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Development of invariant natural killer T cells

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Abstract

Invariant natural killer T (iNKT) cells develop into functionally distinct subsets. Each subset expresses a unique combination of transcription factors that regulate cytokine gene transcription upon activation. The tissue distribution and localization within tissues also varies between subsets. Importantly, the relative abundance of the various subsets is directly responsible for altering several immunological parameters, which subsequently affect the immune response. Here, I review recent advances in our understanding of the molecular regulation of iNKT cell subset development.

Introduction

iNKT cells are a unique subset of T lymphocytes characterized by their recognition of lipid antigens presented by CD1d, a non-polymorphic major histocompatibility complex class I-like antigen-presenting molecule [1–3]. These cells use an invariant T-cell antigen receptor (TCR) made up of a single invariant TCR α chain (V α 14-J α 18 in mice, V α 24-J α 18 in humans) in combination with certain TCR β chains (using V β 8.2, 7 or V β 2 in mice, V β 11 in humans) to engage CD1d. Upon antigenic stimulation, iNKT cells rapidly respond by producing cytokines, such as T-helper type, Th1, Th2 or Th17 cytokines [4]. They can also be potently cytotoxic [5]. Furthermore, it was recently shown that at least some iNKT cells produce cytokines at steady state, which in turn influences the development and activation of surrounding cells [6,7^{**}]. Although iNKT cells are not numerous, their unique properties establish them as a major regulatory cell population that is positioned to influence how immune responses develop. As such, iNKT cells have been implicated in diverse immune reactions, including responses against pathogens and cancer. iNKT cells have also been implicated in several autoimmune diseases and allergy [5]. This functional heterogeneity can be partly explained by the discovery of iNKT cell subsets that produce different cytokines and have distinct tissue localization preferences [6,8,9,10,11]. Recent advances in the area of iNKT cell development and the generation of iNKT cell subsets are the subjects of this review.

iNKT cell subsets

Five major functionally distinct iNKT cell subsets, each producing a different set of cytokines, have been recently identified. In addition to their cytokine secretion pattern, each

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subset can be further characterized by the expression of distinct transcription factors that generally correlate with their cytokine response upon activation. iNKT1 cells express T-bet and secrete predominantly IFN γ ; iNKT2 cells express high levels of GATA3 and PLZF and secrete IL-4 and IL-13 [6]; iNKT17 have intermediate levels of PLZF, are RoR γ t+ and secrete IL-17; iNKT_{FH} (follicular helper) express Bcl-6 and provide help to B cells in an IL-21 dependent fashion [12]; iNKT10 cells represent a unique subset of iNKT cells that are PLZF negative but express *Nfil3* (E4BP4) and secrete IL-10 to create an immunoregulatory environment [13,14*].

It is expected that all iNKT cells are of thymic origin. iNKT1, iNKT2 and iNKT17 cells acquire their functional capacity in the thymus during their development [6,15,16], before they distribute in the peripheral organs in tissues specific manner [7**]. To date, iNKT_{FH} and iNKT10 cells have not been described in the thymus and their origins and developmental cues remain unclear. It remains possible that these two subsets develop toward these phenotypes at very low frequencies in the thymus before migration, or that they may represent distinct states of activation. Indeed, iNKT_{FH} cells are induced in the spleen of mice following injection of lipid antigens [12]. Similarly, 'induced' iNKT10 cells that share common characteristics with the iNKT10 cells found at steady state in the adipose tissues have been described following antigen stimulation [14*,17].

A better understanding of iNKT cell subset development and of the signals that are required for the commitment to these various subsets will be key in providing new insight into how to control and manipulate these cells for therapeutic purposes.

Self-antigen(s) and the positive selection of iNKT cells

Positive selection of iNKT cell precursors in the thymus requires recognition by their TCR of 'self' lipid(s)-CD1d complexes [18]. Furthermore, in many cases the activation of iNKT cells in peripheral organs is believed to also be dependent on the presentation of endogenous lipids by CD1d molecules at the surface of APCs [19]. As such, tremendous efforts have been aimed at identifying potential self-antigen(s) for iNKT cells. This led to the identification of several glycolipids capable of activating iNKT cells *in vitro* and/or *in vivo* [20–22]. Consistent with the commonly held idea that α -linked glycosylceramides could not be enzymatically synthesized in mammalian cells [23], these self-glycolipids were either β -anomers of glycosylceramides (in which the sugar head group is attached to the lipid part through a β -linkage) or phospholipids. However, recent results established that the previous reports of iNKT cell stimulatory activity by mammalian-purified β -glucosylceramide [21] is due to minute traces of contaminating α -linked forms [24]. In subsequent studies using antibodies specific for the complex α -Galcer/CD1d, Kain *et al.* revealed that the endogenous ligands responsible for the activation of iNKT cells and their positive selection in the thymus are in fact α -linked monoglycosylceramide α -galactosylceramide and α -glucosylceramide, respectively [25**,26]. Because these two ligands show preferences in their interaction with iNKT TCRs composed of either V β 8 or V β 7 chains [27], and iNKT cell subsets show preference with regards to the usage of these two V β chains [6], these results suggest that the development of the various iNKT cell subsets could be dependent on the fine specificity requirements of the iNKT TCR-CD1d interaction.

Commitment to become an iNKT cell

These self-lipid/CD1d complexes are expressed by both DP thymocytes and epithelial cells in the thymus. iNKT cells are positively selected at the DP stage by CD1d-expressing DP cells themselves, instead of the epithelial cells that drive the selection of conventional T cells [28]. This unique selection process, involving homotypic interactions across the DP-DP synapse, imparts the selected thymocytes with the unique developmental program of iNKT cells. These generate 'second signals' mediated in part by the cooperative engagement of the homophilic receptors of at least two members of the signaling lymphocytic-activation molecule (SLAM) family (Slamf1 [SLAM] and Slamf6 [Ly108]) [29,30]. Such engagements lead to the downstream recruitment of the adaptor SLAM-associated protein (SAP) and the Src kinase Fyn, which were previously recognized as essential for the expansion and differentiation of the iNKT cell lineage [31].

A role for the TCR?

The engagement by the iNKT TCR of the agonistic antigens α -Galcer and α -Glucer presented by CD1d molecules expressed at the surface of DP thymocytes was expected to provide 'higher than normal' TCR signaling during positive selection compared to conventional T cells. This was directly confirmed *in vivo* by the analysis of nuclear hormone receptor Nur77-reporter mice [32]. Notwithstanding these strong TCR signals, a narrow window of iNKT TCR affinity for self-lipid/ CD1d complexes must exist that allows for the proper development of iNKT cells while avoiding negative selection [33*].

Agonistic TCR signals are associated with high expression of the Ras-dependent [34] and Ca^{2+} -dependent early growth response transcription factors early growth response protein (Egr)-1 and, especially, Egr-2 [35,36]. The expression level of Egr-2 in pre-selection DP thymocytes is potentiated by the co-stimulation through Ly108 [37*]. While Egr-2 directly regulates several genes involved in the development of iNKT cells, its modulation of the expression of the transcription factor PLZF is particularly important. Egr-2 is recruited at the promoter of Zbtb16 (which encodes PLZF) after TCR engagement and co-stimulation with Ly108 [35,37*]. Expression of PLZF then directs the acquisition of effector properties by the developing iNKT cells, specifying their migratory properties to peripheral organs and their ability to produce cytokines immediately upon stimulation [38,39]. This sequence of events suggest that TCR signaling associated with Ly108 signaling would be responsible for instructing the iNKT cell lineage.

It was recently reported that TCR-mediated signaling (with and without Ly108 signaling) was not sufficient to induce PLZF in conventional T cells [40*]. Furthermore, in naïve T cells, the Zbtb16 locus was found predominantly decorated with the histone tail modifications H3K27me3, which are usually associated with regions of the genome that are inactive and gene expression is unlikely [41]. A priori, these data are not consistent with a model where TCR signaling would directly impact the expression of PLZF. In DP thymocytes, however, the PLZF promoter possesses bivalent chromatin, where in addition to the H3K27me3 histone modification, the transcriptionally permissive modification histone H3 lysine 4 trimethylation (H3K4me3) is also found [42,43**]. Furthermore, conditional

deletion in T cells of two major components of the PRC2 complex that catalyzes the methylation of lysine 27 of histone 3 (the H3K27me3 methyltransferase Ezh2 [43**] and the lysine demethylase-like protein Jarid2 [44]) lead to an increase in the frequency of PLZF expressing iNKT cells. Interestingly, in absence of Ezh2, a large increase of PLZF⁺ T cells with a diverse TCR repertoire and iNKT-like properties was also observed. This suggests that in wildtype animals, a chromatin-based mechanism might be responsible for the integration of TCR specificity to commitment into unique T cell lineages. Moreover, Jarid2, which is upregulated upon TCR stimulation and during positive selection in the thymus, was shown to bind to the Zbtb16 promoter and thereby regulate the amount of another repressive histone modification (H3K9me3) at that site. Together, these results suggest that a window of opportunity during T cell development might exist, during which strong TCR signaling coupled with Ly108 signaling (and perhaps other signaling pathways) might allow for the induction of PLZF by reducing the levels of H3K27me3 and H3K9me3 at the bivalent Zbtb16 gene.

Sublineage differentiation of iNKT cells

Following positive selection at the DP stage, a few iNKT cell precursors can readily be characterized as CD69⁺ CD24^{hi} cells that express high levels of Egr-2 (stage 0) [45]. Our current model of iNKT cell development predicts that the cells then continue their maturation and reach what has been defined as stage 1 (Fig. 1) by down-regulating CD24 and CD69. Although these cells appear otherwise phenotypically equivalent to other naïve mature CD4⁺ single positive thymocytes, they are committed to the iNKT cell lineage as they express high levels of PLZF and are actively proliferating [33*,45]. It is likely that the decision to become any of the three major iNKT cell subsets described earlier occurs before or at this stage. Indeed, in PLZF-deficient mice, the few remaining T cells labeled with the α Galcer-CD1d tetramer do not further upregulate any of the master transcription factors associated with the three major iNKT cell subsets and are otherwise blocked at this stage 1 [39]. Furthermore, fate-mapping experiments using PLZF-Cre mice revealed that nearly all iNKT cells (94%) had expressed PLZF in the course of their development [46*]. Finally, stage 1 iNKT cells can be further divided on the basis of IL-17RB expression, which is found to be selectively expressed on mature iNKT2 and iNKT17 but not iNKT1 cells [16]. Expression of the tumor necrosis factor superfamily members CD40-L and RANK-L, which are linked to the development of medullary thymic epithelial cells (mTECs), is also preferentially detected on CD4⁺ stage 1 iNKT cells [47].

Soon after, these stage 1 cells acquire a memory-like CD44^{hi} phenotype (stage 2). It is now appreciated that within this CD44⁺ stage 2 population there exist multiple iNKT cell populations: first, cells that continue to differentiate and progress to what was originally labeled as stage 3. This is manifested by the cessation of proliferation, the acquisition of T-bet and of the NK-like program (NK1.1⁺, CD122^{hi}), and the downregulation of PLZF and GATA3. These cells produce preferentially IFN γ (and some IL-4) upon stimulation and represent the iNKT1 subset. Second, cells that retain PLZF expression, express high levels of GATA3 and produce IL-4 and IL-13 upon stimulation (iNKT2 cells). And finally, cells that upregulate expression of retinoic acid-related orphan receptor RoR γ t, while remaining

intermediary for PLZF and negative for T-bet and produce IL-17 upon stimulation (iNKT17).

Cell intrinsic signals

Over the years, multiple transcription factors and signaling molecules have been reported to be important for the development of iNKT cells. Several comprehensive reviews have been published on the subject [48,49,50] and only the most recent findings will be briefly reviewed here.

Because the generation of iNKT cells is abrogated in Dicer-deficient mice [51,52], a role for microRNAs (miR-NAs) in the development of iNKT cells was suspected. Pobezinsky *et al.* reported that lethal-7 (let-7) miRNAs could target Zbtb16 mRNA and thereby post-transcriptionally control the expression of the PLZF protein and the terminal differentiation of iNKT cells [53^{**}]. IL-15, vitamin D and retinoic acid signaling lead to an upregulation of Let-7 concomitant to PLZF downregulation, favoring the development of iNKT1 cells. By contrast, without upregulation of Let-7 miRNAs, iNKT cells maintained high expression of PLZF and terminally differentiated into iNKT2 and iNKT17 cells.

Another miRNA, miR-181, was shown to set a TCR signaling threshold for agonist selection of iNKT cells by targeting PTEN [54,55], and thus possibly control mammalian target of rapamycin (mTOR) signaling and regulation of global metabolic fitness.

A crucial role for mTOR, mTORC1 and mTORC2 in modulating several aspects of iNKT cell development has been reported [56,57]. Multiple signaling pathways that lead to the activation of the metabolic checkpoint kinase mTOR and negative regulators that control mTOR signaling were found to play differential roles in iNKT cell development. However, the mechanisms by which mTOR, mTORC1 and mTORC2 exert their function on iNKT cells remain to be elucidated. This might involve a fine balance between the mTOR pathways and autophagy. Indeed, the developmental phase of iNKT cells involves a massive cell expansion of the cells as they transition between stage 1 and 2. In addition, T-lymphocyte specific deletion of Atg5 or Atg7, two members of the macroautophagic pathway, leads to a large decrease of the total iNKT cell population, with a major effect on the iNKT1 followed by iNKT17 and finally the iNKT2 population [58].

E protein transcription factors (E2A and HEB) and their negative regulators, the Id proteins (Id2 and Id3), were shown to control the development of iNKT cell sublineage after selection [59,60,61]. E2A and HEB were shown to directly bind the promoter of PLZF and control its expression in stage 1 iNKT cells. In absence of E2A and HEB, iNKT cells failed to progress to stage 2 and 3 and did not proliferate at stage 1. Furthermore, a loss of Id3 led to an increased number of iNKT2, while both Id2 and Id3 were required for the formation of iNKT1 cells.

Specific deletion of Notch 1 and Notch 2 in T cells affected the development of iNKT cells [62]. Several iNKT cell populations seemed affected, as increased numbers of stage 2 and 3 iNKT cells, increased proportion of iNKT17 and altered expression of PLZF in stage 1

iNKT cells was observed. It remains unclear what downstream targets of Notch are involved.

The transcription factor lymphoid enhancer factor (LEF1) was shown to play a role in the post-selection expansion of iNKT cells through a direct induction of the CD127 component of the receptor for interleukin-7 (IL-7) and the transcription factor c-myc [63]. LEF1 was also shown to directly augment the expression of GATA3, and thereby promote the development of iNKT2 cells.

Cell extrinsic signals

From the aforementioned sets of data it is clear that several intrinsic signaling pathways differentially affect the subset lineage differentiation of iNKT cells. Yet, extrinsic clues are also playing a role. For example, the Slamf8-deficient mice have normal iNKT cell numbers but with an enlarged PLZF^{hi} iNKT2 compartment [64]. Because the Slamf8 molecules signal independently of SAP and Slamf8-encoding mRNA is expressed in dendritic cells and fibroblasts in the thymus but not DP thymocytes, it is likely that Slamf8 functions differently than the other Slam-family molecules in affecting iNKT cell development [30,64,65,66]. Instead, these results suggest that DC cells or fibroblasts might provide external signals, which are affected by the absence of Slamf8, to developing iNKT cells. The exact mechanism remains to be determined. Interestingly, iNKT cell development depends on normal thymus medulla development and interaction with mTECs. Expression of the chemokine receptor CCR7 enables developing iNKT cells to enter the thymic medulla [67] and gain access to mTECs, which play an important role by providing IL-15 transpresentation [47] necessary to the complete development of iNKT1 cells. Because iNKT2 and iNKT17 cells were also found within the medulla [7**], it is likely that other chemokine receptors regulate localization as well.

Conclusions

iNKT cell subsets are characterized by expression of different transcription factors and different functional responses, especially in terms of the dominant cytokine secretion profile after activation. Furthermore, each subset colonizes and localizes within peripheral tissues differently. As such, each iNKT cell subset is poised to distinctively influence the immune response.

The 'choice' to become iNKT1, iNKT2 or iNKT17 cells appears to be set in the thymus during development and it remains unclear whether each 'fate' is permanently set or can be further modulated. Therefore, understanding the mechanisms of iNKT cell subset differentiation is a prerequisite to the manipulation of the iNKT cell response for therapeutic purposes.

While tremendous progress has been made in the identification of iNKT cell developmental intermediates and the various signaling pathways and transcription factors involved in their development, the molecular mechanisms that control the differentiation of the different iNKT cell subsets remain mysterious. What are the original signals that determine which

and to what extent a given signaling pathway and/or transcription factor expression is turned on or off to determine which developmental pathway iNKT precursors choose?

The advance of genomics, multicolor flow cytometry and mass cytometry (CyTOF) technology will likely help in further defining the iNKT cell developmental road map and in elucidating the details of the molecular pathways involved.

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Figure 1.

Schematic view of mouse iNKT cell development in the thymus. The model incorporates the previously described linear differentiation model using the proposed stages as well as the newly described model of differentiated subsets. The branch point between the three subsets remains poorly defined. Cell surface phenotypes for each stage and/or associated with a given subset are indicated. Expression of transcription factors as well as the associated cytokine production after activation of a given subset is shown. Molecules, transcription factors and/or signaling pathways involved in the developmental progression are indicated. The amounts of Id3 and PLZF are inversely correlated to the amounts of Id2 and Let-7 in the three main terminally differentiated iNKT subsets. Expression of RANK-L and CD40-L on CD4+ stage 1/2 iNKT cells was shown to influence the development of Aire+ mTECs [48]. The expression of these two tumor necrosis factor family receptors was not examined by iNKT cell subsets. Secretion of IL-4 by iNKT2 cells conditions CD8T cells to become 'memory-like' (CD44 high, CD122+, (Eomes)odermin+) and causes dendritic cells to produce the CCL17 and CCL22 chemokines [6]. In addition to differential transcription factor expression, iNKT2 (predominantly CD4+) and iNKT17 (predominantly DN) subsets can be further differentiated by cell surface expression of CD27 [6], IL-23 receptor and CD103 integrin [9]. It remains unclear when these markers are first expressed on these subsets with regards to their developmental stages (*i.e.* CD44 low versus CD44 high).