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The making of NKT cells

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Abstract

Natural killer T cells acquire their unique phenotype and characteristics during development in the thymus. Evidence suggests that the transcription factor PLZF has a unique function in the development of these cells and their acquisition of ‘innate-like’ characteristics.

Multipotent progenitors from the bone marrow colonize the thymus and differentiate into various T-cell lineages in response to environmental and TCR derived signals that induce specific cell-intrinsic transcriptional programs. The molecular mechanisms driving these lineage-fate decisions have been the focus of intense research recently and have resulted in the identification of several pivotal transcription factors. For example, regulation of the $\gamma\delta$ versus $\alpha\beta$ T cell differentiation is mediated by the transcription factor Sox13¹, while Th-Pok acts as a master regulator of the CD4 versus the CD8 commitment step². Similarly, Foxp3 is indispensable for the differentiation of regulatory T cells³. In this issue of *Nature Immunology*, the laboratory of Sant’Angelo reports an essential role for the promyelocytic leukemia zinc finger transcription factor, PLZF, in natural killer T (NKT) cell development⁴. Although a few iNKT cells can be found in mice deficient for PLZF, they do not appear to acquire the activated and “innate-like” phenotype that is characteristic of NKT cells, suggesting an essential role for PLZF in iNKT cell biology.

Natural Killer T (NKT) cells represent a distinct subset of T lymphocytes that has evolved to recognize glycolipid antigens presented by the non-classical MHC class I-like molecule, CD1d. Upon recognition of glycolipids, NKT cells respond by secreting large quantities of cytokines and chemokines within hours of their initial stimulation, reminiscent of innate rather than adaptive functions. These cells have been implicated in the regulation of immune responses associated with a broad range of diseases, including autoimmunity, infectious diseases and cancer (review ref?).

Type I NKT cells, also named iNKT cells, are the most prevalent NKT cells in mice. They are characterized by the expression of a single invariant TCR α chain (V α 14-J α 18 in mice, V α 24-J α 18 in human) in combination with certain TCR β chains (using V β 8.2, 7 or V β 2 in mice, V β 11 in human). While the iNKT cell population is in fact comprised of a multitude of clones that express different combinations of these TCRs, their uniform reactivity with CD1d tetramers loaded with the glycosphingolipid antigen α -galactosylceramide (α -Galcer) allows for the tracking of the iNKT population as a whole. This technical advantage has permitted the study of iNKT cell development in many genetically modified strains of mice and has led to the identification of several transcription factors and signaling pathways important for this process. Mature iNKT cells have a memory or activated phenotype (CD69⁺CD44^{high} IL-2R β ^{high}) and they also express several markers usually associated with the NK lineage from which their name was derived.

iNKT cells develop in the thymus where they segregate from conventional T cell development at the double-positive (DP) thymocyte stage, coincident with the acquisition of TCR $\alpha\beta$ expression. Like conventional T cells, iNKT cell development requires recognition of their restriction element, CD1d, by the iNKT TCR. Both DP thymocytes and epithelial cells in the thymus express CD1d. However, iNKT cells are positively selected at the DP stage by CD1d-expressing DP cells themselves as opposed to epithelial cells that drive the selection of “conventional” T cells. It is thought that selection by DP cells imparts the unique developmental program of iNKT cells to the selected thymocytes due to homeotypic interactions across the DP-DP synapse that generate “second signals” mediated 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])⁵. Such engagements lead to the downstream recruitment of the adaptor SLAM-associated protein (SAP) and the Src kinase Fyn, both of which are essential for the expansion and differentiation of the iNKT cell lineage. Interestingly, CD4⁺ T cells positively selected by MHC class-II-expressing thymocytes also require these signals and share several features with iNKT cells⁶.

Once iNKT cells have been positively selected, they expand in the thymus and undergo orchestrated maturation that ultimately leads to their acquisition of the activated NK-like phenotype (see figure). This process relies on the proper expression of cytokine receptors, signal transduction molecules, transcription factors, and co-stimulatory molecules (see ref ⁷ for a recent review). However, none of the genes previously shown to participate in iNKT cell development are expressed exclusively in iNKT cells. By contrast, PLZF appears to be unique to iNKT cells. Using a combination of intracellular PLZF staining and measurements of PLZF mRNA expression levels by qPCR, Kovalovsky and colleagues show high expression of PLZF in iNKT cells while other immune cells, such as “conventional” T cells, NK cells, B cells, neutrophils, eosinophils and macrophages, do not express this transcription factor. PLZF expression is highest in HSA^{high} CD44^{low} thymic iNKT cells, a population thought to represent immature iNKT cells that have just undergone positive selection⁸. In agreement with a previous study of gene expression in developing iNKT cells (Affymetrix Probe set 1419874_x_at, ref ⁹), PLZF expression decreases as the cells mature, and remains expressed at high levels in mature iNKT cells relative to other lymphoid populations.

What are the signal(s) that induce PLZF expression in the early development of iNKT cells? We currently know that two signaling events are critical for the making of an iNKT cell. The first one involves recognition of CD1d + self-glycolipid(s) by the semi-invariant TCRs; the second one requires the engagement of SLAM family receptors. Because PLZF is expressed at wildtype levels in the very few iNKT cells that can be found in mice deficient for Fyn or SAP, two signaling molecules that convey the SLAM-mediated signals, Kovalovsky *et al.* concluded that SLAM-mediated signaling was likely not essential for PLZF expression. Although not definitive, these results suggest that perhaps a third, unknown, signal is necessary for the induction of PLZF expression. The surface molecules and cell types that might be involved in the PLZF-inducing pathway will likely be the focus of intense research in the near future.

PLZF is a transcription factor that has been implicated in the regulation of hip development, spermatogenesis, hematopoietic proliferation and differentiation, leukemogenesis and tumorigenesis. It is generally thought of as a transcriptional repressor due to its capacity to recruit mammalian Polycomb family members, which in turn recruit histone deacetylases. In the absence of PLZF very few iNKT cells develop, and the cells that are detected fail to fully upregulate several cell surface markers usually associated with iNKT cell maturation. These “immature” iNKT cells tend to accumulate in the lymph nodes and are deficient in several effector functions, such as the capacity to rapidly secrete IL-4 and IFN γ upon TCR triggering. These results suggest that PLZF controls, either directly or indirectly, several genes that are

important for iNKT cell biology (i.e. cytokine secretion, cytotoxicity, migration). In future studies, it will be important to determine how PLZF acts within iNKT cells to control these various aspects of iNKT cells biology. Recent reports demonstrated that PLZF directly inhibits the expression of at least three different microRNAs (miRs) in melanoma and megakaryocytes^{10,11}. In turn, these single-stranded 20 to 25 nucleotides non-coding RNAs regulate gene expression by targeting protein-coding mRNAs for translational repression and/or degradation. The presence of untranslated IL-4 mRNA in resting iNKT cells represents one of the hallmarks of the population (ref). The absence of this “pre-formed” IL-4 mRNA in resting PLZF-deficient iNKT cells could be consistent with such mechanism of regulation, either through the direct binding of miRs to the 3' untranslated region of the IL-4 mRNA, or alternatively through the indirect regulation of genes involved in regulating message stability.

The localization and migratory behavior of the various cells that populate lymphoid tissues appears to be linked to their unique functions. Determining these parameters for iNKT cells should contribute greatly to our understanding of how these cells participate in the immune response. Just as the generation of the α -GalCer-loaded CD1d tetramer revolutionized and refined how researchers could study iNKT cells *ex vivo*^{12,13}, the identification of PLZF as an iNKT cell-specific gene might be a critical stepping stone in advancing our efforts to study iNKT cells *in vivo*. The identification of PLZF as a signature gene of iNKT cells opens the possibility of developing reagents to use PLZF as a molecular tracer of the population *in vivo*. It is currently unclear whether the new monoclonal antibody directed against PLZF that was developed in the Sant'Angelo laboratory will be useful for immunohistochemistry. However, one can readily envision using PLZF, as has been done with Foxp3 and Treg cells, in “knock-in” and/or BAC transgenic approaches to take advantage of the genomic regulatory regions of the *Zbtb16* gene that codes for PLZF. The expression of genes of interest could be driven or abrogated in an iNKT-cell specific manner to track and/or modulate iNKT cell activities directly *in vivo*. One potential complication to the immediate usefulness of the identification of PLZF as a gene specifically expressed in iNKT cells comes from micro-array data derived from blood monocytes purified on the basis of FCS/SSC and CD11b expression¹⁴, which show high expression of PLZF in this monocyte population. This cell population was not examined in the study by Kovalovsky *et al.* and the results will have to be confirmed before we can formally assign PLZF as an iNKT cell-specific transcription factor.

Nevertheless, the findings now reported by Kovalovsky and colleagues represent an important milestone in our understanding of iNKT cell development and will likely open new areas of investigation of this unique lymphocyte population.

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Figure 1. Schematic view of iNKT cell development

Natural Killer T cells arise in the thymus from uncommitted double negative (DN) precursors. The cells progress to the double-positive CD4⁺ CD8⁺ stage (DP) where they randomly rearrange the TCR. Thymocytes that express a TCR that interacts with CD1d bound to self-glycolipid, expressed by other DP thymocytes, enter the iNKT lineage. Homotypic interactions between SLAM and Ly108 molecules, also expressed at the surface of DP thymocytes, are required for progression into the iNKT cell lineage. After selection, iNKT-cell precursors undergo a series of differentiation steps characterized by the sequential expression of several cell surface markers (HSA, CD44 and NK1.1), as depicted. The earliest iNKT cell precursor found in the thymus expresses the highest level of PLZF. PLZF expression level then decreases as the cell progress further in the differentiation pathway. At least four distinct immature iNKT cell populations have been identified in the thymus. Most iNKT cells that emigrate from the thymus do so as immature (CD44^{high}, NK1.1⁻) and continue their final maturation in the periphery. This final maturation step is controlled by the transcription factor T-bet, whose expression level increases as a function of iNKT cell development. Some mature iNKT cells also migrate to the periphery but many remain as long-term resident of the thymus. In absence of PLZF, the cells fail to fully mature and preferentially accumulate in the lymph nodes instead of the spleen and the liver where wildtype iNKT cells usually reside.