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Making memory at birth: understanding the differentiation of natural killer T cells

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Abstract

Glycolipid reactive natural killer T cells with an invariant TCR α -chain (*i*NKT cells) are a conserved population of T lymphocytes with a distinct anatomical distribution and functional properties. The differentiation pathway of *i*NKT cells branches off from mainstream thymocyte differentiation at the double positive stage, and recent work has revealed how signaling events early in the *i*NKT cell pathway imprint a memory-like behavior on these cells. Additionally, unique molecular interactions governing *i*NKT cell development and tissue distribution have been uncovered recently, building up our knowledge of the complex network of interactions that form this population. Novel autologous antigens for these cells have been identified, although it has not yet been resolved if there is single endogenous antigen responsible for both positive selection and/or peripheral activation.

Introduction

Two populations of cells within the double positive (DP) thymocyte pool have a developmental pathway different from most CD4⁺ and CD8⁺ T lymphocytes. These thymocytes exhibit specificity for determinants presented by non-classical class I antigen-presenting molecules, and they express T cell antigen receptors (TCRs) with an extremely limited α chain diversity [1,2]. Upon maturation, cells within these two populations express some markers characteristic of NK cells and/or memory T lymphocytes. Hence they are frequently labeled “NKT cells”, although it has long been recognized that T cells without invariant α chains also can express NK receptors.

The best studied of these two populations are known in the mouse as V α 14 invariant NKT cells (V α 14i NKT cells). As the name implies, V α 14i NKT cells express an invariant TCR α chain, formed by rearrangement of the V α 14 and J α 18 segments, together with a limited range of β chains, primarily V β 8.2, 7, 2 or 8.1. The CDR3 β regions of these TCRs are diverse, however, and therefore this TCR is sometimes called semi-invariant [1]. V α 14i NKT cells recognize lipid antigens presented by the non polymorphic class I molecule CD1d, and they are typically detected using CD1d tetramers loaded with the strong TCR glycolipid agonist α -galactosyl ceramide (α GalCer). A homologous population in humans, V α 24i NKT cells, utilizes V α 24, a V α 14 ortholog, rearranged to J α 18, expressed together with a β chain containing V β 11. The interspecies conservation between these cells is such that mouse V α 14i NKT cells can be stimulated by α GalCer presented by human CD1d, and

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vice-versa [3]. While recent work in mice and humans has characterized cells with a similar specificity that do not express the invariant α chainTCRs, these are much less numerous [4,5].

A second population of cells with an invariant TCR is the mucosal associated invariant T cells (MAIT) [2]. They are reactive to MR1, a class I antigen presenting molecule whose heavy chain is encoded by a conserved gene linked to the CD1 locus. These cells express a *Va19-Ja33* rearrangement in mice and an orthologous *Va7-Ja33* rearrangement in humans. While there are interesting parallels between MAIT and *i*NKT cells, MAIT will not be considered further in this review.

The immediate response of *i*NKT cells is distinguished by the production of Th1 and Th2 cytokines, even in the absence of intentional priming. Hence, *i*NKT cells constitute a natural memory lymphocyte population. Thymic differentiation of *i*NKT cells involves stages of selection, proliferation and maturation, with the latter stages bearing similarity to a peripheral immune response. Therefore *i*NKT cells exhibit “memory at birth”, meaning that they acquire an antigen-experienced phenotype in the thymus. Genetic analyses have revealed a number of factors that regulate these processes, such as the tyrosine kinase Fyn, NF- κ B transcription factors and IL-15, which for most T cells are more important for an immune response or memory formation than for thymus differentiation [6]. While elements uniquely important for the differentiation of *i*NKT cells have been described in previous reviews, this article is intended to provide a summary of the most recent advances in our understanding of *i*NKT cell differentiation.

Factors governing positive selection

The differentiation of *Va14i* NKT cells diverges at the DP stage, due to their antigenic specificity and instruction by the positively selecting cell type. The frequency of DP cells that express the *Va14i* TCR depends on random TCR rearrangement and has been estimated at 1 in 10^6 [7,8]. The *Ja18* segment is located towards the distal end of the *Ja* locus, relative to the *Va* segments. Like other distal *Ja* segments, it only becomes accessible to rearrangement late during the lifespan of DP thymocytes, as they undergo secondary *Ja* rearrangements (Figure 1). Thus, mutations in genes that are necessary to support full DP viability have a major impact on *Va14i* NKT cell number, which can be corrected by ectopic expression anti-apoptotic proteins, such as Bcl- x_L or Bcl-2. This had been shown previously for ROR γ T, a transcription factor that promotes Bcl- x_L expression in DP thymocytes [9,10]. Now, a recent report has demonstrated that DP viability, and consequently *Va14i* NKT development, are compromised when thymocytes lack the E protein transcription factor HEB [11•]. HEB is required for expression of both ROR γ T and Bcl- x_L in DP cells, and DP viability and *Va14i* NKT numbers in HEB-deficient thymocytes were restored by enforced expression of Bcl- x_L .

Despite their low precursor frequency in the DP pool, *Va14i* NKT thymocytes typically comprise 0.5% of all cells in the adult mouse thymus. Their expansion is accompanied by the acquisition of markers of peripheral memory T populations, such as high levels of CD44. It has long been thought that exposure to true TCR agonists, rather than to selecting ligands with a low TCR affinity, drives intrathymic proliferation. As *i*NKT cells exhibit self-reactivity under some circumstances, the same endogenous agonists expressed in the thymus may also participate in the activation of peripheral, mature cells. However, antigens necessary to support *Va14i* NKT development have yet to be conclusively identified. The lysosomal glycosphingolipid (GSL) isoglobotrihexosylceramide (iGb3), is a mammalian agonist for *Va14i* NKT cells. Although genetic evidence suggested that iGb3 might be necessary to support *Va14i* NKT cell differentiation, subsequent studies indicated that iGb3

is not essential [12,13]. A recent report identified forms of the GSL β -D-glucopyranosylceramide (β -GlcCer), particularly one with a C24:1 fatty acid, as important self-antigens for both mouse and human *i*NKT cells [14••]. Notably, this study demonstrated that stimulatory forms of β -GlcCer were detectable in mouse thymus and spleen, that β -GlcCer could activate $V\alpha 14i$ NKT cells, and that $V\alpha 14i$ NKT cell autoreactivity was sensitive to chemical or siRNA-mediated manipulation of enzymes important for β -GlcCer synthesis. Furthermore, β -GlcCer increases after signals, such as TLR stimulation of antigen presenting cells, reported to induce self-reactivity. Thus, β -GlcCer has the properties expected from a major self-antigen that activates $V\alpha 14i$ NKT cells, although its role in positive selection remains to be established. While β -GlcCer was previously found to be non-antigenic, this discrepancy could be due to differences in the lipid structures of the compounds, as the form with a C24:1 fatty acid was much more stimulatory [14,15].

A recent study has analyzed a $V\alpha 14i$ TCR with a β chain having a CDR3 region optimized for self-reactivity [16••]. This engineered TCR could mediate responses to a wide range of self-glycolipids presented by CD1d, including GSLs and phospholipids, based on germ line-encoded TCR interactions with CD1d. It was not reactive to all CD1d presented compounds, however, with reactivity to glycolipids with larger, oligosaccharide head groups, being disfavored. Based on these data, the authors proposed that a variety of lipids with varying TCR affinities could participate not only in CD1d-mediated positive selection of $V\alpha 14i$ NKT cells, but also in the self-antigen mediated activation of these cells in the periphery.

SLAM family interactions

CD1d expression by DP thymocytes rather than thymic epithelial cells is required for the positive selection of $V\alpha 14i$ NKT precursors [17]. DP thymocytes also deliver essential signals due to their expression of two members of the SLAM family of receptors, SLAM and Ly108 [18]. Signal transduction upon SLAM family interactions requires the SH2 domain-containing adaptor protein SH2D1A/SAP (SAP), which is known to recruit Fyn. Deletion of either SAP or Fyn, results in profound blocks at the earliest stages of $V\alpha 14i$ NKT differentiation, and SAP has been shown to be required for the differentiation of $V\alpha 24i$ NKT cells [18,19]. The absence of Fyn has a less severe effect than loss of SAP, and a point mutation in SAP that prevents the interaction with Fyn has only a moderate inhibitory effect on the generation of $V\alpha 14i$ NKT cells [20]. Thus redundant mechanisms may exist to support SAP-mediated SLAM family signaling, some of which may be independent of Fyn.

The Ras/Mapk pathway

A surprising early finding with regard to $V\alpha 14i$ NKT cell differentiation was the report that interference with Ras-Map kinase signaling by dominant-negative transgenes did not perturb their differentiation [21]. These data are at odds with the known role for the Ras-Erk pathway in TCR signal transduction. Now a recent report has established that interference with the Ras-Mapk pathway through dominant negative inhibition results in markedly reduced numbers of $V\alpha 14i$ NKT cells in the thymus and periphery [22•]. Consistent with this, inactivation of the Erk target protein Egr2 was found to negatively impact $V\alpha 14i$ NKT cells, with defects in both selection and terminal maturation [22,23]. The effects of Egr2 deficiency were $V\alpha 14i$ NKT cell-intrinsic, but were not corrected by extending DP lifespan, implying that they were not due to reduced secondary TCR α rearrangements. Very recently, it has been shown that Egr2 plays multiple roles in $V\alpha 14i$ NKT cell differentiation [24••]. It is required for activating the promoter of the PLZF gene (see below), which is important for the acquisition of effector functions. However, it is also activates the *Il2rb* promoter, which is part of the IL-15 receptor. IL-15 is important for the terminal stages of $V\alpha 14i$ NKT cell maturation. Deletion of RasGrp1, an activator of the Ras-Erk pathway, also led to severe,

V α 14i NKT cell-intrinsic defects in development and maturation [25•]. These reports suggest that the early stages of thymic selection of V α 14i NKT cells require typical TCR mediated signaling pathways as well as signals through SLAM/Ly108-SAP-Fyn.

On the other hand, excessive TCR signaling can also be deleterious to V α 14i NKT development, as first suggested by studies in which CD1d was over expressed or when agonists were added to thymic organ cultures [26]. Mice doubly deficient for the diacylglycerol kinases DGK α and DGK ζ , which attenuate signaling by diacylglycerol (DAG) produced from the TCR signaling cascade, exhibit a profound deficit in V α 14i NKT cells [27••]. Interestingly, a recent report indicates that DGK α negatively regulates SLAM-SAP signaling in human T cells, suggesting that diacylglycerol kinases allow for iNKT cell differentiation by limiting signaling by both the TCR and the SLAM-mediated pathways [28]. Furthermore, enforced activation of either of the two signaling pathways negatively regulated by the DGK proteins, Ras-Erk or NF- κ B, resulted respectively in moderate or severe inhibition of V α 14i NKT cell development [27••]. Mutation of the deubiquitinase CYLD also has effects of V α 14i NKT cell differentiation that are related to excessive signaling of NF- κ B proteins. V α 14i NKT cells in the mutant mice can mature, but they have increased NF- κ B activity, appear to be hyper activated, and undergo massive apoptosis at the early stage of thymus maturation. The excessive NF- κ B function causes decreased ICOS expression and decreased expression of the IL-7 receptor α chain, two factors known to be important for V α 14i NKT cell homeostasis [29••]

Genes required for thymic V α 14i NKT maturation

Several genes, including those encoding the T box factor T-bet, the Tec kinases Itk and Rlk, and the vitamin D receptor (VDR) are required for the developmental progression and expansion of V α 14i NKT cells [6,30]. Itk, Rlk and VDR all act to support T-bet expression, which in turn is required for expression of the IL15R subunit CD122 that is necessary for the survival of mature V α 14i NKT cells [31,32]. IL-15 is important not only for inducing Bcl-X_L expression, required for survival, but also for the acquisition of effector functions in thymic V α 14i NKT cells [33•].

Recently it has been shown that deletion of the Med1 gene, which encodes a component of the Mediator complex that links nuclear hormone receptors with the transcriptional machinery, results in V α 14i NKT cell maturation defects that are similar to those observed in T-bet, VDR and Itk/Rlk-deficient mice [6,34•]. Because the VDR has been identified as one of the nuclear receptors that can interact with the Mediator complex, it appears likely that Med1 functions to support V α 14i NKT cell maturation through facilitating the regulation of VDR target genes. Curiously, while mice deficient for vitamin D have reduced numbers of V α 14i NKT cells, in contrast to VDR-deficient mice, terminal maturation is not affected [35]. Furthermore, a lack of functional vitamin D during gestation led to irreversible deficits in V α 14i NKT numbers that could not be corrected by dietary supplementation later [35].

PLZF and the acquisition of effector function

The development of V α 14i NKT thymocytes is divided into stages based on the expression of CD24, CD44 and NK1.1, with the most mature, stage 3 cells being CD44^{high}, NK1.1⁺. Recent thymic emigrants are mostly stage 2 (CD44^{high}, NK1.1⁻) cells, which also express neuropilin [36•], a protein initially described to be involved in axonal guidance. Thymocytes deficient for the BTB-ZF family transcription factor *Zbtb16* or PLZF are blocked at stage 1 (CD44^{low}, NK1.1⁻) with a decreased population size due to impaired expansion. They also display properties of naïve T cells, including preferential localization to the peripheral LN [37,38]. The V α 14i NKT cell maturation defect is observed even in heterozygous *Zbtb16*

mutant mice [39]. Additionally, enforced expression of PLZF promoted the expression of a memory phenotype in conventional T cells, independent of agonist selection [38,40•,41]. PLZF expression is not dependent upon SLAM-SAP-Fyn signaling, nor is enforced expression of PLZF able to support V α 14i NKT cell differentiation in the absence of SAP or Fyn [38,40•], demonstrating that PLZF and SLAM-SAP-Fyn signaling operate independently.

PLZF and tissue localization

V α 14i NKT cells preferentially localize to the liver, spleen and bone marrow. A recent study has demonstrated that liver and splenic V α 14i NKT cells recirculate very slowly, but that recirculation could be induced by blockade of the integrin LFA-1[42••]. V α 14i NKT cells express higher levels of LFA-1 than recirculating lymphocytes. Furthermore, enforced expression of PLZF increases the expression of LFA-1 by conventional T lymphocytes, and prevents the recirculation of liver resident CD4 T cells.

Co-receptor expression by NKT cells

V α 14i NKT cells are CD4⁺ or CD4, CD8 double negative (DN), and the absence of CD8 expression was originally attributed to negative selection of CD8⁺ cells[43]. An early analysis of transgenic mice with forced expression of CD8 $\alpha\beta$ in T lymphocytes showed they lacked V α 14i NKT cells, which supported this hypothesis [43]. However, subsequently an interaction between CD8 and CD1d could not be demonstrated, and a more recent analysis of the same transgenic line showed that mice hemizygous for the transgene in fact do have functional CD8⁺ V α 14i NKT cells [44••]. The near absence of V α 14i NKT cells in mice homozygous for the CD8 $\alpha\beta$ construct was due to unexpected and not fully explained effects of the transgene, including a reduced survival of DP thymocytes as well as a reduction in CD1d expression.

A second member of the BTB-POZ family, *Zbtb7b/c-krox* or Th-POK, was first identified as being essential for CD4 SP thymocyte differentiation [45,46], and similar to its effects of MHC class II reactive thymocytes, V α 14i NKT cells in these mice are either CD8SP or DN [44••,47]. Interestingly, the Th-POK mutant V α 14i NKT cells were defective in their in vivo response to α GalGer, with the production of IL-4 and IL-13 more profoundly inhibited than IFN γ and TNF [44••]. Because Th-POK is expressed at all stages of V α 14i NKT cell differentiation, the data suggest that Th-POK silencing of CD8 expression in V α 14i NKT cells is a by-product of the requirement for this transcription factor for their functional differentiation.

Genes with multiple effects on V α 14i NKT development

Recently several factors have been identified that promote V α 14i NKT differentiation through multiple mechanisms. The transcription factor c-myb was found to be necessary to support DP survival, and hence secondary TCR α rearrangement [48••]. However, c-myb also was required in a V α 14i NKT cell-extrinsic manner, likely through its effects on the expression of CD1d, SLAM, Ly108 and SAP. Expression of myb is partially regulated by micro RNA miR-150. Ectopic expression of miR-150 in transgenic mice was shown to inhibit the developmental progression of V α 14i NKT cells, which could be due in part to its effects on myb [49].

Bcl11b, a zinc finger transcription factor required for the positive selection of MHC class I and class II reactive T cells, also has been shown recently to have both cell-intrinsic and cell-extrinsic roles in V α 14i NKT cell differentiation [50••]. The V α 14i NKT-extrinsic functions of Bcl11b are likely associated with defects in DP thymocyte glycolipid

processing and loading. In contrast to c-myb, Bcl11b does not appear to be required for secondary TCR α rearrangements. The V α 14i NKT-intrinsic roles of this gene may instead be related to TCR signaling defects in Bcl11b-deficient DP thymocytes [51].

Functional subsets

Although the majority of iNKT cells produce both Th1 and Th2 cytokines in response to TCR stimulation, increasing evidence supports the existence of functional subpopulations [52]. The best-defined subpopulation secretes Th17 cytokines, and although these so-called NKT-17 cells represent only a small fraction of the V α 14i NKT cells in the spleen and liver, they are a much larger fraction in the peripheral LN and skin [53]. NKT-17 cells express the Th17 master regulator ROR γ T and are known to arise from a distinct population in the thymus. Intriguingly, recent reports have demonstrated that at least a fraction of V α 14i NKT cells can be differentiated into either follicular helper T cells or FoxP3⁺ regulatory T cells *in vivo* [54-56]. Taken together, these data suggest new avenues to explore in the search for ways to manipulate responses of V α 14i NKT cells.

Concluding remarks

Studies have shown that not only is the pathway of iNKT cell differentiation very different from other thymocytes, but it is more complex, in that many more genes are required. This intrathymic process, similar in some ways to an immune response, allows the generation of a memory-like population. As more genes are discovered that are critical for iNKT cell differentiation, it is becoming increasingly possible to categorize them into pathways and networks. Given the powerful tools at hand, we can expect even more progress in understanding these pathways in the coming years.

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Highlights

- V α 14i NKT cells are a semi-invariant T cell population.
- These cells recognize glycolipids presented by CD1d.
- V α 14i NKT cells have phenotypic properties of memory T and NK cells.
- The genetic requirements for V α 14i NKT cell development are complex.

Figure 1.

Diagram showing approximate limits of accessibility of the TCR $J\alpha$ locus to rearrangement at early and late stages of the lifespan of a DP thymocyte, the developmental potential of early and late DP thymocytes with respect to conventional and $V\alpha 14i$ NKT differentiation, and some factors known to regulate DP lifespan. The position of the $J\alpha 18$ segment that is required for formation of the invariant NKT TCR is indicated in bold type. Secondary rearrangements occur if the primary rearrangement does not support positive selection. The exact boundary of rearrangement in early DP thymocytes is uncertain, and more likely represents a gradient of accessibility, with distal $J\alpha$ rearrangements occurring in longer lived DP thymocytes. [17]