). To test the possibility that this small but apparently activated population of cells could be producing IL-4, we stimulated total

FIGURE 5. Naive WT CD8+ T cells acquire a memory phenotype in the presence of Ndfip1−/− T cells. (A) Representative histograms of CD124, Eomes, CD44, and CD49d expression on WT CD45.1+ CD8+ T cells transferred to cKO or control recipients, isolated from blood (D9) and spleen (D14). (B) CD124, Eomes, CD44, and CD49d expression on these T cells based on flow cytometric analysis. The p values were determined by unpaired Student t test.

for surface markers with no tetramer (center), used to set tetramer+ gate; and positive control cells from an OTI+ Rag1−/− mouse stained for surface markers and SIINFEKL-loaded tetramer (right). Cells in black are gated on CD8+ T cells, whereas cells in gray are gated on CD4+ T cells from the same sample. (B) Representative histograms of CD124, CD44, CD122, CD124, Eomes, and CD49d expression on splenic WT and Ndfip1KO tetramer+ CD8+ T cells. (C) CD44, CD122, CD124, Eomes, and CD49d expression on splenic WT and Ndfip1KO tetramer+ CD8+ T cells based on flow cytometric analysis. (D) Representative histograms of CD44, CD122, CD124, Eomes, and CD49d expression on splenic IL-4KO and DKO tetramer+ CD8+ T cells. (E) CD44, CD122, CD124, Eomes, and CD49d expression on splenic IL-4KO and DKO tetramer+ CD8+ T cells based on flow cytometric analysis. The p values were determined by unpaired Student t test.
splenocytes with anti-CD3 overnight and analyzed IL-4 secretion by ELISA. As expected, neither WT nor control MHCII−/− cells produced detectable levels of IL-4, whereas Ndfip1−/− cells produced a great deal of this cytokine (Supplemental Fig. 3D). Notably, although IL-4 was greatly reduced in Ndfip1−/− cells, it was still present at levels ~50 times the limit of detection (Supplemental Fig. 3D). Although this is much more IL-4 than was observed when invariant NKT cells were specifically stimulated with glycolipid for 4 d (Supplemental Fig. 2D), it is possible that this result represents IL-4 production by NKT cells. Next, we analyzed intracellular cytokine production after stimulation with PMA and ionomycin. Ndfip1 analyzed intracellular cytokine production after stimulation with that this result represents IL-4 production by NKT cells. Next, we analyzed intracellular cytokine production after stimulation with PMA and ionomycin. Ndfip1−/− cells were significantly more likely to be IL-4+ than control MHCII−/− cells (Supplemental Fig. 3E). Taken together, these data suggest that Ndfip1 restricts IL-4 production even in nonclassical CD4+ T cells (including, potentially, NKT cells), and the IL-4 produced in Ndfip1−/− mice is sufficient to induce a memory-like phenotype in CD8+ T cells.

Ndfip1 KO CD8+ T cells are IL-4–dependent VM cells

Ndfip1−/− CD8+ T cells displayed characteristics of both innate and VM CD8+ T cells in the previous experiments. One major difference between these two cell types is that VM cells typically arise in the periphery, whereas innate CD8+ T cells arise in thymus (11). To determine the origin of memory-phenotype Ndfip1−/− CD8+ T cells, we sacrificed mice shortly after birth and compared CD8+ T cells isolated from thymus and spleen. Because Ndfip1 inheritance is non-Mendelian (V. Kurzweil, A. LaRoche, and P.M. Oliver, unpublished observations), to generate more KOs we bred in Ndfip1−/− mice to Ndfip1−/− littermates as controls. In Fig. 3A and 3B, it is apparent that splenic CD8+ T cells in Ndfip1−/− mice begin to take on a memory phenotype as early as 8 d after birth. In contrast, Ndfip1−/− CD8 single-positive thymocytes do not display a memory phenotype even at 4 wk of age. This is consistent with results of IL-4 ELISA (Fig. 3C), which indicate very little IL-4 production in the thymus compared with the spleen. Thus, it appears that both IL-4 production and appearance of memory-phenotype CD8+ T cells occur primarily in the periphery, not the thymus, of Ndfip1−/− mice.

To distinguish VM cells from Ag-experienced memory cells, it is helpful to use tetramer staining to identify cells specific for nominal Ag in unimmunized mice. To do this, we stained Ndfip1−/− spleen and lymph node cells with SIINFEKL-H2Kb tetramer to identify endogenous OVA-specific CD8+ T cells. Tetramer+ CD8+ T cells from Ndfip1−/− mice displayed elevated levels of CD44, CD122, CD124, and Eomes and decreased expression of CD49d (Fig. 4B, 4C). This phenotype was absent in Ndfip1−/− IL-4−/− cells (Fig. 4D, 4E). Thus, OVA-specific CD8+ T cells in naive Ndfip1−/− mice have the same memory phenotype observed in bulk CD8+ T cells, and this phenotype is dependent on IL-4. To further confirm that the memory phenotype can be induced in the absence of overt Ag stimulation, we next sorted for naive (CD44lowCD62Lhigh CD25−) CD8+ T cells from WT mice expressing the congeneric marker CD45.1. Naïve CD8+ T cells were then transferred to cKO (Ndfip1fl/fl CD4-Cre+) or control (Ndfip1fl/fl CD4-Cre−) recipients. Mice were bled 9 d after transfer, at which point WT CD45.1 CD8+ T cells isolated from cKO recipients expressed higher levels of CD49d and CD124 than Eomes than T cells isolated from control recipients but did not yet display statistically significant differences in CD44 or CD49d expression (Fig. 5). At 14 d posttransfer, mice were sacrificed and lymphocytes were isolated from spleens. At this time point, WT CD45.1 CD8+ T cells that had been transferred to cKO recipients not only clearly expressed more CD124 and Eomes than WT cells transferred to control recipients but also expressed more CD44 and slightly less CD49d (Fig. 5). Expression of CD122 was not significantly different between T cells transferred to cKO and control recipients at either time point (data not shown). This experiment demonstrates that naive, WT CD8+ T cells in the presence of Ndfip1−/− T cells acquire a phenotype similar to VM cells. This suggests that the increase in memory-phenotype CD8+ T cells in Ndfip1−/− mice could be driven at least in part by increased phenotypic conversion of naive cells, rather than solely by proliferation of existing memory-like cells.

The previous results establish that memory-phenotype Ndfip1−/− CD8+ T cells have the characteristics of VM cells. However, it was recently suggested that IL-4 plays a minor role in the development of the VM population of WT mice and that VM cells arise primarily in response to IL-15 (11). To determine whether IL-4 affected the VM cells in Ndfip1−/− mice, we examined the CD44highCD49dlow VM cell population in WT and Ndfip1−/− mice and their IL-4–deficient counterparts. Consistent with previous findings (10, 11), we observed a small but significant decrease in the CD44highCD49dlow VM cell population in IL-4–deficient mice compared with WT mice (Fig. 6A, 6B).

**FIGURE 6.** Virtual memory cells in Ndfip1−/− mice are IL-4 dependent. (A) Representative contour plots of CD44 and CD49d expression on splenic WT, Ndfip1KO, IL-4KO, and IL-4KO Ndfip1KO (DKO) CD8+ T cells. (B) VM cells (CD44highCD49dlow) as a percentage of total CD8+ T cells based on flow cytometric analysis. (C) VM cells (CD44highCD49dlow) as a percentage of total tetramer+ CD8+ T cells based on flow cytometric analysis. The p values were determined by unpaired Student t test. Data are representative of three independent experiments.
Importantly, the majority of VM cells in Ndfip1−/− mice are absent in Ndfip1−/− IL-4−/− mice. When endogenous OVA-specific CD8+ T cells were isolated from unimmunized mice, we again observed a large increase in CD44hiCD49dlow VM cells in Ndfip1−/− mice that was ablated in the absence of IL-4 (Fig. 6C). In contrast, the presumptive Ag-experienced population (CD44hiCD49dlo) is a similar percentage of total T cells regardless of the presence of IL-4 (Fig. 6A, data not shown). Together, this indicates that the VM cell population expands when exposed to increased IL-4 levels.

Discussion
Our data demonstrate that IL-4 in the periphery can lead to an expanded VM population. Although thymic IL-4 has a well-characterized role in the generation of innate CD8+ T cells, to our knowledge this is the first study to link overproduction of IL-4 to an increase in VM cells. Our data are consistent with recently published data (10, 11) showing that the naturally occurring VM population in WT mice is partially decreased in the absence of IL-4. One explanation for the comparatively minor role for IL-4 observed in those studies is that they primarily described VM cells in WT C57BL/6 mice, which have very low IL-4 levels. Indeed, it is possible that multiple cytokines contribute to the formation of the normal VM population, although clearly IL-15 has a crucial role, as described previously (11). However, our data show that when IL-4 is produced at high levels in peripheral compartments, it is an important driver of VM cells, leading to a fairly specific increase in VM cells compared with conventional Ag-experienced cells (Fig. 6). Our results indicate that the IL-4-rich Ndfip1−/− host environment has the potential to induce phenotypic conversion of naive CD8+ T cells (Fig. 5), but it is likely that the large endogenous VM population in Ndfip1−/− mice represents a combination of both increased conversion of naive cells and increased proliferation of existing VM cells.

By establishing that high IL-4 levels can lead to increased VM cells, our data also suggest a possible link between innate CD8+ T cells and VM cells. The primary differences that have been described in these two memory-phenotype populations are their relative reliance on IL-4 and their origins in the thymus versus the periphery. Both these differences may actually stem from differences in the experimental systems in which they were described. Unlike VM cells, innate CD8+ T cells have been described primarily in systems with elevated percentages of IL-4+ PLZF+ T cells. These IL-4+ PLZF+ T cells are, by and large, nonconventional T cells that become activated in and localize to the thymus. In contrast, Ndfip1−/− mice have greatly increased IL-4 levels compared with WT mice, but the IL-4 is produced by cells in the periphery. It is thus not surprising that the expansion of memory-phenotype CD8+ T cells in Ndfip1−/− mice occurs in the periphery in an IL-4–dependent manner. This result may suggest that innate CD8+ T cells and VM cells are not fundamentally different cell types but rather reflect different pathways leading to acquisition of the same phenotype. Further characterizations of these cell types, including direct comparisons of functional properties, are necessary to address this possibility.

Development of both VM cells and innate CD8+ T cells is dependent on Eomes (11, 24). Sosinowski et al. (11) pointed out that this links memory-phenotype cells with IL-15, as IL-15 increases Eomes expression, which in turn increases CD122 expression and therefore sensitivity to IL-15 (25, 26). However, IL-4 also induces Eomes expression in CD8+ T cells (12, 27, 28), as does IL-2 (29). Moreover, the timing of phenotypic changes to naive WT cells transferred to eKO recipients (Fig. 5) is consistent with a model in which IL-4 exposure, indicated by increased CD124, leads to an increase in Eomes expression, which then promotes acquisition of a memory phenotype. In light of this, it seems probable that memory-phenotype CD8+ T cells can arise in response to localized increases in any of several cytokines.

It has been speculated that unconventional memory-phenotype CD8+ T cells may be important in early responses to infection (15, 30). However, our data suggest that these cells may be especially relevant in autoimmune or allergic disorders, which frequently involve increased local production of IL-4. For example, CD8+ T cells can play a role in the pathology of asthma, and some of this may be due to bystander effects of IL-4. In mouse models of allergic airway disease, CD8+ T cells that develop in the presence of IL-4+ CD4+ cells worsen lung pathology (31, 32). Additionally, increased bronchial CD8+ cell infiltrate in human asthma patients is predictive of decreased lung function (33). Future research should address the question of whether memory-phenotype CD8+ T cells can be generated in a disease setting and, if so, whether they contribute to the pathology of inflammatory diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplementary Figure 1. Loss of Ndfip1 in T cells is sufficient for increase in memory-phenotype CD8+ T cells. (A) Representative histograms of CD44, CD122, CD124, Eomes, and CD49d expression on splenic control (Ndfip1^{fl/fl} CD4-Cre^-) and cKO (Ndfip1^{fl/fl} CD4-Cre^+) CD8+ T cells. (B) CD44, CD122, CD124, Eomes, and CD49d expression on splenic control and cKO CD8+ T cells based on flow cytometric analysis. (C) IL-4+ cells as a percentage of total CD4+ T cells after stimulation with PMA and ionomycin for 4 hrs. p values, unpaired Student’s t-test. Data are representative of two independent experiments. Each dot represents an individual mouse.
Supplementary Figure 2. Ndfip1KO mice do not have increased NKT or TCRγδ populations. (A) Representative contour plots of spleen and thymus cells gated on CD3+ CD8− populations. Cells were stained with anti-TCRγδ antibody and fluorochrome-labeled CD1d tetramer loaded with PBS-57 (α-GalCer analogue) or unloaded CD1d tetramer as a control. (B) Percentages of NKT (PBS57-CD1d tetramer+) and TCRγδ+ T cells in WT and Ndfip1KO spleen and thymus. (C) PLZF expression on NKT cells from WT and Ndfip1KO spleen and thymus based on flow cytometric analysis. (D) IL-4 production by WT and Ndfip1KO NKT cells. Splenocytes (n=4) were stimulated in vitro with PBS44 (α-GalCer analogue) for 4 days and culture supernatants were assayed for IL-4 by ELISA. p values, unpaired Student’s t-test. NS, not significant.
Supplementary Figure 3. Ndfip1KO mice lacking MHC Class II retain some IL4+ CD4+ T cells and memory-phenotype CD8+ T cells. (A) Representative histograms of CD44, CD122, and Eomes expression on splenic MHCIKO and Ndfip1KO MHCIKO (DKO) CD8+ T cells. (B) CD44, CD122, and Eomes expression on splenic MHCIKO and DKO CD8+ T cells based on flow cytometric analysis. (C) CD4+ cells as a percentage of total lymphocytes in the spleen. (D) IL-4 ELISA on culture supernatants harvested from splenocytes stimulated overnight with anti-CD3. (E) IL-4+ cells as a percentage of total CD4+ T cells after stimulation with PMA and ionomycin for 4 hrs. p values, unpaired Student's t-test. Data are representative of two independent experiments. NS, not significant.