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The transcriptional programs of iNKT cells

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Abstract

Invariant natural killer T (iNKT) cells are innate T cells that express a semi-invariant T cell receptor (TCR) and recognize lipid antigens presented by CD1d molecules. As part of innate immunity, iNKT cells rapidly produce large amounts of cytokines after activation and regulate the function of innate and adaptive immune cells in antimicrobial immunity, tumor rejection and inflammatory diseases. Global transcriptional profiling has advanced our understanding of all aspects of iNKT cell biology. In this review, we discuss transcriptional analyses of iNKT cell development, functional subsets of iNKT cells, and global comparisons of iNKT cells to other innate and adaptive immune cells. Global transcriptional analysis revealed that iNKT cells have a transcriptional profile distinct from NK cells and MHC-restricted T cells, both during thymic development and in the periphery. The transcription factors EGR2 and PLZF (and microRNA like miR-150) are key regulators of the iNKT cell transcriptome during development. PLZF is one of several factors that control the homing and maintenance of organ-specific iNKT cell populations. As in MHC-restricted T cells, specific transcription factors are characteristic of functional subsets of iNKT cells, such as the transcription factor T-bet in the NKT1 subset. Exciting future directions for global transcriptional analyses include iNKT cells in disease models, diverse NKT cells and human studies.

Keywords

NKT cell development; NKT cell subset; Global transcriptome profiling; Global transcriptional profiling; PLZF

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1. Introduction

Invariant natural killer T (iNKT) cells, also known as type I or classical NKT cells, are innate $\alpha\beta$ T cells expressing a semi-invariant T cell receptor (TCR) that recognizes lipid antigens presented by CD1d [1]. As part of the innate immune system, iNKT cells rapidly produce large amounts of cytokines just minutes after activation. These cytokines profoundly modulate the innate and adaptive arms of the immune system in infection, tumor rejection and inflammatory disease. In humans, iNKT cells generally express an invariant V α 24-J α 18 TCR α chain paired with V β 11. In mice, iNKT cells generally express a semi-invariant TCR of V α 14-J α 18 paired with a limited V β repertoire (usually V β 2, V β 7, or V β 8.2). iNKT cells are a well-studied representative of the growing family of innate T lymphocytes, and comparisons of iNKT cells with MHC-restricted T cells illuminate the principles that govern the interaction of the innate and adaptive immune systems. Global transcriptional profiling (*via* RNA microarray or RNA-sequencing [RNA-Seq]) has revolutionized research into the unique combination of innate and effector memory-like functions in iNKT cells. With a global view of innate and adaptive programs, transcriptional analyses address the question, “What is an iNKT cell?” To answer this question, we will review the transcriptional analyses of iNKT cell development, the transcriptional programs that regulate functional subsets of iNKT cells in the periphery, and the innate (“NK cell-like”) character of the iNKT cell transcriptome. We conclude with future directions for global transcriptional analyses of iNKT cells.

2. Transcription factors and microRNA in iNKT cell development

2.1. Distinct transcriptional profile for iNKT cells in development

Global transcriptional analyses have advanced our understanding of the transcription factors involved in iNKT cell development. iNKT cells develop in the thymus, where the first iNKT cell precursors arrive at post-natal day 5 [2]. iNKT cells diverge from MHC-restricted T cells in the double positive (DP, CD4⁺CD8⁺) thymocyte pool [3]. A subset of DP thymocytes express the transcription factor retinoic acid receptor-related Orphan Receptor γ (ROR γ t) and rearrange their V α 14 and J α 18 segments to form the invariant TCR α chain. The semi-invariant T cell receptor on iNKT cell precursors recognizes lipid antigens presented by CD1d, a homologue of major histocompatibility complex (MHC) [4]. These cells then down-regulate CD8 and enter stage 0 (CD44⁻NK1.1⁻CD4⁺). iNKT cells proceed through three subsequent stages of development in the thymus. Our laboratory performed global transcriptional analysis of thymic iNKT cell subsets in C57BL/6J mice as part of the Immunological Genome Project (Imm-Gen, www.immgen.org) [5]. ImmGen is a multi-center consortium of immunologists and computational biologists characterizing the immune system transcriptome using a systematic approach. By microarray analysis, the transition from DP to stage 1 (CD44⁻ NK1.1⁻) changed expression in 1850 genes; the transition from stage 1 to stage 2 (CD44⁺ NK1.1⁻), 155 genes; and the transition from stage 2 to stage 3 (CD44⁺ NK1.1⁺), 697 genes [5]. Focusing on genes known to regulate the development of iNKT cells, our data demonstrated that thymic iNKT cells had a distinctly different profile than MHC-restricted thymic T cells (Fig. 1). For example, *Zbtb16*, *Tbx21*, and *Gata3* showed opposite patterns of expression across all iNKT cell thymic subsets compared to all

MHC-restricted T cell thymic subsets. Other transcription factors, such as *Egr2* or *Slamf6*, showed a mixed pattern of expression in NKT cell and MHC-restricted T cell subsets. We will focus on two transcription factors in iNKT cell development, EGR2 and PLZF.

2.2. EGR2

The transcription factor EGR2 is an important regulator of iNKT cell development. Studies on *Egr2* are elegant examples of global transcriptional analysis paired with chromatin immunoprecipitation with deep sequencing (ChIP-seq). Calcineurin B1 and NFAT signaling drive transcription of the *Egr* family (*Egr1*, *Egr2*, and *Egr3*). In the *Egr* family, only *Egr2*-deficient mice have a significant reduction in thymic and peripheral iNKT cell numbers compared to wild-type mice, although studies show some subtle defects in *Egr1*-deficiency [6,7]. *Egr2*-deficient mice are not viable due to defective brain development and so are studied as chimeras. *Egr2*-deficient fetal liver chimeric mice had impaired progression from stage 1 (CD44⁺ NK1.1⁺) to stage 2 (CD44⁺ NK1.1⁺) [6] while *Egr2*-deficient bone marrow chimeric mice showed arrest at stage 2 [7]. In contrast, MHC-restricted T cells have grossly normal development in *Egr2*-deficient chimeric mice. To screen for targets of EGR2, Seiler et al. used four criteria [7]: (1) upregulation in the global transcriptome of thymic NKT cells, as compared to CD4⁺ single-positive thymocytes in our ImmGen dataset. There were 614–983 genes with increased relative expression in each iNKT stage 1, 2 or 3; (2) binding by EGR2 in the ChIP-seq of thymic NKT cells from Vα14 transgenic mice. There were 33–43 genes in iNKT stages 1–3; (3) binding by EGR2 in the ChIP-seq of thymocytes from wild-type mice treated with anti-TCRβ antibody. There were 13–17 genes in iNKT stages 1–3; (4) conservation of the binding site in human and mouse. There were 5–7 genes in iNKT stages 1–3. The nine genes that met all four criteria were *Zbtb16*, *Il2rb*, *FasI*, *Ccnd2* (cyclin D2), *Slc9a9* (ion exchange protein in late endosome), *Ptpn22* (transmembrane tyrosine phosphatase involved in cell cycle), *Hivep3* (transcription factor that may bind the recombination signal sequences flanking the V, D, and J regions of T cell receptor), *Tbkbpl* (adaptor protein likely involved in TNF and NFκB signaling), and *Fam46a*. Electrophoretic mobility-shift assays and *in vivo* studies confirmed EGR2 transactivation of *Il2rb* and, notably, *Zbtb16*, which encodes the key transcriptional factor PLZF [7].

2.3. PLZF

The discovery that iNKT cells highly express promyelocytic leukemia zinc finger (PLZF, encoded by the gene *Zbtb16*) was a key insight into iNKT cell development and function [8,9]. PLZF is a member of the BTB-POZ-ZF (broad complex, tramtrack, bric-a-brac or poxvirus and zinc finger) family of transcription factors that control many functions of the immune system. Initially thought to be specific for iNKT cells, PLZF is expressed in other innate T cell subsets (*i.e.*, mucosal-associated invariant T (MAIT) cells and a population of γδ T cells [10,11]) and innate lymphoid precursor cells in the fetal liver and bone marrow [12]. PLZF is induced immediately after positive selection of iNKT cells [8,9] (Fig. 1) and is required for both iNKT cell development and effector functions. In the absence of PLZF, the number of iNKT cells is dramatically reduced to approximately 5–10% of normal levels. In addition, the trafficking of iNKT cells to tissues depends on PLZF. In PLZF-deficient mice, iNKT cells accumulated in the lymph nodes, rather than peripheral organs, likely due to high expression of CD62L compared to wild-type iNKT cells [9]. Mature iNKT cells require

PLZF for normal effector function. After activation, PLZF-deficient iNKT cells failed to secrete high levels of IFN γ and IL-4 and lacked an activated or memory phenotype [8,9]. Ectopic expression of PLZF was sufficient to confer an iNKT-like phenotype to MHC-restricted T cells [8,9]. More generally, PLZF is linked to innate effector functions in several cell types besides iNKT cells.

To identify downstream effects of PLZF, Kreslavsky and colleagues compared gene expression profiles of iNKT cells from WT and PLZF-deficient mice. PLZF controlled key aspects of development, survival, homeostasis and trafficking [13]. PLZF regulated ThPOK (encoded by *Zbtb7b*), which is required for iNKT cell development [14]. PLZF also regulated Id2, which is critical for iNKT cell survival in the liver [15]. PLZF also regulated the expression of genes coding for chemokine receptors (*Ccr2* and *Ccr10*) and cytokine receptors (*Il12rb1* and *Il18r1*) [13]. To explain the molecular mechanism of PLZF, Mathew et al. used immunoprecipitation to show that PLZF associates with the E3 ubiquitin ligase cullin 3 (CUL3) [16]. PLZF transports CUL3 to the nucleus, where they both associate with a chromatin-modifying complex. The ability of PLZF to drive the ubiquitination of key nuclear proteins helps to explain its diverse effects on the iNKT cell transcriptome. These effects of PLZF on the transcriptome help explain the profound NKT cell abnormalities in PLZF-deficient mice [8,9].

2.4. MicroRNA

MicroRNA (miRNA) are 22 nucleotide, single-stranded RNA that modulate the stability of RNA post-transcription. Global transcriptional analysis is a natural fit for studies of miRNA, since miRNA can have wide-ranging effects on the transcriptome. For example, ectopic expression of miRNA *Lin28* in bone marrow hematopoietic stem/progenitor cells (HSPC) reprograms HSPC to skew toward the innate lymphocyte pathway and increased production of iNKT cells [17]. In mice with the conditional deletion of Dicer (*Dicer^{flox/flox} × Cd4-Cre* mice), DP thymocytes can still express mature miRNAs, but single positive thymocytes have a profound reduction in functional miRNA [18]. This conditional deletion of Dicer reduced thymic iNKT cell number by over 90%, with similar decreases in peripheral iNKT cell number. Thymic iNKT cells had a distinct miRNA profile (by miRNA microarray) when compared to thymic MHC-restricted T cells. Of 70 miRNA detected, thymic iNKT cells differently expressed 17 miRNA compared to MHC-restricted thymic T cells. Real-time PCR confirmed that thymic iNKT cells had higher expression of *miR-21* and lower expression of *let-7g*, *miR-106a*, *miR-34b-3p*, *miR-17*, *miR-30c*, *miR-106b*, *miR-16*, *miR-467a*, *miR-467b*, *miR-690*, *miR-15b*, *miR-669f* and *miR-150* (in order of increasing magnitude of fold-difference). The expression patterns of miRNA in iNKT cell subsets is unknown and may be informative. For example, *miR-150* was down-regulated in iNKT cells (compared to MHC-restricted T cells) in the thymus. One might conclude that *miR-150* is less important in development. However, the miRNA microarray was done in pooled thymic iNKT cells. The pooling masked that *miR-150* expression increased significantly from thymic stage 1 to stage 3. Correspondingly, *miR-150*-deficient mice have a defect in later stages of iNKT cell differentiation [18,19].

Global transcriptional analysis greatly facilitates assessment of the down-stream effects of miRNA. For example, mice deficient in *miR-181a1b1* have a nearly complete loss of iNKT cells at the DP stage. Global transcriptional profiling of double-positive thymocytes by RNA-Seq showed more than 80% of genes had lower expression in *miR-181a1b1*-deficient mice as compared to wild-type mice. These down-regulated genes included several metabolic pathways (*e.g.*, glycolytic, pentose phosphate and nucleotide synthesis) [20]. The 146 upregulated genes included *Pten*, a negative regulator of the PI3K pathway, a key regulator of metabolism. PTEN may drive the broad metabolic dysregulation in developing iNKT cells from *miR-181a1b1*-deficient mice, as inhibition of *Pten* expression rescued iNKT cell development [20]. Global transcriptome analyses of both miRNA and mRNA have illuminated iNKT cell development in the thymus.

3. iNKT cell subsets

3.1. Tissue-specific subsets of iNKT cells

iNKT cells leave the thymus and move to the periphery at stage 2 (CD44⁺ NKT1.1⁻) or stage 3 (CD44⁺ NKT1.1⁺) of development. At baseline, iNKT cells are most abundant in the liver and spleen, but iNKT cells can dramatically increase their numbers in a range of organs during inflammation or infection, such as the lung in asthma and chronic bronchitis [21,22], the pancreas in type I diabetes [23,24] and in other situations. From organ to organ, iNKT cells have varying numbers and phenotypes, even different frequencies of TCR V β chain usage [25]. Organ-specific differences in iNKT cells likely derive from both characteristics acquired before entering the organ and the effects of the organ environment on iNKT cells. Organ-specific chemo-attractants and integrins select for specific subsets of iNKT cells, and these interactions help generate organ-specific iNKT cell populations. For example, splenic iNKT cells home to the spleen in part because of their response to BCA-1 (CXCL13), a chemokine that mediates B and T cell homing to follicular regions of lymphoid tissue [26]. In contrast, hepatic iNKT cells do not respond to BCA-1. For hepatic iNKT cells, expression of LFA-1 is necessary to retain these iNKT cells in the liver sinusoids, but LFA-1-deficient mice have normal numbers of iNKT cells in the spleen and other organs [27,28]. One regulator of organ-specific differences is the transcription factor PLZF. When PLZF was over-expressed in MHC-restricted CD4⁺ T cells, these cells up-regulated LFA-1 and accumulated in the liver [27]. PLZF-deficient mice had altered homing and increased numbers of iNKT cells in lymph nodes [9].

Other factors besides homing regulate organ-specific populations of iNKT cells. The steady-state equilibrium reflects homing and proliferation balanced by recirculation and cell death. iNKT cells have very different patterns of recirculation compared to other T cells. Parabiotic congenic mice demonstrated that iNKT cells have markedly reduced recirculation in tissues compared to recirculation by other T cells, as first shown by the Bendelac lab in liver, lung and spleen [27] and by our laboratory in adipose tissue [29]. Within two weeks of parabiosis, B cells and MHC-restricted T cells achieved almost complete chimerism in the liver, lung, spleen, and adipose tissue of parabiotic mice. In contrast, iNKT cells had minimal recirculation, and 90–95% of organ-resident iNKT cells in these tissues were endogenous and not derived from the parabiotic partner mouse [29]. With reduced recirculation, local

regulation of iNKT cell survival takes on increased importance. Multiple signals from the organ environment regulate iNKT survival. Mice with a hepatocyte-specific deficiency in expression of IL-7 had a significant decrease of iNKT cells in the liver (but normal numbers in thymus and spleen) [30]. In another example, intra-vital microscopy revealed that CXCR6 is not necessary for normal recruitment and patrolling behavior. CXCR6 is required, however, for a normal number of iNKT cells in the liver sinusoids, and so may enhance survival of iNKT cells in the liver [31].

In addition to cell number, the phenotype of iNKT cells varies from organ to organ. Antigen presenting cells (APCs) regulate many of these organ-specific phenotypes. In one demonstration, both thymic and splenic iNKT cells had increased production of IFN γ after incubation *ex vivo* with splenic APCs, as compared to after incubation with thymic APCs [32]. The co-stimulatory molecules on APCs may mediate their organ-specific influences. For example, expression of SLAM on DCs skews NKT cells toward a “Th-2-like” phenotype [33], and different genetic haplotypes at the *Slam* locus correlate with different iNKT behavior in several mouse strains [34,35]. In theory, APCs in different organs could present different endogenous antigens to skew NKT cell behavior; however, this mechanism has not been clearly demonstrated *in vivo*.

To examine how iNKT cells subsets differ from organ to organ, our laboratory performed a global transcriptional analysis that compared CD4⁺ and CD4⁻ iNKT cells from liver, spleen and lung of naïve C57BL/6J mice. iNKT cell gene expression patterns showed significantly more variability in CD4⁺ versus CD4⁻ iNKT subsets in lung than liver (261 differently expressed genes in lung, 159 in spleen, 17 in liver) [5]. Underscoring the value of an unbiased approach, the differently regulated genes have a broad range of functions, from NK receptors (*e.g.*, *Klra3*) and chemokine receptors (*e.g.*, *Ccr7* or *Ccr9*) to genes of uncertain significance (*e.g.*, *Hist1h2bc* or *Snord1c*). The interaction of iNKT cell subsets with different organ environments is a key topic for further study.

3.2. iNKT cells have distinct phenotypic and functional subsets

Despite having a limited TCR repertoire, “invariant” NKT cell subsets are quite heterogeneous in phenotype and function. In mice, cell surface markers, such as CD4, NK1.1 and IL-17RB, can help to distinguish functional subsets [2,36–38]. For example, NK1.1⁻ iNKT cells and CD4⁺ iNKT cells tend to produce IL-4 and little IFN γ , while NK1.1⁺ iNKT cells and CD4⁻ iNKT cells tend to skew in the opposite direction [37]. Alternatively, one can sub-divide populations by expression of CD4 and IL-17RB. IL17RB⁻ iNKT cells produce IFN γ ; CD4⁺IL17RB⁺ iNKT cells produce Th1, Th9 and Th17 cytokines in response to IL-25, and CD4⁻IL17RB⁺ iNKT cells also express ROR γ t and produce IL-17 in response to IL-23 [38]. One problem for some surface receptors is their strain dependence. For example, Balb/c mice do not express NK1.1 [39]. Instead, programs of gene expression may more accurately identify iNKT cell subsets. Analogous to helper T cells, iNKT cells can be divided into NKT1, NKT2, NKT17, NKT-Follicular Helper (NKT_{FH}), and NKT10 subsets based on transcription factors and cytokine production [40]. The Hogquist lab proposed an intracellular staining protocol for transcription factor expression to classify Th1-like, Th2-like, and Th17-like iNKT cell subsets [39].

Interestingly, the “key regulators” of the NKT1, NKT2, NKT17, NKT_{TH}, and NKT10 subsets are analogous to equivalent MHC-restricted T cell subsets.

3.3. NKT1

NKT1 cells, as their name suggests, primarily produce the Helper T helper type 1 (Th1) cytokine IFN γ , but NKT1 cells also produce IL-4 in some situations [39]. NKT1 cells express high levels of T-bet, the Th-1-associated transcription factor, but also express low levels of GATA3 [39,41]. NKT1 cells lack IL17RB expression, generally express NK1.1 (in C57BL/6 mice), and require IL-15 for homeostasis [38]. In C57BL/6 mice, the most abundant subset is NKT1 cells; NKT1 cells are much reduced in Balb/c mice. In terms of location, the NKT1 subset dominates in the liver, even in Balb/c mice. The enrichment of the NKT1 subset in the liver may account for the enhanced cytotoxic function of CD4⁻ iNKT cells in the liver compared to iNKT cells elsewhere [42].

3.4. NKT2

NKT2 cells produce IL-4 and little to no IFN γ when stimulated with the potent lipid ligand α -galactosylceramide (α -GalCer) *in vivo*. NKT2 cells and NKT17 cells both express high levels of T helper type 2 (Th2)-like transcription factors GATA3 and IRF4. GATA-3 is a zinc-finger transcription factor that plays an essential role in T cell development and Th2 cell differentiation [43,44]. ChIP-Seq analysis in T cell subsets identified genome-wide GATA3 binding sites. Some GATA-3 binding sites, such as the *Ctla4* and *Icos* genes, are widely shared among T cell subsets. However, some GATA-3 binding motifs and target genes are specific to particular T cell subsets [41]. In most T cells, WGATAA was the top binding motif, and the Runx motif was the top secondary binding motif. However, in iNKT cells, TCF11 was the top secondary binding motif [41]. This difference in secondary-motifs partially explains the different activity of GATA-3 in different T cell subsets. In mice, NKT2 cells are the most predominant subset in the thymus and spleen of Balb/c mice. Overall, NKT2 cells are approximately eight times more abundant in the Balb/c strain compared to C57BL/6 [39]. This difference in NKT2 cell number may contribute to differences between the Balb/c and C57BL/6 strains in some experimental models, such as allergic asthma. In humans, CD4 expression on iNKT cells may predict Th2-like iNKT cells; and the relative abundance of Th2-like iNKT cells varies widely in humans [36,37].

3.5. NKT17

NKT17 cells predominantly produce Th17 cytokines such as IL-17 and IL-22. NKT17 cells were discovered as a subset of hepatic NK1.1⁻ iNKT cells producing abundant IL-17 (and less IL-4 and IFN γ) after stimulation by α -GalCer or a microbial lipid antigen [45]. The transcription factor ROR γ t drives development of NKT17 cells in the thymus [45]. Global transcriptional profiling revealed that the transcription factor Th-POK represses the development of NKT17 cells. Thus, in Th-POK deficiency, ROR γ t expression is increased, and iNKT cells produce IL-17 [14]. In mice, NKT17 cells are abundant in the lymph nodes, lungs, and skin and have proven important in several disease models, such as airway neutrophilia induced by α -GalCer [45].

3.6. NKT_{FH}

Like MHC-restricted T Follicular Helper (T_{FH}) cells, a distinct population of iNKT_{FH} cells can provide cognate help to antigen-specific B cells [46–48]. iNKT_{FH} cells provide help to lipid-reactive B cells that internalize lipid antigens *via* their B cell receptor. iNKT_{FH} cells can initiate germinal center reactions faster than MHC-restricted T cells, but iNKT_{FH} cells did not generate long-lived plasma cells. As in MHC-restricted cells T_{FH}, BCL-6 was the transcription regulator of iNKT_{FH} cells and induced expression of PD-1 and CXCR5 [49]. Also like MHC-restricted T_{FH} cells, NKT_{FH} cells produced IL-21, which drove B cell help. 80% of NKT_{FH} cells expressed CD4 and were generally NK1.1[−]. NKT_{FH} cells are another example of NKT cell heterogeneity driven by a key transcription factor influencing localization and function.

3.7. NKT10

A population of regulatory iNKT cells has recently been described [38,50–52] with characteristic production of IL-10. IL-10 is a regulatory cytokine that can downregulate inflammatory responses. Motomura et al. reported that the transcription factor E4BP4, encoded by *Nfil3*, is responsible for IL-10 production in T helper cells and iNKT cells [50]. Following stimulation with γ -GalCer, a small proportion of splenic iNKT cells upregulated E4BP4 and produced IL-10. Moreover, iNKT cells from E4BP4^{−/−} mice did not produce IL-10. IL-10 producing iNKT cells were in the CD4⁺IL-17RB⁺ subset of iNKT cells [38]. IL-17RB is a receptor of IL-25, and treating cells with IL-25 induced E4BP4 and IL-10 production, among other cytokines, including IL-13 [38,50]. Our laboratory has found that regulatory IL-10 producing iNKT cells are enriched in adipose tissue [51]. Adipose iNKT cells are protective in obesity induced inflammation and metabolic disorder [51,53–55]. The protective role of adipose iNKT cells is likely due to a regulatory phenotype and production of IL-10, which dampened inflammation in macrophages (and possibly in adipocytes themselves) [51,54]. Using IL-10 reporter mice, Sag et al. recently confirmed the enrichment of IL-10 producing iNKT cells in adipose tissue. The “NKT10” cell subset is also a naturally occurring population in human peripheral blood mononuclear cells (PBMCs) [52]. Further studies are required to determine whether iNKT cells produce IL-10 after TCR-driven stimulation and represent a post-activation or anergy-like state; or whether IL-10 producing E4BP4⁺ iNKT cells are a thymically determined subset that traffics to adipose tissue.

4. What is an iNKT cell?

Since the term “natural killer” in the moniker “iNKT cell” is a historical term based on expression of NK1.1, it was open to question whether NKT cells functionally deserved the description as “NK cell-like.” Global transcriptional analysis revealed that iNKT cells occupy a distinct space “in between” MHC-restricted T cells and NK cells. Our laboratory performed global transcriptional profiling of thymic iNKT cell subsets through ImmGen, and our analysis showed that iNKT cells progressively acquire NK cell-like character as the iNKT cells diverge from MHC-restricted T cells during development. For example, in thymic stages 2 and 3, maturing iNKT cells coordinately upregulate a broad spectrum of NK cell inhibitory and activating receptors (Fig. 2). To assess the “innate” and “adaptive” character of iNKT cells, we compared the global transcriptome of our iNKT subsets (from

spleen, liver and lung) to NK cell subsets (from spleen and liver) and naïve or memory MHC-restricted T cell subsets (from spleen or lymph node) (Fig. 3). Principal component analysis placed iNKT cells in a unique transcriptional space, separate from other innate and adaptive lymphocytes.

Recent work extends our analysis by comparing iNKT cells to specific subsets of NK cells. Daussy et al. focused on Eomesodermin (Eomes)-negative NK cells. T-bet drives the development of Eomes⁺ NK cells in the liver. Global transcriptional profiles of liver iNKT cells, Eomes⁺ NK cells and Eomes⁻ NK cells demonstrated that liver iNKT cells, which lack Eomes expression in mice, more closely resemble Eomes⁻ NK cells [56]. iNKT cells and Eomes⁻ NK cells share similar expression patterns for integrins (increased *Itga1*, *Itgav*, *Itgb3*; decreased *Itga2*, *Itgam*, *Itgb7*), metallo-proteinases (increased *Mmp9*, *Adam13*), and chemokine receptors (highly increased *Cxcr3* and *Cxcr6*; decreased *Cx3cr1*). This analysis suggests that NKT cells and the Eomes⁻ NK cells may have functional similarities, such as in trafficking. The work of our laboratory and others reinforce the idea that the “natural killer” moniker in NKT cells is indeed quite meaningful. Studies on additional gene expression datasets have been informative. For example, Vahl et al. explored whether conditional activation or deletion of the alpha chain of the semi-invariant T cell receptor in iNKT cells (Vα14i-TCR) confers “NKT cell phenotype”. To assess the “NKT cell phenotype” of CD8⁺ T cells expressing transgenic Vα14i-TCR, Vahl et al. measured expression of selected transcription factors (*i.e.*, PLZF, EGR2, ThPOK) and seven NK cell receptors/markers (*e.g.*, NKG2D, NKG2A). Based on these selected genes, they concluded that transgenic expression of Vα14i-TCR does not confer “NKT cell identity”. Similarly, they concluded that deletion of the Vα14i-TCR in NKT cells does not remove their “NKT cell identity” because the cells could still rapidly produce cytokines. However, these analyses could be extended with a global transcriptional analysis to characterize the acquisition or loss of “NKT cell identity”.

5. Future directions

In just the past decade, investigators have greatly advanced our understanding of the NKT cell transcriptome. They have identified key transcription factors and characterized their global effects on development, function and NKT cell subsets. Global transcriptome analyses revealed that, in line with their unique functions, iNKT cells have a unique transcriptome distinct from that of NK cells and MHC-restricted T cells. A promising future direction is the NKT transcriptome in dynamic situations, such as inflammation, infection, and models of human disease. With a comprehensive database of the NKT cell transcriptome during dynamic situations, the NKT community could perform a bioinformatic analysis similar to that done by Novershtern et al. on 38 human hematopoietic lineages, which identified hundreds of “transcriptional modules” and their cis-regulatory circuits [57]. A second promising future direction is the transcriptome of immune cells regulated by NKT cells. NKT cells are powerful transactivators of the immune system and control the activation of neutrophils, NK cells, $\gamma\delta$ T cells, macrophages, dendritic cells, B cells and MHC-restricted T cells [1]. However, we lack global transcriptome analyses of cells after transactivation by NKT cells. The literature holds just a few examples, such as dendritic cells after co-culture with NKT cells [58,59]. It would be invaluable to have a systematic

analysis of the global transcriptome of neutrophils, NK cells, $\gamma\delta$ T cells and macrophages after transactivation by iNKT cells.

This review focuses on invariant NKT cells in mouse models. The transcriptomes of diverse NKT cells and human NKT cells are additional important areas for the next decade. Diverse NKT cells, also known as type II NKT cells, have a more diverse repertoire of TCRs and are not limited to the V α 14 TCR alpha chain used by iNKT cells. Diverse NKT cells do not recognize the lipid antigen α -GalCer, the canonical lipid antigen for invariant NKT cells [1]. Therefore, CD1d tetramer loaded with α -GalCer identifies invariant NKT cells but does not bind to diverse NKT cells. With heterogeneous antigen specificity, diverse NKT cells often cannot be identified *in vivo* and are challenging to isolate for transcriptional analysis. Human studies have their own challenges, such as the low numbers of iNKT cells in human peripheral blood (*i.e.*, often less than 0.1% [60]). New technologies, such as single cell transcriptional profiling by RNA-Seq, may help meet these challenges in diverse NKT cell and human studies. Human studies will also benefit from creative approaches to translational science. For example, Crawford et al. used a mouse model of a human mutation in a “bedside-to-bench” study [61]. DOCK8 is a guanine nucleotide exchange factor for CDC42 [62]. Human patients with a rare congenital defect in DOCK8 have an 80% decrease in peripheral NKT cell number, which may contribute to their susceptibility to infection and malignancy [61]. To investigate this clinical presentation, Crawford et al. studied DOCK8-deficient mice that mimicked the human phenotype. Global transcriptome analysis of thymic iNKT cells identified a candidate gene, *Itgae* (integrin α E, CD103) for continued study. This study is just one example of the creative possibilities in marrying murine and human immunology. Clearly, the transcriptional space of NKT cells is a space that we have just begun to explore.

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Abbreviations:

α-GalCer	α -galactosylceramide
BTB-POZ-ZF	broad complex, tramtrack, bric-a-brac or poxvirus and zinc finger
CCR2	C-C chemokine receptor type 2
CCR10	C-C chemokine receptor type 10
ChIP-seq	chromatin immunoprecipitation with deep sequencing
CUL3	cullin 3
DOCK8	dedicator of cytokinesis 8
DP	double positive

EGR2	early growth response protein 2
Eomes	eomesoder-min
ImmGen	Immunological Genome Project
iNKT	invariant natural killer T cell
iNKT_{FH}	invariant natural killer cell follicular helper
IFNγ	interferon-gamma
IL-4	interleukin-4
IL-17RB	interleukin-17 receptor B
IRF4	interferon regulatory factor 4
LFA-1	lymphocyte function-associated antigen 1
MAIT	mucosal-associated invariant T
MHC	major histocompatibility complex
miRNA	microRNA
NFIL3	nuclear factor, interleukin-3 regulated
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NKG	natural killer cell group
PBMC	peripheral blood mononuclear cell
PLZF	promyelocytic leukemia zinc finger
RNA-Seq	RNA-sequencing
RORγt	retinoic acid receptor-related orphan receptor γ
T-bet	T-box expressed in T cells
TCR	T cell receptor
T_{FH}	T follicular helper
Th1	T helper type 1
Th2	T helper type 2
TNF	tumor necrosis factor

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Fig. 1.

Expression profile of thymic T cell subsets: selected genes involved in iNKT cell development. T cell precursors, MHC-restricted T cells and stage 1, 2, and 3 iNKT cells were sorted by flow cytometry from the thymi of C57BL/6J mice, and global transcriptional analysis was performed by RNA microarray.

Fig. 2. Expression profile of thymic T cell subsets: Natural Killer Receptor (NKR) genes. T cell precursors, MHC-restricted T cells and stage 1, 2, and 3 iNKT cells were sorted by flow cytometry from the thymi of C57BL/6J mice, and global transcriptional analysis by RNA microarray was performed (Fig. 1E from [5]).

Fig. 3.

Principal component analysis of iNKT, NK and MHC-restricted T cell subsets. iNKT cells subsets (CD4^{+/-}) from spleen, liver and lung; NK cell subsets (CD49c^{+/-}, CD49H^{+/-}) from spleen and liver; and MHC-restricted T cells in naïve CD4⁺ and CD8⁺ subsets (CD25^{Lo}CD44^{Lo}CD62L^{Hi}), memory CD4⁺ subsets (CD25⁻CD44^{Hi}CD122^{Lo}) and CD8⁺ subsets (CD25⁻CD44^{Hi}CD122^{Hi}) from spleen and lymph nodes were sorted by flow cytometry from C57BL/6J mice. Global transcriptional analysis was performed by RNA microarray and transformed by principal component analysis.