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Transcriptional regulation of NKT cell development and homeostasis

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Summary

NKT cells comprise a distinct T cell subset that acquires effector function during development and prior to antigen exposure. NKT cells are of limited antigen specificity but possess the ability to be recruited into an immune response without the need for further differentiation or proliferation and thus may be considered to function as memory cells or as part of the innate immune system. While the development and maturation of NKT cells share some similarities with conventional T cell populations, many transcriptional regulators and signaling molecules are known to be uniquely required for NKT cell development. Recently, new transcription factors that specify NKT lineage and effector function and novel roles for previously identified transcriptional regulators in the differentiation of the NKT cell population have been discovered.

Introduction

Upon activation, NKT cells participate in the early phases of the immune response through production of great quantities of numerous cytokines that can influence the activation of other immune cells such as NK cells, macrophages, dendritic cells, B and T lymphocytes. Thus, NKT cells can influence a wide range of immune responses and disease states. The NKT population can be divided into three main subsets [1,2]: Type I are the well-studied and most abundant V α 14 (mouse) or V α 24 (human) invariant subset which can be activated through interaction of their TCR with glycolipid antigens, such as α -galactosylceramide (α GalCer), presented by the MHC class I-like molecule CD1d. This population of NKT cells will be referred to as *i*NKT cells and are the focus of this review. Type II include CD1d-reactive NKT cells with diverse non-V α 14 TCR usage. Type III are CD1d-independent NKT cells, which have diverse TCR rearrangements. The majority of NKT cells are type I and express a semi-invariant TCR with a canonical TCR V α 14-J α 18 rearrangement associated with TCR V β 8, V β 7, or V β 2 chains and can be identified using CD1d-tetramers loaded with α GalCer. In the C57BL/6 background, most *i*NKT cells can also be identified by expression of NK1.1 and TCR β , however not all functionally mature NKT cells express NK1.1 [3].

*i*NKT cells develop in the thymus, where they differentiate from CD4⁺CD8⁺ double positive (DP) thymocytes and proceed through a number of key stages at which various factors are essential for their development [1,2]. Rearrangement of the canonical V α 14-J α 18 TCR by developing DP thymocytes mediates recognition of CD1d on neighboring thymocytes and

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initiates the positive selection process of *i*NKT cells. Signals initiated by TCR and SLAM family receptors allow progression to the earliest detectable subset of *i*NKT cell, which can be termed stage 0, where cell surface molecules CD24 and CD69 are expressed [4] (Fig. 1). Subsequently, *i*NKT cells progress through three more developmental stages: CD24^{lo}CD44^{lo}NK1.1⁻ (stage 1), CD44^{hi}NK1.1⁻ (stage 2), and CD44^{hi}NK1.1⁺ (stage 3) [1, 2]. This last maturation step for many *i*NKT cells often occurs in the periphery after CD44^{hi}NK1.1⁻ NKT cells have exited the thymus. During maturation, *i*NKT cells upregulate expression of many markers of T cell activation (including CD44, CD69, CD122) and surface molecules also expressed by NK cells (such as KLRG1 and NK1.1).

Transcriptional regulation of *i*NKT cell differentiation from DP thymocytes

The divergence of *i*NKT cell development from conventional T cell development appears to be initiated by the rearrangement of the TCR α gene segments V α 14 to J α 18, which allows recognition of their selecting antigen in the context of CD1d, when combined with the appropriate TCR β chain rearrangements. All DP cells must successfully rearrange a TCR α chain to continue maturation, with rearrangements first occurring between proximal V α and J α segments. If such primary rearrangements fail to generate a productive TCR α chain, secondary rearrangements between V α and J α segments that are more distal from each other will then proceed. It is well established that the canonical *i*NKT TCR, utilizing V α 14 to J α 18 rearrangements, requires such secondary rearrangements and thus the development of *i*NKT cells is dependent on factors that promote distal rearrangements. One such transcription factor, ROR γ t, is required for *i*NKT cell development [5,6] due to the fact that it is essential for the induction of expression of the anti-apoptotic molecule Bcl-x_L [7,8]. Bcl-x_L allows DP thymocytes to survive long enough for distal TCR α rearrangements to occur. ROR γ t-deficient thymocytes show decreased Bcl-x_L expression, diminished survival, and an absence of distal TCR α rearrangements, including the canonical V α 14-J α 18 chain necessary for *i*NKT cell development [9] (Fig. 1a). Once committed to the *i*NKT lineage, numerous transcription factors, cytokines, and signaling molecules have been identified that are uniquely required for *i*NKT cell maturation, but dispensable for conventional T cell development. In this review, we focus on recent advances identifying transcriptional regulators that intrinsically control the generation and homeostasis of the *i*NKT cell population. In Figure 1, the stage(s) of *i*NKT cell development affected by specific transcriptional regulators are noted, with the most recent highlighted.

A role for the E protein transcription factor HEB

Recently, we have discovered that the E protein transcription factor, HEB, is essential for *i*NKT cell development. HEB is a member of the type I basic helix-loop-helix family and, in conjunction with its family member E2A, binds consensus E-box regulatory sites and influences numerous aspects of T cell development [10–13]. Using conditional knock-out mice, in which HEB was deleted in DP thymocytes, we found that *i*NKT cells failed to develop at all in the absence of HEB [14••]. This defect was severe and deficiency in HEB blocked *i*NKT cell development at their earliest developmental stage (CD24^{hi}CD44⁻NK1.1^{lo}). Loss of HEB diminished survival of developing DP thymocytes, and analysis of TCR α rearrangements revealed that all HEB-deficient thymocytes lacked distal J α gene rearrangements, including the canonical rearrangement required for *i*NKT cell development [14••]. However, the development of conventional CD4⁺ and CD8⁺ T cells was apparently normal in spite of a limited TCR repertoire. Interestingly, loss of HEB expression resulted in diminished levels of ROR γ t (which is likely a direct target of HEB) and Bcl-x_L mRNA. Furthermore, *i*NKT cell development was rescued by expression of a rearranged V α 14 TCR transgene, providing further evidence that HEB influences *i*NKT cell development by regulating thymocyte survival and thus distal rearrangements of the TCR α chain [14••]. These data revealed a surprising role

for HEB, distinct from that of its sister protein E2A, in influencing *i*NKT cell development from DP thymocytes and in controlling the total T cell repertoire.

PLZF in specification of the *i*NKT effector program

Recently, two groups reported the identification of a new transcriptional regulator, promyelocytic leukemia zinc-finger transcription factor (PLZF), as a key factor in establishing the *i*NKT cell lineage and effector functions [15•,16•]. PLZF was expressed rather selectively by developing *i*NKT cells after positive selection (beginning at stage 0). Strikingly, its deficiency lead to a block in *i*NKT maturation at the transition from stage 1 to stage 2, resulting in a dramatic reduction in the frequency and number of *i*NKT cells in thymus, spleen and liver. The few *i*NKT cells that did accumulate did so only in the lymph nodes where they were phenotypically immature and produced significantly less IL-4 and IFN γ (as well as other cytokines) upon activation [15•,16•]. Hence, PLZF is required for the early stages of *i*NKT cell maturation and acquisition of the “innate-like” production of cytokines. The factors controlling PLZF expression have not yet been identified, although it appears that SLAM-mediated signals are not required for PLZF expression as mRNA levels were normal in both Sap- and Fyn-deficient cells [15•,16•]. Interestingly, forced PLZF expression converted CD4⁺ and CD8⁺ T cells to a memory phenotype and promoted their ability to produce effector cytokines instead of IL-2 when activated [16•,17]. In the case of another innate T cell subset, $\gamma\delta$ T cells, PLZF was shown to be expressed by the V γ 1⁺ subset where it similarly functioned to support cytokine production [18], particularly dual secretion of IFN- γ and IL-4 as was observed for *i*NKT cells. More recently it was shown that PLZF-deficient mice have impaired anti-viral immunity, likely due to defective NK cell activation [19•]. This is of particular interest as PLZF was initially known as a transcriptional repressor; however, here it was shown that IFN signaling lead to activation of PLZF, its association with histone deacetylase 1 and promyelocytic protein, and direct activation of a subset of interferon response genes. Thus, in multiple contexts, PLZF supports the development and acquisition of effector capability by lymphocytes with innate functional activity.

Calcium signaling and Egr2

Selection of conventional T cells from DP thymocytes requires TCR-mediated signals, which induce numerous intracellular events including increased intracellular calcium levels and subsequent activation of calcineurin. Activation of calcineurin leads to dephosphorylation of cytosolic NFAT family members, leading to their accumulation in the nucleus and induction of their gene targets, including early growth response (Egr) transcription factors. Recently, this pathway was found to also regulate *i*NKT cell development [20,21•]. Thymocyte-specific deletion of calcineurin B1 led to a significant loss in both thymic and peripheral *i*NKT cell numbers. When potential gene targets of calcineurin activation were explored, Egr2 emerged as one that specifically affected *i*NKT cell development [21•]. While loss of Egr1 or Egr3 is known to affect development of conventional T cells, their loss did not impact the generation of *i*NKT cells. In contrast, Egr2-deficient fetal liver chimeras showed dramatically impaired development and accumulation of *i*NKT cells. Egr2 appeared to be important after positive selection of V α 14 DP cells into the *i*NKT cell lineage as percentage and numbers were diminished subsequent to the CD24⁺ stage 0 (Fig. 1a). The residual Egr2-deficient *i*NKT cells displayed increased evidence of apoptosis and marginally impaired cytokine production. Together, these data provide evidence for calcineurin-NFAT-Egr2 pathway in support of *i*NKT cell generation/homeostasis.

The nuclear factor thymocyte selection-associated HMG box protein (TOX), which is induced by calcineurin-mediated TCR signaling in DP thymocytes, was also recently shown to be essential for *i*NKT cell development [22•]. Mice lacking TOX showed a severe block in the late stages of positive selection of conventional CD4⁺ T cells, generated significantly fewer

regulatory T cells and failed to generate *i*NKT cells. While the specific stage of *i*NKT development affected by TOX-deficiency has not yet been determined, the severe paucity of CD1d-tetramer⁺ cells observed suggests a relatively early block (Fig. 1a). The mechanism by which TOX regulates development of these T cell lineages is still under investigation; however, these results highlight the fact that *i*NKT cell development is dependent on numerous nuclear factors, some in common with conventional T cells and others uniquely essential for the *i*NKT lineage.

c-Myc

The bHLHZip transcription factor myelocytomatosis oncogene (c-Myc) has well appreciated roles in cell growth via its regulation of proliferation and survival. Interestingly, two groups have now found a highly specific role for c-Myc in the development of mature *i*NKT cells [23••,24••]. With deletion of c-Myc in DP thymocytes, no mature *i*NKT cells were generated with a block reported between stages 0 and 1 [23••] or stages 1 and 2 [24••]. While there were some differences in the two reports, reduced proliferation was observed by c-Myc-deficient *i*NKT cells at stage 0 compared to their wild-type counterparts [23••] and development could not be rescued by transgenic expression of Bcl-2, suggesting survival was not impaired [23••,24••]. Interestingly, c-Myc binds to E-box regulatory sites as a heterodimer to promote proliferation and has been shown to regulate proliferation of conventional CD4⁺ T cells in response to antigen as well as thymocyte proliferation in response to pre-TCR signaling [25]. Furthermore, the very few *i*NKT cells that developed in the absence of c-Myc upregulated markers of differentiation such as PLZF, GATA-3 and IL-4, arguing that c-Myc does not regulate expression of such targets. At this time it is not known which signals regulate c-Myc expression or activity during *i*NKT differentiation, however, it has been suggested that its induction may require NF-κB family members downstream of either TCR or Sap-mediated signals [25].

Additional transcription factors

Runt related transcription factor 1 (Runx1) plays key roles in many aspects of hematopoiesis and thymocyte development and is also essential for *i*NKT cell development from DP thymocytes. In its absence, *i*NKT cell development is blocked at the earliest detectable *i*NKT committed subset (stage 0) [6] (Fig. 1a). However, Vα14-Jα18 rearrangements can be detected by DP cells in the absence of Runx1, indicating that its activity is required during positive selection or for expansion at subsequent stages [6]. At this point, little is known about how Runx1 expression is regulated or which gene targets are relevant by cells committing to the *i*NKT lineage.

NF-κB family members are also implicated in *i*NKT cell development downstream of SLAM-Sap-Fyn signaling cascade and/or TCR-mediated signals [2]. Mutations in various molecular components of the NF-κB pathway result in both intrinsic and extrinsic defects in the *i*NKT cell populations at various stages. Loss of classical NF-κB signaling due to over-expression of dominant-negative form of IκBα or due to deletion of NF-κB1 reveals intrinsic, defective progression from stage 2 to stage 3 [26,27]. Interestingly, loss of another family member, RelA also yields a dramatic block at the same stage of *i*NKT maturation, perhaps in part due to its regulation of IL-15 responsiveness [28]. Notably, interference of the classical NF-κB pathway using an alternative strategy, T cell-specific deletion of IKK2, lead to a loss of *i*NKT cells at an apparently earlier stage [29]. Thus, there is significant evidence supporting a key function for the classical NF-κB pathway with individual components impacting different stages of *i*NKT maturation.

The T box transcription factor, T-bet, is essential for *i*NKT cell development during the last stage of thymic maturation (stage 2 to 3) [30,31]. In the absence of T-bet, *i*NKT cells

accumulate at stage 2 and fail to acquire effector functions (i.e. ability to produce IFN- γ and kill targets) [30,31]. Interestingly, IL-15 (along with IL-12 and IL-18) may induce T-bet expression [31,32] and T-bet (along with its related family member Eomesodermin) have been implicated in promoting expression of the IL-15-receptor component, CD122 [30,33]. Thus, a positive-feedback loop may be mediated by these two molecules during the IL-15-dependent development/expansion phase that occurs during thymic development [34,35].

Transcriptional regulation of peripheral *i*NKT cell homeostasis and survival

The molecular factors controlling peripheral maturation and survival of *i*NKT cells have not been extensively investigated. It is known that like memory T cells, *i*NKT cells undergo a slow, IL-15-dependent proliferation and are independent of their selecting ligand for survival [35, 36]. However, little is known about the transcriptional regulation of *i*NKT homeostasis. Recently, we found that Id2, a natural antagonist of E protein transcription factors, is required for survival and maturation of peripheral *i*NKT cells [37••](Fig. 1b). The absence of Id2 expression by hematopoietic cells resulted in a severe, cell-intrinsic defect in the percentage and number of *i*NKT cells in the liver, one of their primary sites of accumulation. Id2-deficient hepatic *i*NKT cells displayed increased cell death as well as impaired up-regulation of some activation markers. Interestingly, Id2-deficient *i*NKT cells expressed significantly lower levels of the chemokine receptor CXCR6 compared to wild type *i*NKT cells, and CXCR6 is known to regulate accumulation of hepatic *i*NKT cells [38,39]. However, Id2-deficiency leads to a more severe defect in hepatic NKT cells than observed for CXCR6-deficient mice, suggesting Id2 regulates expression of additional targets. Ultimately, the failure of Id2-deficient NKT cells to accumulate in the liver is likely due primarily to a survival defect as both mature and immature cells are lost and Id2-deficient hepatic *i*NKT cells expressed lower levels of the anti-apoptotic molecules Bcl-2 and Bcl-x_L [37••]. Furthermore, the removal of the pro-apoptotic molecule Bim rescued the *i*NKT cell population on the Id2-deficient background. Thus, Id2 mediates the survival of peripheral hepatic *i*NKT cells.

To address why the Id2 defect was liver specific, we evaluated expression of multiple Id family members by NKT cell populations from different tissues. We found that splenic *i*NKT cells expressed mRNA for both Id2 and another Id family member, Id3, while hepatic *i*NKT cells expressed only Id2 mRNA [37••]. We hypothesize that Id-mediated inhibition of E proteins by Id2 and/or Id3 is essential for survival of post-selection *i*NKT cells. Thus, Id2-deficiency specifically impairs the hepatic *i*NKT population as Id2 is the only Id protein expressed, whereas splenic *i*NKT cells survive due to compensatory activity of Id3. Experiments are needed to address whether Id proteins impact *i*NKT cell homeostasis entirely due to their ability to diminish E protein activity and whether E proteins, such as HEB, regulate mature *i*NKT cell homeostasis. Consistent with a role for Id2 in attenuating E protein function, it is known that high levels of E protein activity promote cell death and that Bim expression is induced by E protein activity [40,41]. Notably, the requirement for expression of Id proteins by *i*NKT cells in the periphery implies that E proteins must be negatively regulated during mature *i*NKT cell homeostasis. Curiously while we found that the E protein HEB was required for Bcl-x_L expression during *i*NKT cell development, the loss of Id2 expression by peripheral homeostasis (presumably resulting in great E protein activity) also led to a loss of Bcl-x_L expression. This implies that either E protein transcription factors differentially regulate their target genes based on developmental stage (perhaps due to differences in expression of cofactors) or that a different E protein maybe targeting Bcl-x_L expression by mature *i*NKT cells. Further experiments identifying the relevant E proteins and their targets in mature *i*NKT cells will be necessary to resolve these issues.

Interestingly, Id2 is similarly necessary for terminal NK maturation and for survival of CD8⁺ effector cells and memory T cell formation [42,43], suggesting a common role for Id2

in regulating accumulation of mature effector lymphocyte populations by promoting their survival. It is worth raising the point that multiple factors associated with the activation in response to antigen and formation of memory subsets by mature, conventional T cells, including Id2, T-bet, c-Myc, and NF- κ B family members, prove to be essential during *i*NKT cell maturation. The parallel requirements for these factors may indicate common transcriptional regulation of the acquisition of effector functions such as the ability to rapidly produce cytokines, lyse targets and to perform these functions without the need for proliferation or differentiation upon antigen exposure. For *i*NKT cells, all of these functions are obtained during the process of thymic differentiation, which may explain why molecules that impact conventional T cell activation are required *i*NKT development.

In another report addressing transcriptional control of *i*NKT cell homeostasis, mice lacking the zinc finger transcription factor GATA-3 were shown to have a loss of peripheral *i*NKT cells, where numbers of both splenic and hepatic populations were significantly reduced, but thymic numbers were unaffected [44••] (Fig. 1b). These mice show also a dramatic block in development of conventional CD4⁺ T cells and the CD4⁺ subset of *i*NKT cells was largely absent in all tissues including the thymus. Further investigation is ongoing into the differences and similarities between CD4⁺ and DN *i*NKT cells and it is likely that GATA-3 plays a role specifically in the differentiation of CD4⁺ *i*NKT cells. While the remaining CD4⁺ GATA-3-deficient *i*NKT cells were able upregulate NK1.1, CD69 expression was significantly lower than wild type *i*NKT cells in the periphery (but not in the thymus) and these cells failed to produce cytokines or acquire an activated phenotype in response to α -GalCer. Interestingly, bypassing proximal TCR signaling by administration of PMA and ionomycin restored IFN- γ production and upregulation of CD69 by GATA-3-deficient *i*NKT cells, while production of IL-4 and IL-13 was not rescued, indicating defects at multiple levels. This is consistent with the fact that GATA-3 has been described as a specific activator of cytokine gene expression in CD4⁺ T cells [45] and to play a role in chromatin remodeling of cytokine gene loci [46]. It will be of interest to address the role of GATA-3 as both a chromatin-remodeling factor and as a direct transactivator of cytokine genes in the context of *i*NKT cell development and homeostasis.

Conclusions

While many individual transcription factors are now known to provide essential roles in *i*NKT maturation, what controls expression of these factors and how their activity is coordinated to promote positive selection, lineage specification, acquisition of functional activity and homeostasis remain poorly understood. Future studies will place these factors into transcriptional networks that will begin to clarify how *i*NKT lineage specification and maturation are accomplished through the activity of molecules both in common with and distinct from other lymphocyte lineages.

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Figure 1.

(a) Transcriptional regulators demonstrating intrinsic control over thymic maturation of *i*NKT cells. (b) Transcriptional regulators controlling homeostasis of the *i*NKT cell population after thymic maturation. Factors most recently found to be involved in *i*NKT cell development and discussed in this review are outlined in blue. For a comprehensive review of additional molecules regulating NKT cell development see [2].