Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY REVIEW ARTICLE

Control of early stages in invariant natural killer T-cell development

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doi:10.1111/j.1365-2567.2011.03463.x
Received 13 May 2011; revised 17 May 2011; accepted 31 May 2011.
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Summary

Natural killer T (NKT) cells develop in the thymus from the same precursors as conventional $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells, $CD4^+$ $CD8^+$ double-positive cells. In contrast to conventional $\alpha\beta$T cells, which are selected by MHC–peptide complexes presented by thymic epithelial cells, invariant NKT cells are selected by lipid antigens presented by the non-polymorphic, MHC I-like molecule CD1d, present on the surface of other double-positive thymocytes, and require additional signals from the signalling lymphocytic–activation molecule (SLAM) family of receptors. In this review, we provide a discussion of recent findings that have modified our understanding of the NKT cell developmental programme, with an emphasis on events that affect the early stages of this process. This includes factors that control double-positive thymocyte lifespan, and therefore the ability to generate the canonical Vα rearrangements that characterize this lineage, as well as the signal transduction pathways engaged downstream of the T-cell receptor and SLAM molecules.

Keywords: cell fate; development; natural killer T cells; signal transduction; survival

Introduction

Natural Killer T (NKT) cells are a subset of $\alpha\beta$ T cells that are not restricted by classical MHC molecules, and that respond to antigen recognition with a 'cytokine storm'; the secretion, within hours, of large quantities of T helper type 1 and type 2 cytokines and chemokines, reminiscent of innate rather than adaptive functions. In fact, the rapid and dual production, at a single cell level, of interferon-γ and interleukin-4 (IL-4) in response to stimulation is a characteristic of many of these cells.[1] Through this cytokine and chemokine production, NKT cells influence the behaviour of many other cells in the immune system, including NK cells, macrophages, other $\alpha\beta$ T cells, dendritic cells and neutrophils (reviewed in ref. 2), and have been implicated in multiple processes, including microbial immunity and tumour rejection. Similarly, they play a role in the pathogenesis of autoimmune processes, atherosclerosis and allergy (reviewed in ref. 3).

Although there are different types of NKT cells (see ref. 4 for a review), the most common, and better studied are type I NKT cells, also called invariant NKT cells (iNKT). These cells are characterized by the expression of an invariant T-cell receptor (TCR) α chain (Vα14-Jα18 in mice or Vα24-Jα18 in humans) in combination with certain TCR-β chains (using Vβ8.2, Vβ7 or Vβ2 in mice, or Vβ11 in humans). The iNKT cells in mice can be $CD4^+$ or double-negative (DN; $CD4^-$ $CD8^-$), generally have a 'memory' or 'activated' phenotype ($CD69^+$ $CD62L^-$ $CD44^{hi}$ IL-2Rβ^{hi}) and express markers characteristic of NK cells, including NK1.1, NKG2D and Ly49. They are found mainly in the liver and bone marrow, but also in the thymus, spleen and blood. The numbers of NKT cells are highly variable between individuals and mouse strains.[5–9] Given their role in immunoregulation this may be relevant for the pathogenesis of autoimmune diseases, and deficiencies in NKT cell numbers have been identified in pathologies such as systemic lupus erythematosus.[10]

Invariant NKT cells recognize the glycosphingolipid α-galactosylceramide presented by CD1d, and are best identified using a CD1d tetramer bound to this antigen.[11] Other ligands for NKT cells have been identified, both endogenous and derived from pathogens, but the nature

of the ligand(s) that mediate positive selection of this lineage in the thymus remains elusive.[12,13]

The iNKT cells develop in the thymus from the same precursors as conventional $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells, $CD4^+$ $CD8^+$ double-positive (DP) cells.[14] In contrast to conventional $\alpha\beta$ T cells, which are selected by MHC–peptide complexes presented by thymic epithelial cells, iNKT are selected by lipid antigens presented by the non-polymorphic, MHC I-like molecule CD1d, present on the surface of other DP thymocytes (reviewed in refs 3,15,16).

The differentiation of iNKT cells in the thymus has been divided into a series of stages, based on phenotypic markers, and sensitivity to different mutations (reviewed in refs 11,16–18) (Fig. 1). Once a DP thymocyte expresses the invariant TCR and receives signals through the signalling lymphocytic–activation molecule (SLAM)/SLAM-associated protein (SAP) and TCR (Control Point 1) it starts down-regulating Heat Stable Antigen (HSA) (CD24) and up-regulating first CD44, and later DX5. These cells are small, $NK1.1^-$ and not cycling. Some of these cells can exit the thymus and mature to $NK1.1^+$ iNKT cells in the periphery, while others mature in the thymus. The transition from $NK1.1^-$ immature iNKT cells to $NK1.1^+$ iNKT is accompanied by a proliferative burst (Control Point 2). Mature iNKT cells can be $CD4^+$ or $CD4^-$ (i.e. DN), and these subsets are functionally different, at least in humans. $CD4^+$ iNKT cells have a T helper type 0 cytokine profile while DN iNKT cells are more T helper type 1 biased. The relationship between these two subsets is unclear, although some genetic manipulations, like the deletion of GATA-3 at the DP stage, affect them differentially.[19] Recent data suggest that some of the $NK1.1^-$ iNKT cells found in the periphery are a distinct mature population.[20] There is also some evidence[21,22] for a process of negative selection, induced by high-affinity ligands, although it has not been well characterized. In the periphery, iNKT cells are long-lived, have a slow turnover, are dependent on IL-15, and do not seem to require low-affinity interactions of their TCR with CD1d for survival.[23]

Numerous recent reviews have addressed the mechanisms that control NKT cell development and function.[4,11,16,18,24–26] Here, we will focus on recent findings that impact our understanding of the early stages of iNKT development (Control Point 1).

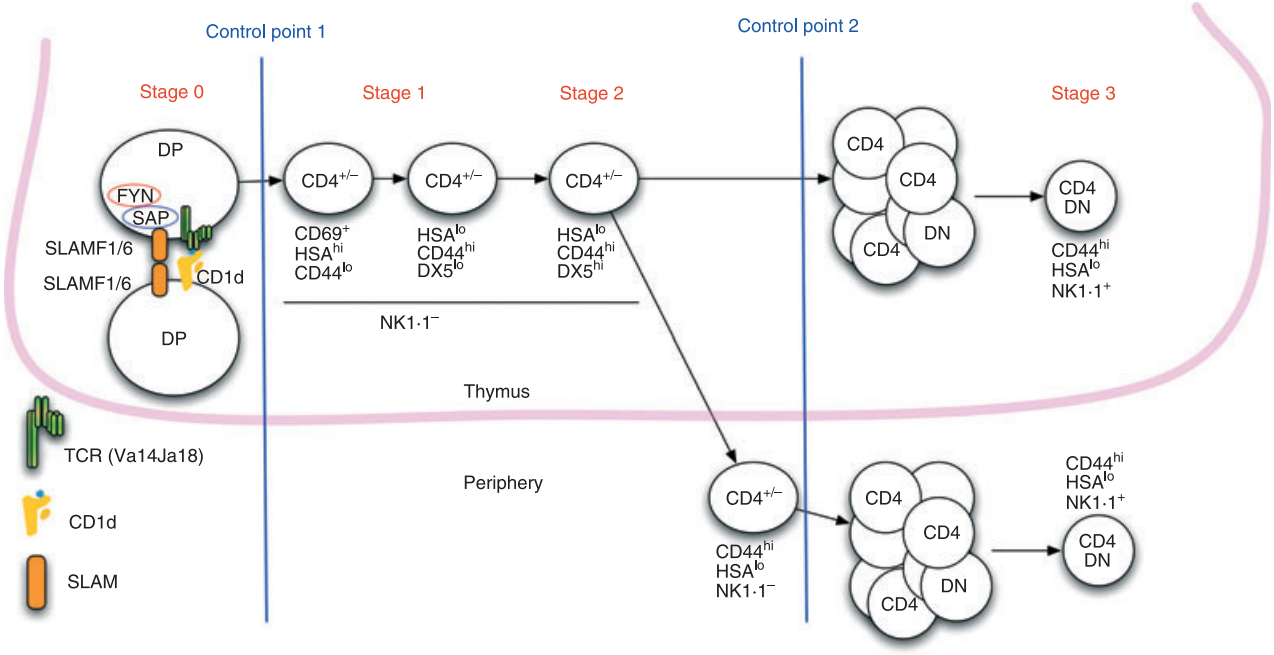

Figure 1. Stages of invariant natural killer T (iNKT) cell development. The NKT cells develop in the thymus from $CD4^+$ $CD8^+$ double-positive (DP) thymocytes. Thymocytes that express a T-cell receptor (TCR) that interacts with CD1d bound to self glycolipid, expressed by other DP thymocytes, enter the NKT-cell lineage. Signals from the signalling lymphocytic–activation molecule (SLAM) co-receptors are also required. Once selected, NKT-cell precursors undergo a series of differentiation steps that ultimately result in the NKT-cell pool. The initial selection event is called Control Point 1. Four distinct stages of differentiation have been defined through differential expression of CD24, CD44 and DX5. The NKT cells then undergo an actively regulated maturation step, which ultimately results in a range of functional and phenotypic changes, including expression of NK1.1. This maturation step is called Control Point 2. Many NKT emigrate from the thymus as immature cells and undergo final differentiation in the periphery. Some mature thymic NKT cells also migrate to the periphery, but many remain as long-term thymus-resident cells. Both immature and mature NKT cells include $CD4^+$ and $CD4^-$ (DN) subsets.

Control of DP thymocyte lifespan

The first prerequisite for the generation of iNKT cells is the stochastic rearrangement of the invariant Vα14-Jα18 TCR-α chain. Given the orderly sequence of rearrangements in the TCR-α locus (proximal to distal), and the distal position of Jα18 in the Jα region, Vα14-Jα18 rearrangements are always secondary.[27] Therefore an extended DP lifespan is necessary for the development of NKT cells, as first evidenced by the defects in NKT development observed in RAR-related orphan receptor γ_T ($ROR\gamma_T$) -deficient and Bcl-x_L-deficient mice.[28–30] In contrast with this model, it has also been proposed that some early αβ T-cell precursors are pre-committed to the iNKT lineage at the DN stage.[31] It is unclear how these results can be reconciled with multiple lines of evidence that suggest that a long DP lifespan is a requirement for iNKT cell development.

Double-positive thymocytes have a short average lifespan (3–4 days).[32–34] The mechanisms that regulate this defined lifespan are not completely understood. Although DP thymocytes are exquisitely sensitive to elevations in glucocorticoid levels, as those induced by stress,[35,36] the role of normal levels of glucocorticoids in determining DP thymocyte lifespan is controversial.[37–39] $ROR\gamma_T$, a retinoid-related orphan nuclear receptor, was shown to control DP lifespan,[40] and analysis of $ROR\gamma_T$-deficient knockouts provided the first evidence for the requirement of long DP lifespans for iNKT generation. The $ROR\gamma_T$ affects DP survival through the regulation of Bcl-x_L, an anti-apoptotic member of the Bcl-2 family. Although this basic relationship has been known for a number of years, a number of other factors that also contribute to DP survival have been recently identified.

HEB, a member of the E-box family of transcription factors, is required for iNKT cell development because of its effect on controlling expression of $ROR\gamma_T$ and, therefore, Bcl-x_L.[41] Similarly, DP-specific deletion of the transcription factor c-Myb results in a decrease in DP thymocyte lifespan,[42] and a blockade of iNKT cell development.[43] The alteration in DP lifespan is the result of its effect on Bcl-x_L expression, but, in contrast with HEB, this effect is independent of $ROR\gamma_T$ expression. The effect on Bcl-x_L does not seem to be direct, or, at least, c-Myb cannot be detected by ChiP on three predicted c-Myb binding sites present in the Bcl-x_L promoter.[42] Furthermore, although expression of Bcl-x_L rescues DP lifespan, and distal Vα-Jα rearrangements, this is not sufficient to rescue NKT cell development in c-Myb-deficient thymocytes[43] (see below). Other pathways that regulate of DP lifespan through control of Bcl-x_L levels include the Wnt-β-catenin-TCF-1 pathway,[44–46] and the energy sensor LKB1, through activation of AMP-activated protein kinase.[47]

Another genetic alteration that results in decreased DP thymocyte survival, and biased TCR-α rearrangements, is the deletion of the transcriptional repressor histone deacetylase 7 (HDAC-7).[48] Presumably this mutation should also block NKT development, although this was not tested. In this case the effect is apparently independent of alterations in $ROR\gamma_T$ or Bcl-x_L, because their expression is not altered in HDAC-7-deficient thymocytes. The relationships between all these different pathways, and how they cooperate to maintain homeostasis of the DP population, are subjects of interest that will require additional experiments in the future (see Fig. 2).

Signals that regulate Control Point 1

It is thought that selection by DP cells imparts the unique developmental programme of iNKT cells, and other 'innate-like' αβ T cells such as T-CD4 T cells,[49,50] by the cooperative engagement of the TCR and at least two members of the SLAM family (SLAMF1 and SLAMF6),[51] although it is unclear if this cooperative engagement alters the signal that a positively selected DP thymocyte receives from its TCR, or whether the SLAM/SAP-derived signals are required to block negative selection induced by strong TCR-derived signals.[52] Below we discuss the known contributions of different signal transduction pathways downstream of the TCR and the SLAM axis. In some

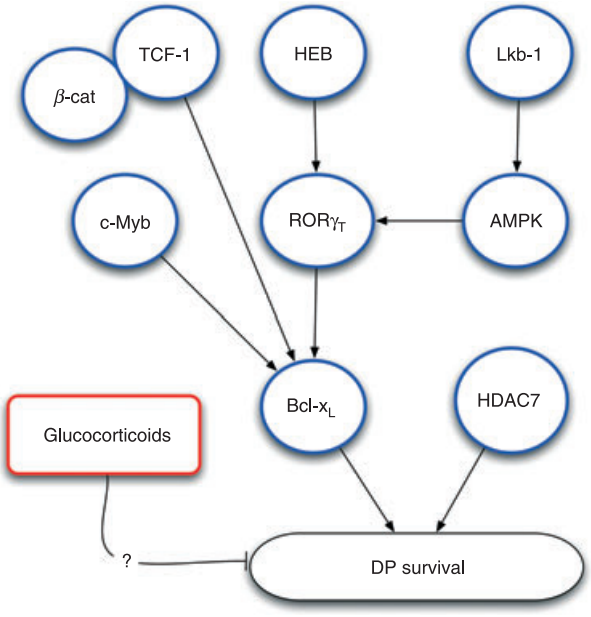

Figure 2. Control of double-positive (DP) thymocyte lifespan. $CD4^+$ $CD8^+$ DP thymocytes have a short lifespan, and in the absence of signals from the T-cell receptor (TCR) die in 3–4 days. Factors that shorten this lifespan dramatically affect development of invariant natural killer T cells, because the Vα14-Jα18 TCR-α rearrangement that characterizes them is always secondary. In this diagram we illustrate some of the known factors that influence DP lifespan, and their interactions.

cases, most notably the nuclear factor-κB (NF-κB) pathway, both inputs are able to activate the same pathway (see Fig. 3).

Signals from the TCR

Selection of conventional αβ T cells from DP thymocytes requires TCR-mediated signals, which induce numerous intracellular events including activation of the Ras/mitogen-activated protein kinase (MAPK) cascade,[53] increased intracellular calcium levels and subsequent activation of calcineurin[54] and activation of itk family kinases.[55,56] A number of recent papers has explored the contribution of these pathways to the selection of iNKT cells.

Activation of the Ca^{2+}/calcineurin pathway is required for iNKT cell development. Deletion of calcineurin B1 in DP thymocytes produced a significant loss in both thymic and peripheral iNKT cell numbers.[57] In the same work, the authors identified Egr2 as an important mediator of this pathway. Egr2-deficient fetal liver chimeras showed impaired development of iNKT cells, starting at the earliest stages of positive selection (Control Point 1). Although the evidence presented in this work suggested that neither Egr1, nor Egr3, played a role in iNKT cell development, more recent experiments, using competitive mixed bone marrow chimeras, and double Egr1-Egr2 knockouts, have shown that both Egr1 and Egr2 play a role in NKT cell development.[58] Contrary to the situation in conventional αβ T-cell development, where Egr1 and Egr2 play a quantitatively similar role,[59,60] during iNKT cell development Egr2 seems to play a much more central role. The mechanism that underlies these functional differences is not yet characterized. Another molecule induced by calcium signals, the high-mobility group box transcription factor TOX was also recently shown to be essential for iNKT cell development.[61] The specific stage of iNKT development affected by TOX-deficiency was not determined, but the dramatic decrease in the percentage of CD1d-tet$^+$ cells observed in these mice suggests an early block.

Although the Ras/MAPK cascade plays a central role in positive selection of conventional αβ T cells[62,63] its involvement in iNKT cell development has not been studied in detail until recently, in part because an early report suggested that the number of NK1.1$^+$ cells in the thymus of mice over-expressing dominant negative Ras and Mek-1 was normal.[64] This result, together with data showing that cross-linking of SLAMF1 resulted in recruitment of RasGAP,[65] resulted in a model where the role of the SLAM-derived signal would be to limit Ras/MAPK activation (see refs 4,24,25,66). However, a recent re-examination of iNKT populations in dnRas transgenic mice shows that inhibition of this pathway profoundly perturbs iNKT cell development, with a blockade at the early stages of positive selection (Control Point 1).[58] Therefore, as with conventional αβ T cells the Ras/MAPK pathway plays a critical role during positive selection of iNKT cells. The Itk family kinases, which are important for the selection of conventional αβ T cells (reviewed in refs 55,56), also seem to play a role in iNKT cell development,[67] but this effect seems to be evident first at later stages than those from mice defective in Ras or Egr.

The contribution of the NF-κB pathway to positive and negative selection of conventional αβ T cells is a controversial topic. In peripheral T cells, TCR triggering

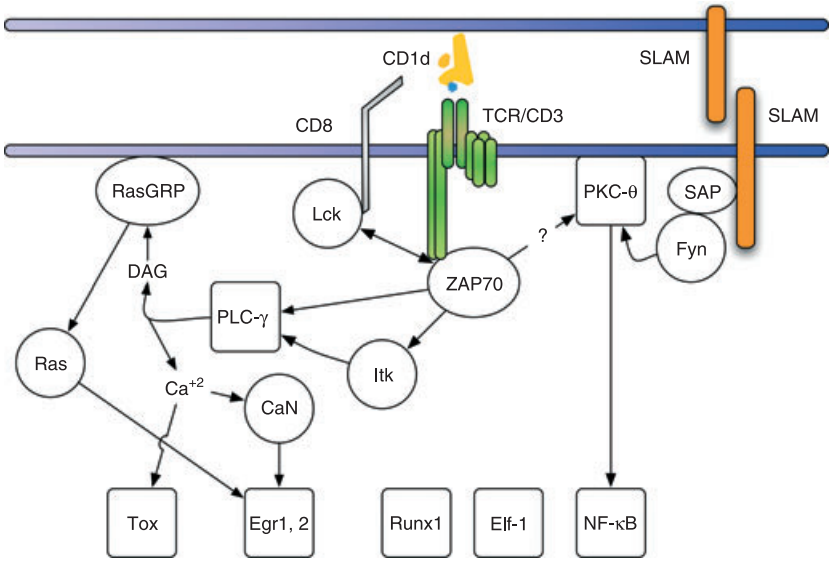

Figure 3. Signals that drive Control Point 1. Positive selection of invariant natural killer T (iNKT) cells from double-positive (DP) thymocytes requires signals from the T-cell receptor (TCR) and the signalling lymphocytic–activation molecule (SLAM) co-receptors. This diagram shows some of the signal transduction pathways involved in this early stage of iNKT cell development, as well as some transcription factors that are required for further development.

results in NF-κB activation mediated by protein kinase C-θ, Bcl10 CARMA-1 and Malt1, but gene knockout of these components does not alter positive selection of conventional αβ T cells.[68–70] However, other alterations further downstream from this pathway result in changes in T-cell development.[71] In contrast, activation of the NF-κB pathway seems to be more important for the NKT cell lineage, because positive selection of NKT, but not conventional αβ T cells, is blocked in mice with a T-cell-specific deletion of IKK2.[72,73] However, mice deficient in CARMA-1 and Bcl10 have normal NKT development.[73,74] Protein kinase C-$\theta^{-/-}$ mice show a partial defect in thymic development, with accumulation at immature stages, but almost normal populations in the periphery.[73,75]

SLAM/SAP and NKT cell development

The SLAM family consists of a number of related proteins including (Slamf1), CD48 (Slamf2), 2B4 (Slamf4), Ly9 (CD229, Slamf3), CD84 (Slamf5), NTB-A (Slamf6), Cracc (Slamf7), BLAME (Slamf8) and SF2001 (Slamf9). These receptors are expressed on the surface of a wide variety of haematopoietic cells, have diverse functions, including roles in regulating co-stimulation, T-cell cytokine production, adhesion between haematopoietic cells, the development of innate T lymphocytes (SLAM and Ly108) and others (see refs 76,77 for recent reviews on this family of receptors). Although DP thymocytes express high levels of SLAMF1, 2, 3, 5 and 6, and lower levels of SLAMF4, 7, 8 and 9,[43,77,78] the only SLAM family members that seem to play a role in iNKT cell development are SLAMF1 and SLAMF6. Interestingly their function is redundant. Single knockouts have a partial phenotype,[79,80] but a series of elegant mixed bone marrow chimeras clearly demonstrated that the combined lack of SLAMF1 and SLAMF6 causes a dramatic decrease in NKT cell numbers.[51] This finding fits nicely with data obtained from NOD mice, a mouse strain that has been used as a model for autoimmune diabetes.The NOD mice have decreased levels of NKT cells.[81] This phenotype was mapped to the SLAM locus,[82] and a comparison of the expression pattern of SLAM family members in DP thymocytes showed a decrease in expression of SLAMF1 and SLAMF6, but no other SLAM family members.[78] Recently, we found that the transcription factor c-Myb plays a role in NKT cell development in part by modulating expression of SLAMF1 and SLAMF6.[43] Interestingly, the SLAM family expression patterns in DP thymocytes from mice carrying the NOD SLAM locus and in mice defective in c-Myb are very similar,[78] suggesting that the defective expression of SLAM1 and SLAM6 may be the result of alterations in c-Myb binding.

It is well established that SLAM engagement recruits the adaptor SLAM-associated protein (SAP) and the Src kinase Fyn, both of which are essential for the selection of the iNKT cell lineage,[83–87] but the signal transduction pathways engaged downstream from the SLAM/SAP/FYN module that are relevant for NKT cell development are not well defined.

In a T-cell line SLAMF1 cross-linking induces recruitment and phosphorylation of of SHIP, Dok1, Dok2 and Ras-GTPase-activating protein (Ras-GAP)[65] and this evidence has been used to propose a model where the SLAM-derived signals would inhibit activation of the Ras/MAPK cascade induced by the TCR.[66] As discussed above,[58] recent results argue against this model. SLAM-SAP triggering induces other signal transduction pathways. SLAM cross-linking on CD4 T cells results in AKT activation[88] and prolongs protein kinase C-θ recruitment to the site of antigen-presenting cell contact, as well as influences Bcl-10 phosphorylation and patterns of NF-κB activation.[89] It is unclear whether this effect on NF-κB activation by SLAM/SAP is important for NKT cell development. Activation of this pathway is important, as shown by the almost complete block in NKT cell development observed in IKK2$^{-/-}$ mice,[73] and in mice expressing a degradation-resistant Iκ-Bα,[90,91] but the contribution of different upstream activators is not clear.

Whether SLAM/SAP and TCR-derived signals cooperate to activate NF-κB during positive selection of DP thymocytes is unclear, although they do in mature CD4 T cells.[89] Over-expression of a constitutively active IKKβ kinase in SAP$^{-/-}$ mice fails to rescue NKT cell development,[92] suggesting that additional pathways downstream of SLAM/SAP are required. In over-expression studies, SAP also bound to PAK-interacting exchange factor (PIX), leading to synergistic NFAT (nuclear factor of activated T cells) activation in conjunction with ionomycin in Jurkat T cells[93] and to NCK1 (non-catalytic region of tyrosine kinase 1).[94] The possible contribution of these pathways to NKT cell development has not been explored.

Many factors important for the initial stages of NKT cell development have been recently identified. However, we still do not understand the specific contribution of the SLAM/SAP signal to this process, and how it complements TCR-derived signals. The DP-specific knockouts of other transcription factors such as Runx1,[29] Fra-1[95]or Elf-1[96] also result in alterations at early stages in NKT cell development, but it is unclear whether signals from the TCR or SLAM affect the activity or expression of these transcription factors in this process, or how all these signals cooperate to set in motion the NKT-specific developmental programme.

Acknowledgement

This work was supported by grant AI059302 (NIH/NIAID) to J.A.I.

Disclosures

The authors declare no competing interests.

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