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Transcriptional regulation of the NKT cell lineage

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Abstract

How expression of canonical semi-invariant TCRs leads to innate-like effector differentiation is a central enigma of NKT cell development. NKT thymic precursors undergo elevated TCR signals leading to increased *Egr2*, which directly induces their signature transcription factor, PLZF. PLZF is necessary and sufficient to induce a multipotent, unbiased effector program that precedes terminal differentiation into T-bet^{high} NK1.1⁺ (NKT1) cells and recently identified NKT2 and NKT17 sublineages. Major variations in polarized NKT sublineages have been uncovered in different mouse strains and in several mutants, with direct impact on NKT cell function but also, unexpectedly, on the development and function of conventional T cells.

Introduction

Innate-like T cell lineages are defined by their evolutionarily conserved, semi-invariant $\alpha\beta$ or $\gamma\delta$ TCRs and their expression of the signature transcription factor PLZF [1,2]. The best characterized population is composed of $\alpha\beta$ T cells specific for microbial lipids presented by MHC class I-like CD1d molecules, which mostly (but not exclusively) express canonical V α 14-J α 18 TCR α chains associated with V β 8, V β 7 or V β 2 [3,4]. Other PLZF-expressing lineages include V γ 1-V δ 6.3 T cells [5,6], whose ligand is unknown, and mucosal associated invariant T (MAIT) cells expressing V α 19-J α 33 TCRs specific for microbial vitamin B metabolites presented by MR1 [7,8].

Because expression of these canonical TCRs is sufficient to induce PLZF expression and innate-like differentiation [5,9], studies of TCR signaling and co-signaling events during thymic selection have provided critical insights into the mechanisms of NKT lineage decision. Recent advances in this area are the subject of this review.

Elevated TCR signaling during thymic selection

The V α 14 TCR recognizes several endogenous agonist lipid ligands [10–13] presented by CD1d on the surface of cortical thymocytes [14]. Accordingly, various studies support the notion that NKT thymocytes undergo elevated TCR signaling. For example, although NKT precursors activate Ras/MAP kinase [15] and calcineurin/NFAT [16] as conventional thymocytes, they exhibit more elevated *Egr1* and *Egr2* downstream of these respective pathways at their CD69⁺CD24^{high} ‘transitional’ stage following TCR engagement (so-called ‘stage 0’) compared with conventional thymocytes, and these levels are sustained in later stages [17]. Consistent with increased TCR signaling, stage 0 and stage 1 cells also express

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higher levels of CD5 and PD-1 and they express more GFP in Nur77-GFP reporter mice [18].

How NKT precursors escape negative selection despite agonist signaling may be due to the lack of B7 expression by cortical thymocytes [19,20] and to the alternative engagement of homophilic interactions between Slam family molecules such as Slamf1 and Slamf6 [21], which signal through SAP and Fyn to induce NF- κ B and promote NKT cell survival.

PLZF expression induced by Egr2

PLZF induction begins at stage 0, when Egr2 levels are highest, and is expressed by 100% of NKT thymocytes at the subsequent stage. Egr2 ChIP-Seq of NKT thymocytes revealed binding of Egr2 to a single canonical Egr binding site in the proximal promoter of PLZF [17]. Furthermore, injection of anti-TCR β antibody as an agonist ligand *in vivo* led to an immediate increase of Egr2 followed by PLZF mRNA induction in conventional thymocytes. Other Egrs may also bind the PLZF promoter and it is difficult to dissect their respective contribution because Egr2-deficient thymocytes had a compensatory increase in Egr1. Nevertheless, Egr1/2 double deficient NKT thymocytes did not express PLZF at stage 1 and, likewise, injection of anti-TCR β antibody in these double mutant mice failed to induce PLZF in thymocytes [17]. The induction of PLZF as a consequence of elevated TCR signaling and Egr2 may be a general feature of innate-like T cell lineages, as V γ expressing, anti-TCR antibodies induced PLZF expression during development of the corresponding V γ -expressing subset in OP9-DL1 cultures [5]. Furthermore, in a model of transgenic MHC class II expression on cortical thymocytes where MHC class II-restricted CD4 T cells acquire innate-like effector differentiation, PLZF expression was observed on the fraction of cells with the highest level of Nur77-GFP [22].

Egr2 and PLZF direct different branches of NKT lineage differentiation

In general, Egr2 appears to promote cell division and survival through direct activation of *Ccnd2*, *Bcl2* and *FasI* [17], while PLZF directs the acquisition of effector properties [23]. In the absence of PLZF, NKT cells revert to naïve-like cells recirculating between lymph nodes and blood and producing IL-2 rather than IL-4 or IFN- γ . Conversely, transgenic expression of PLZF under the *Cd4* promoter is sufficient to induce the upregulation of CD44 and the downregulation of CD62L, the upregulation of LFA-1 and the homing to liver and lung, the ability to secrete a mixture of IL-4 and IFN- γ at the single cell level as well as IL-17 [1,2,23–25]. Thus, PLZF induces a mixed, unpolarized cytokine program. PLZF is a member of the BTB-ZF family of transcription factors that binds a corepressor complex containing NCoR, Sin3a and HDAC1, as well as the E3 ligase cullin3 whose ablation phenocopies the PLZF deficiency [26]. The molecular details of the transcriptional program and the direct targets of PLZF are under investigation. Nevertheless, it is notable that this program can unfold in the absence of cell division or agonist signaling, as shown in PLZF transgenic mice, demonstrating that acquisition of effector properties can be uncoupled from strong TCR signaling and division [23,24]. Consistent with this view, PLZF-deficient NKT thymocytes still exhibit the same rate of EdU incorporation as their wild-type counterparts [1,2], which is presumably driven by Egr2 and other TCR signals. However, the mutant cells do not expand, suggesting increased cell death. In that regard, while anti-apoptotic signals arise from Slamf-SAP-Fyn-PKC θ -NF- κ B signaling, PLZF may also exert anti-apoptotic properties, as suggested by the partial rescue of Mtv-reactive thymocytes from negative selection in PLZF transgenic mice [23]. Importantly, Egr2 appears to contribute to the initiation of NKT1 polarization as it plays an essential role in binding and activating *Il2rb* [17], a key component of the IL-15 receptor that is critical to this terminal differentiation pathway. Consistent with this role of Egr2, low levels of IL-2R β and NK1.1 can be observed in a fraction of PLZF-deficient NKT thymocytes [1,2]. Furthermore, it has been reported

that continuous exposure to CD1d was required for NK1.1 expression [27,28], which may be due to the need for sustained Egr2.

Terminal differentiation of polarized NKT sublineages

T-bet^{high} NKT1 cells capable of producing large amounts of IFN- γ are the main terminal differentiation product in B6 mice [27,29]. It should be stressed, however, that these cells are not fully polarized in that they produce both IFN- γ and IL-4 on a single cell basis, although the amount of IL-4 is comparatively low compared with previous developmental stages. Furthermore, it is the NK1.1⁺ subset that is responsible for the rapid release of IL-4 upon stimulation of NKT cells *in vivo* [30]. Recent studies, however, have uncovered alternatively polarized NKT sublineages, including GATA3^{high} NKT2 [31,32] and ROR γ t⁺ NKT17 cells [33–35]. These sublineages are rare in B6 mice but can be dominant in other strains or in various mutant mice. NKT2 cells, which are more abundant in BALB/c mice, predominate in the lung; NKT17 cells are rare and mostly found in peripheral lymph nodes draining the skin; NKT1 cells predominate in spleen and liver. These polarized sublineages have the typical profiles of transcription factors, cytokines and chemokine receptors found in their Th1/Th2/Th17 or ILC1/ILC2/ILC22 counterparts, although their bias seems to be less absolute because, for example, NKT1 cells also produce IL-4.

These new findings warrant a modification of the early model of NKT cell development and raise the question of the branch point at which NKT2 and NKT17 cells emerge. Intracellular staining for GATA3 and ROR γ t indicate that these subsets are included within the previously termed stage 2 NKT thymocytes, which also contain the precursors to NKT1 cells, as demonstrated by intrathymic cell transfers. In search of a surface marker differentiating true stage 2 precursors from terminally differentiated NKT2 and NKT17, it has been suggested that IL-17RB, a key component of the receptor for IL-25, was selectively expressed in NKT2 and NKT17 precursors but not in NKT1 precursors [32]. These conclusions were derived based on transfers of NKT thymocyte subsets into D-guanosine treated fetal thymus cultured *in vitro*, or intravenously into J α 18-deficient mice. Strikingly, mice lacking IL-17RB lacked both NKT2 and NKT17, but not NKT1 thymocytes, suggesting that commitment to these sublineages required signaling through this receptor [32] (Dr. Watarai, personal communication). As IL-17RB is expressed as early as in stage 1 cells, the commitment to NKT1 vs NKT2 or 17 sublineages may occur very early. Because ~50% of stage 1 and 75% of stage 2 cells are IL-17RB⁺ in the B6 background, the implication of these findings is that there are more thymic precursors to the NKT2 and NKT17 lineage than to the NKT1 lineage, which is in apparent conflict with the overall predominance of mature NKT1 cells in B6 mice both in thymus and in periphery. One possibility is that the NKT2 and NKT17 cells are short-lived compared with NKT1 cells, which exhibit IL-15-driven self-renewing properties [36]. Indeed, most NKT17 cells in the periphery express neuropilin 1, a marker of recent thymic NKT emigrants, and rapidly disappear after thymectomy [37].

Transcriptional regulation of polarized NKT sublineages

Studies of mice lacking T-bet, GATA3 and ROR γ t, the signature transcription factors of NKT sublineages have shed partial light on the mechanisms regulating the NKT sublineage decisions. T-bet-deficient [38] and IL-15-deficient mice [36] exhibited a block in the development of NKT1 thymocytes at a IL-2R β -NK1.1⁺ and IL-2R β ^{low}NK1.1^{low} stage 2, respectively. Interestingly, they also exhibited a relative increase in NKT2/NKT17 cells [32,38]. In mice lacking the BLIMP-1 homolog Hobit, which is specifically expressed in mouse NKT1 cells, a relative accumulation of NKT2 or stage 2 thymocytes was observed, along with defects in NKT1 cell number and function affecting expression of Ly49 receptors, granzyme B and IFN- γ [39]. Whether the apparent crossregulation between NKT

subsets reflects accumulation of arrested precursors or antagonism between the different sublineages remains to be investigated.

The respective impact of GATA3 and ROR γ t has been more difficult to evaluate because these mutations have widespread effects on thymocyte development. For example, in the absence of ROR γ t, distal V α 14-J α 18 rearrangements did not occur, due to the shorter lifespan of cortical thymocytes, and NKT cells were consequently totally absent [40,41]. GATA3^{fl/fl} Cd4-cre mice exhibited a complex phenotype whereby stage 1 and 2 thymocytes were drastically diminished, despite the presence of apparently normal numbers of NKT1 cells in the thymus [42]. These NKT1 cells were not merely cells that escaped GATA3 deletion and accumulated in the thymus, because their expression of ThPOK, a target of GATA3, was also impaired [43]. There were fewer and dysfunctional NKT1 cells in the periphery, however, implying that GATA3 impacted the development and/or survival of all NKT sublineages.

As transgenic studies have established that PLZF expression is sufficient to prime developing thymocytes for the production of an unbiased mixture of IL-4, IFN- γ and IL-17, an appealing scenario is that PLZF promotes a mixed pattern of gene expression by multilineage transcriptional priming, similar to PU-1 in myeloid progenitors [44], prior to the segregation of polarized transcriptional programs driven by antagonistic regulatory circuits. In that regard, it is intriguing that PLZF is partially downregulated in NKT17 (PLZF^{int}) and NKT1 (PLZF^{low}) compared with early NKT precursors and NKT2 cells (PLZF^{high}). As previous reports have ruled out a role for STAT4 and STAT6 in NKT cell development [45,46], novel mechanisms that determine the terminal polarization of NKT1, NKT2 and NKT17 remain to be elucidated.

Dysregulation of NKT2 and NKT17 sublineages in inbred and mutant strains

Intriguingly, several apparently unrelated mutations were recently found to produce closely related phenotypes consisting of the transformation of conventional naïve thymocytes into effector/memory cells prior to emigration. Hogquist et al. investigated the phenotype of mice lacking KLF-2, a transcription factor that controls CD62L and S1P₁ expression after positive selection and was previously associated with the maintenance of lymphocyte quiescence. Mixed bone marrow chimeras established that the ‘innate effector/memory’ phenotype of conventional T cells in KLF2 deficient mice was in fact the bystander consequence of a cell-intrinsic dysregulation of NKT2 (or stage 2) NKT thymocytes releasing IL-4 in the steady state [47,48]. Furthermore, an expansion of IL-4 producing V γ 1-V δ 6.3 T cells was also observed, indicating a general dysregulation of PLZF-expressing thymic lineages [49]. A similar mechanism operates in mice lacking the Tec kinases Itk or Rlk [47,50,51], or bearing the Y146F mutation in the scaffold protein SLP756 [6], which impairs the binding of these kinases after TCR signaling, as well as in mice lacking the E protein inhibitor Id3 [6,52,53] or the histone acetyl transferase CBP [54]. At present, it is unclear whether these genes belong to the same signaling pathway or whether they impact different regulatory pathways that ultimately converge to cause an expansion of NKT2-like cells and the steady-state secretion of IL-4.

While the physiological significance of these mutations is unclear, it is remarkable that a related phenotype, including the effector/memory conversion of thymic CD8 cells, was recently reported in wild-type BALB/c mice, which harbor a spontaneous expansion of NKT2 cells [47,55]. In the absence of CD1d, BALB/c CD8 T cells reverted to a naïve phenotype, demonstrating that NKT cells exert bystander regulation of the thymic microenvironment with substantial impact on T cell functional properties. Notably, BALB/c NKT thymocytes lost their exaggerated NKT2 phenotype in the absence of the transcription factor KLF-13, although this factor did not appear to be dysregulated in BALB/c cells [55].

ThPOK, a critical regulator of the CD4 lineage is also expressed in all NKT cells, whether CD4⁺ or CD4⁻, but is not required for their development. However, closer analysis revealed that ThPOK-deficient mice exhibited not only partial defects in NKT1 differentiation and frequency, but also a reciprocal increase in ROR γ t-expressing NKT17-like cells, suggesting a degree of antagonism between ThPOK and ROR γ t [43,56,57].

NKT-fh and NKT-reg

NKT cells can readily express Bcl-6 and become follicular helper cells upon activation by their microbial lipid ligands [58,59], and they can express Foxp3 and suppress CD4 T cell proliferation following treatment with TGF β [60,61]. However, these pathways do not seem to operate during development in the thymus, at least in normal mice.

Role of the PI3K/mTOR pathway

As NKT thymocytes, unlike conventional CD4 and CD8 T cells, undergo blasting and cell division during development, it is anticipated that their metabolic needs will be tightly regulated by the PI3K/mTOR pathway. Mice lacking, or expressing a catalytically inactive form of phosphoinositide dependent kinase 1 (PDK1), which is required for activation of Akt showed NKT developmental arrest between stages 0 and 1, with defective blasting and curtailed expansion of NKT precursors [62], a phenotype similar to the c-Myc mutant NKT thymocytes [63,64]. Mice lacking Tsc-1, a suppressor of mTORC1, exhibited defective NKT cell development due to apoptosis [65]. How the mTOR pathway is recruited and whether it can also differentially regulate the polarized NKT sublineages, as is the case for T-helper lineages [66], are areas of investigation.

Conclusion

Recent advances have elucidated, in great detail, the TCR signaling pathway and the transcriptional network governing NKT lineage development. The classic 'stage 0-1-2-3' model has been enriched by the discovery of alternative NKT2 and NKT17 sublineages which not only impact NKT cell responses to microbial pathogens, but also regulate in the steady state previously unsuspected aspects of conventional T cell development and function. Many new exciting areas are open for future investigations, including a genomic and biochemical understanding of PLZF function with identification of its direct targets and its broader transcriptional regulatory network. Multilineage priming by a BTB-ZF transcription factor and its subsequent resolution into cytokine polarized sublineages, as apparent in PLZF-expressing innate-like T cell lineages, may be a shared mechanism across lymphoid cells such as CD4 T helper cells and innate lymphoid cells.

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Highlights

- PLZF is the master transcription factor of the NKT cell lineage.
- PLZF is induced during thymic development by Egr2 downstream of TCR/NFAT signaling.
- PLZF directs acquisition of the innate-like effector program whereas Egr2 and other TCR signals/co-signals induce cell division.
- In addition to the major T-bet^{high} NKT1 subset, novel NKT sublineages include NKT2 and NKT17.
- Different mouse strains exhibit wide variations in the relative representation of NKT1, NKT2 and NKT17.

Figure 1. Thymic developmental pathway of the NKT cell lineage

The current model incorporates the newly discovered NKT2 and NKT17 subsets side by side with NKT1 cells (all of which correspond to developmental stage 3). The branching points between the NKT1, NKT2 and NKT17 sublineages at stage 1 and 2 are still under study. The thymic emigrants are still cycling and immature. Note, that Tbet⁺ NKT1 cells do produce IL4, albeit in smaller amounts than NKT2 cells. Some NKT1 precursors fail to emigrate, possibly due to premature downregulation of S1P₁ [67], and they accumulate in the thymus of aging mice (not depicted). NKR, NK lineage receptors including NK1.1, Ly49, NKG2D, CD94, DX5.