Control points in NKT-cell development

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Abstract | CD1d-dependent natural killer T (NKT) cells are a unique T-cell subset with the ability to regulate the immune system in response to a broad range of diseases. That low NKT-cell numbers are associated with many different disease states in mice and humans, combined with the fact that NKT-cell numbers vary widely between individuals, makes it crucial to understand how these cells develop and how their numbers are maintained. Here, we review the current state of knowledge of NKT-cell development and attempt to highlight the most important questions in this field.

α-GalCer–CD1d tetramers
Tetrameric (or multimeric) forms of CD1d molecules bound to α-galactosylceramide (α-GalCer), which have sufficient affinity for the T-cell receptor (TCR) of type I natural killer T (NKT) cells to allow the detection of invariant NKT cells by flow cytometry.

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CD1d is an MHC-class-I-like molecule that presents glycolipid antigens to a specialized subset of T cells known as natural killer T (NKT) cells. At least two classes of CD1d-dependent NKT cells have been defined. Type I NKT cells (also known as invariant or semi-invariant NKT cells (*i*NKT cells)) express an invariant T-cell receptor α-chain (TCRα; Vα14-Jα18 in mice, Vα24-Jα18 in humans) in combination with certain Vβ chains (Vβ8.2, Vβ7 or Vβ2 in mice, Vβ11 in humans). Type I NKT cells recognize the glycosphingolipid antigen α-galactosylceramide (α-GalCer) and are best defined using CD1d tetramers loaded with this antigen (α-GalCer–CD1d tetramers)[1]. Type II NKT cells are also CD1d dependent, but they express more diverse TCR Vα chains. Much less is known about type II NKT cells because we lack specific reagents to directly identify them. This Review focuses on type I NKT cells (herein referred to simply as NKT cells).

NKT cells are a thymus-dependent T-cell subset, but are developmentally and functionally distinct from mainstream $CD4^+$ and $CD8^+$ T cells. NKT cells are multifunctional, they can enhance microbial immunity and tumour rejection, suppress autoimmune disease and promote tolerance. Yet NKT-cell activity can also be deleterious to the host, by exacerbating atherosclerosis, allergy and some autoimmune diseases[2] (FIG. 1). The impact of NKT cells appears to be related to their overall number and functional competence, so understanding the factors that regulate NKT-cell development and homeostasis represents an important goal for immunologists.

The origin of NKT cells

For many years, the developmental origin of NKT cells was a matter of debate. Some studies suggested that they developed very early in ontogeny, independently of the thymus, and before the appearance of conventional T cells[3,4]. However, there is now overwhelming evidence that NKT cells are a thymus-dependent population. They are absent from nude mice[5,6]; do not develop in thymectomized mice[7–9] and first appear in the thymus slightly later than most other T-cell subsets[6,10–13]. There is also convincing evidence that NKT cells segregate from conventional T-cell development at the double positive (DP; $CD4^+CD8^+$) thymocyte stage in the thymic cortex[13–15].

Although NKT cells always express the invariant TCR Vα14-Jα18 chain[16], their non-transcribed TCRα allele shows clear evidence of random recombination[17]. Furthermore, although the complementarity-determining region 3 (CDR3) of the α-chain is invariant at the amino acid level, this is not the case at the nucleotide level, in which non-templated nucleotide additions contribute to the invariant amino-acid sequence[16]. Taken together, these data suggest that the development of NKT cells is a selective (rather than instructive) event stemming from the random production of a TCR that recognizes CD1d.

How to select an NKT cell

Positive selection of conventional T cells requires TCR recognition of peptide antigens presented in the context of MHC class I or II molecules expressed by thymic cortical epithelial cells. By contrast, NKT-cell selection requires their ligation to glycolipid antigens presented by CD1d on DP cortical thymocytes. This was shown using bone-marrow chimaeras or transgenic restoration of CD1d expression in *Cd1d*$^{-/-}$ mice, to restrict expression of CD1d to the haematopoietic or stromal compartments of mice. NKT cells are only selected when CD1d is expressed by haematopoietic cells, and more specifically, by DP thymocytes[8,18–22]. In a recent study, when expression of a *Cd1d* transgene was restricted mainly to DP thymocytes in *Cd1d*$^{-/-}$ mice by placing it under the control of the *Lck* promoter[23], NKT-cell development, thymic emigration and establishment of a

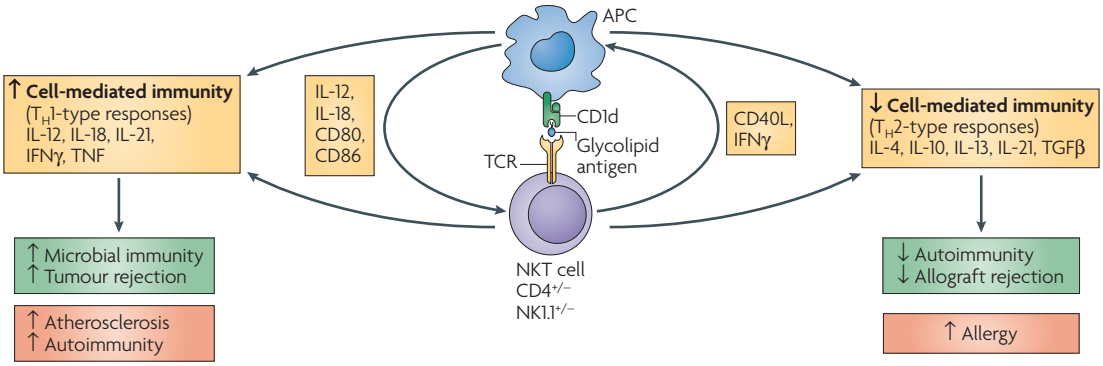

Figure 1 | **NKT-cell function.** Natural killer T (NKT)-cell recognition of glycolipid antigens presented by CD1d leads to a cascade of events involving cytokines and co-stimulatory molecules that results in the activation of antigen-presenting cells (APCs) and many other bystander cells (including NK, T and B cells; not shown). The diversity and extent of cytokine production can have a broad range of effects, ranging from enhanced cell-mediated immunity (T helper 1 (T_H1)-type responses) to suppressed cell-mediated immunity (T_H2-type responses). In most experimental cases, the response is advantageous to the host (indicated in green), however, in some cases, the response can be deleterious (indicated in red). CD40L, CD40 ligand; IFNγ, interferon-γ; IL, interleukin; TCR, T-cell receptor; TGFβ, transforming growth factor-β; TNF, tumour-necrosis factor.

peripheral NKT-cell pool were essentially normal. This is interesting because thymic selection of conventional T cells is usually mediated by epithelial cells. Epithelial cells also express CD1d[21,22], which raises the important question of why they cannot select NKT cells. The most probable explanation is that DP thymocytes provide (or epithelial cells lack) signals that are necessary for NKT-cell development, or that DP thymocytes are uniquely capable of presenting the glycolipids required for NKT-cell selection. Selection by DP thymocytes, rather than by thymic stromal cells, may promote some of the unique characteristics associated with NKT cells. Consistent with this, $CD4^+$ T cells selected by interaction with MHC class II on thymocytes rather than stromal cells adopt some characteristics that are reminiscent of NKT cells (that is, intermediate TCR expression, and increased expression of CD44, NK1.1 and CD69)[24].

It is important to stress that the presence of CD1d alone is not sufficient for NKT-cell selection. Several studies have shown that CD1d also needs to be able to recycle through the intracellular endosomal and/or lysosomal pathway[25]. In the absence of this trafficking, even though CD1d can be expressed at the cell surface, NKT-cell selection is absent or greatly impaired, which strongly suggests that, just as conventional T cells require MHC and peptide antigen for efficient intrathymic selection, NKT-cell selection requires CD1d to be loaded with an endogenous endosome- or lysosome-derived antigen(s). Many recent studies have identified the factors involved in this CD1d trafficking and loading pathway (FIG. 2).

Glycolipid antigens and NKT-cell selection

The past few years have seen many important developments in the search for the NKT-cell-selecting antigen(s). α-GalCer is the most potent known antigen, but it was never considered to be a potential selecting ligand because it is not a natural mammalian product. Nevertheless, mammalian cells contain many glycosphingolipids with structural similarities to α-GalCer, which made this class of molecules an obvious candidate. Consistent with a role for glycosphingolipids in NKT-cell selection and self recognition, Stanic *et al.* reported that cells lacking β-D-glucosyltransferase (and therefore β-glucosylceramide — a precursor for mammalian glycosphingolipid biosynthesis) lost the ability to stimulate NKT-cell hybridomas[26]. With this in mind, Zhou and colleagues[27] set about narrowing down the candidate glycosphingolipid families by examining mice with deficiencies in particular glycosphingolipid synthesis or degradation enzymes. This approach identified an important block in NKT-cell development in mice deficient in β-hexosaminidase B, a lysosomal enzyme responsible for the degradation of globo/isoglobo-series glycosphingolipids. This enzyme produces globotrihexosylceramide (Gb3) and isoglobotrihexosylceramide (iGb3) in lysosomes, and further experiments pinpointed lysosomal iGb3 as the key candidate. Since then, studies reported in several papers from independent groups have provided additional evidence supporting the role of iGb3 as a self antigen for NKT-cell selection in mice[28–32]. In comparison to α-GalCer, iGb3 is a relatively weak agonist ligand, but nonetheless, it can clearly activate mature mouse and human NKT cells, and indirect evidence has suggested that iGb3 recognition as a self antigen is also important for the NKT-cell response to infection with *Salmonella* spp.[33].

Zhou *et al.*[27] suggested that iGb3 was the main glycosphingolipid responsible for NKT-cell selection, but not all investigators agree. One study[34] argued that it was not the lack of lysosomal iGb3 that disrupted NKT-cell development in β-hexosaminidase-B-deficient mice, but rather, a general disruption in glycosphingolipid processing (a phenomenon known as lysosomal storage disease). A similar conclusion was derived from a very recent study of two other lysosomal storage disease models[35]. This is a very controversial subject because the study by Zhou *et al.* reported that cells from

Globo/isoglobo-series glycosphingolipids
One arm of the glycosphingolipid family, which is characterized by an α-linked galactose sugar in the third sugar position. Globotrihexosylceramide (Gb3) has an α1–4 linked galactose sugar, whereas isoglobotrihexosylceramide (iGb3) has an α1–3 linked galactose sugar. Additional β-linked sugars bound to these base structures yield Gb4 and iGb4.

Agonist ligand
A ligand that results in cell activation and proliferation. Agonistic activity is often, but not always, associated with high-affinity and/or high-avidity binding of the T-cell receptor.

β-hexosaminidase-B-deficient mice could process and present other glycolipids to NKT cells, which suggested that their lysosomal processing capabilities were generally intact[27]. This controversy has been extensively discussed in a recent commentary article[36]. Two recent papers have further challenged the significance of iGb3 in NKT-cell selection. Direct biochemical analysis of glycolipids isolated from mouse or human thymi or dendritic cells failed to detect iGb3 (REFS 37,38). However, the lower limit of the HPLC (high-performance liquid chromatography) assay used in these studies was approximately 200 molecules per cell and therefore this result does not in itself exclude a biologically significant role for iGb3, particularly if it is only present in a small subset of thymic cells. The strongest evidence against a role for iGb3 in NKT-cell selection was that mice deficient in iGb3 synthase (also known as A3GALT2) have no obvious NKT-cell defect[37]. Given that iGb3 synthase is thought to be essential for iGb3 biosynthesis, this is a more direct appraisal of the importance of iGb3 and

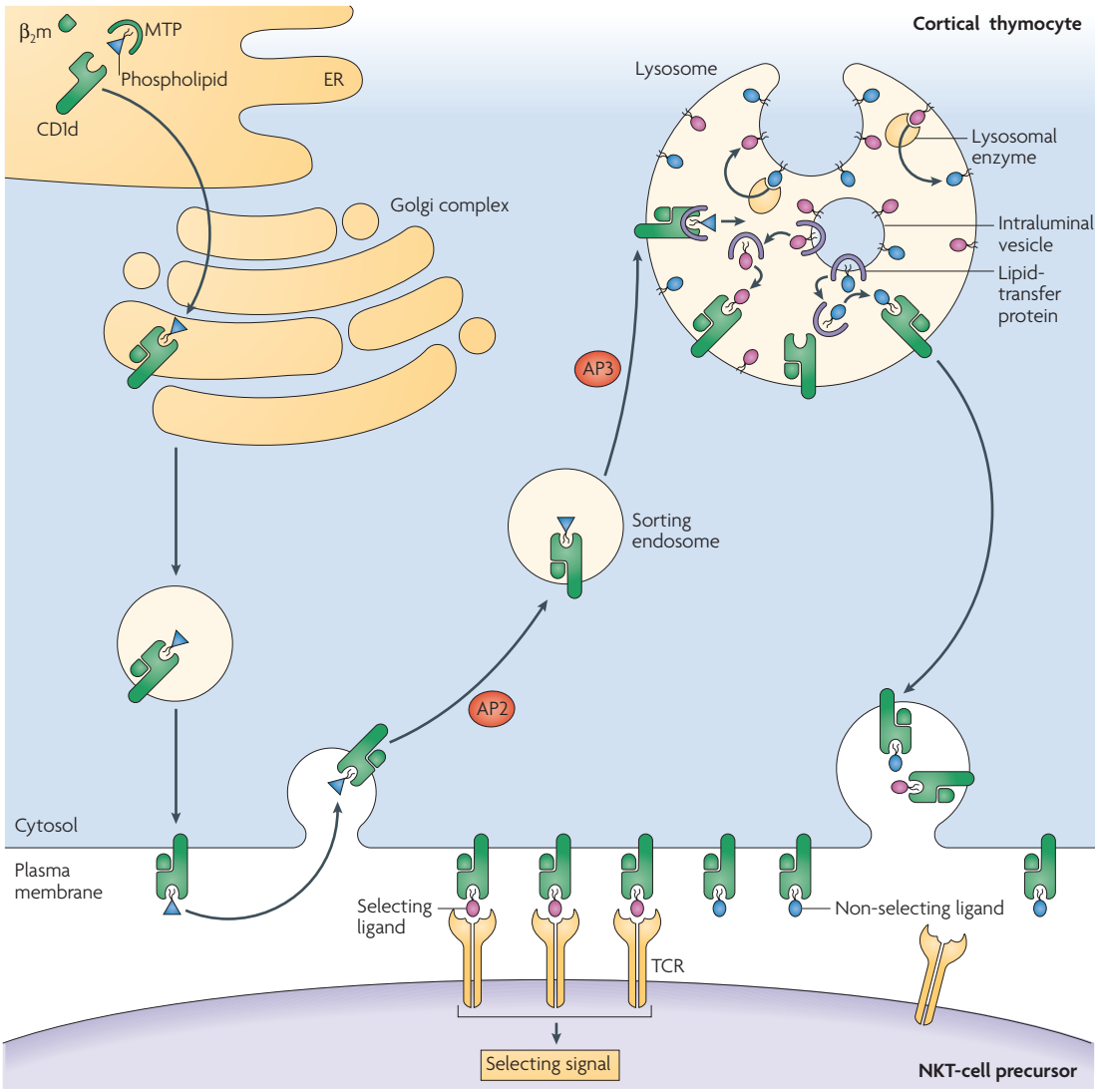

Figure 2 | **CD1d processing and antigen presentation.** CD1d is assembled with β_2-microglobulin (β_2m) in the endoplasmic reticulum (ER), and loaded with lipid ligands (mainly glycosylphosphatidylinositol[99]) possibly under the control of microsomal triglyceride transfer protein (MTP)[100,101]. These loaded CD1d molecules then traffic through the Golgi complex to the cell surface, and subsequently are internalized through the endosomal/lysosomal pathway, under the control of adaptor protein 2 (AP2) and AP3 (REF. 25), to lysosomes, where lipid-transfer proteins, such as saposins and Niemann-pick type C2 protein (NCP2)[102,103], facilitate the exchange of ER-derived lipids for lysosomal lipid antigens. Lysosomal lipid antigens include glycolipids that have been processed and degraded by lysosomal enzymes; various degradation products, including agonist and non-agonist glycolipids, are loaded onto CD1d before CD1d traffics back to the cell surface. This lysosomal trafficking step is essential for CD1d to acquire agonist glycolipid antigens, such as isoglobotrihexosylceramide (iGb3), for presentation to natural killer T (NKT) cells. A possible caveat to some aspects of this pathway is that some of the mouse mutant models used for these studies have associated lysosomal storage diseases, which may be a confounding factor in the interpretation of the results[34,35]. TCR, T-cell receptor.

more strongly suggests that iGb3 is not essential for NKT-cell development. That said, this does not exclude a redundant role for this glycolipid in normal mice, and the possibility remains that other glycosyltransferases may contribute to iGb3 biosynthesis in the thymus. This is the most controversial area in the field of NKT-cell development at the moment, with compelling evidence both for and against iGb3 as an important ligand for NKT-cell selection. Further studies are clearly necessary to resolve this issue.

In light of the debate about the role of iGb3 in NKT-cell selection, we are reminded of the importance of not dismissing other mammalian glycolipids that could potentially have a role in the selection of NKT cells. These include the weakly agonistic β-glycosylceramides[39] and the more complex derivatives of these compounds (such as lactosylceramide and Gb3). It might also be misleading to disregard candidate ligands simply because they lack strong agonist activity, as conventional MHC-restricted T cells are typically selected by non-agonist peptides[40]. Furthermore, there might be some level of antigen redundancy in NKT-cell selection (as there is for conventional T cells); so, when eliminating individual glycosphingolipid types, the lack of a significant impact on NKT-cell numbers cannot be regarded as proof that these molecules have no significance in the development of NKT cells.

The TCRβ repertoire and NKT-cell selection?

It is well established that an invariant TCRα is required for the development and selection of NKT cells (with one or two possible exceptions[41]), yet there is also strong evidence that NKT cells are collectively capable of recognizing a range of different antigens, most probably due to diversity of their TCRβ. In mice, the TCR Vβ chain usage is heavily biased to Vβ8.2, Vβ7 and Vβ2, but the Jβ genes and CDR3β regions are diverse[16,42]. In humans, TCR Vβ usage by NKT cells is almost entirely limited to Vβ11, but, similar to mice, diverse Jβ genes are used and there is no canonical CDR3β usage that predominates[43,44]. So, although only some Vβ genes allow NKT-cell selection, there is clearly a degree of TCRβ plasticity. This is supported by models showing that TCRβs from conventional T cells can support NKT-cell development, providing they contain the Vβ8.2 region[45,46], and also by structural studies that suggest that flexibility in CDR3β might allow recognition of a range of antigens presented by CD1d[47]. A recent study[29] examined Vα14-Jα18 TCR-transgenic *Cd1d*$^{-/-}$ mice, in which the invariant Vα14-Jα18 chain paired with random Vβ chains in the absence of CD1d-dependent selection. This report showed that many different Vβ chains could pair with the invariant Vα14-Jα18 TCR, and surprisingly, a diverse range of Vβ chains in this unselected repertoire allowed αGalCer–CD1d recognition (including Vβ6, Vβ9, Vβ10 and Vβ14, in addition to Vβ8.2, Vβ7 and Vβ2 that characterize the selected NKT-cell repertoire). By contrast, the Vβ8.2, Vβ7 and Vβ2 chains were required for the recognition of iGb3. This report suggested that the selecting glycolipid antigen determines TCRβ usage, and also provided further support for the involvement of iGb3, or a closely related molecule, in thymic NKT-cell selection[29]. Even among the selected Vβ8, Vβ7 and Vβ2 chains, some appear to confer higher affinity for glycolipid ligand bound to CD1d than others. Whereas Vβ8.2 is expressed by approximately 50% of NKT cells and provides the highest affinity for α-GalCer–CD1d recognition[48], Vβ7 is overrepresented in NKT cells compared with conventional T cells, suggesting a stronger selection bias[29]. Vβ7^{+} NKT cells are preferentially selected under competitive conditions, for example, in *Cd1d*$^{+/-}$ mice or in human Vα24 TCR-transgenic mice, in which the NKT cells have a lower affinity for CD1d[28], and Vβ7^{+} NKT cells are also preferentially expanded by CD1d-expressing DP thymocytes pulsed with iGb3, or even by DP thymocytes alone, presumably presenting an undefined self antigen[28].

NKT-cell negative selection

Given that NKT cells develop following random TCR gene rearrangements, with diverse TCRβs, it is possible that some clones will have a very high affinity for self-ligand–CD1d complexes and be potentially self-reactive. Indeed, the fact that iGb3, a candidate ligand for positive selection, is a known NKT-cell agonist, raises the important question of whether NKT cells are susceptible to negative selection during their development. Two studies demonstrated that the presence of α-GalCer during NKT-cell development *in vitro* or *in vivo* abrogates NKT-cell development[49,50], which is consistent with negative selection. Chun and colleagues also showed that overexpression of CD1d by dendritic cells (DCs), in the absence of exogenously added agonist ligands, also prevented NKT-cell development. This suggested that the overall avidity for a natural self ligand presented in the context of higher levels of CD1d was increased to the point that NKT cells were negatively selected. This is also interesting because it shows that although thymic stromal cells (including DCs) cannot mediate NKT-cell positive selection, they may be capable of driving NKT-cell negative selection, which is consistent with the role of DCs in the negative selection of conventional T cells. Interestingly, thymocytes appear to be capable of mediating both positive and negative selection of NKT cells[125]. It needs to be stated that these studies have really only shown a failure of NKT-cell development in the presence of strong agonist signals. They did not directly demonstrate the deletion of pre-formed NKT cells, and no CD1d-tetramer-positive NKT-cell subset has been directly shown to be susceptible to negative selection. This is in contrast to conventional T cells, for which there is a well-defined stage during which thymocytes are susceptible to negative selection[51]. Further studies are required to understand if, how and when NKT-cell subsets might be cleansed of highly self-reactive clones during their intrathymic development. The identification of a small subset of CD24^{hi} (also known as heat stable antigen (HSA)^{hi}) NKT cells that express NUR77 (REF. 52), a transcription factor associated with negative selection, may point to a window of susceptibility, but this remains to be formally investigated.

A developmental pathway for NKT cells

NKT-cell development defined by NK-cell marker expression. Early definitions of NKT cells were based on the co-expression of the NK-cell marker NK1.1 and αβTCR. However, with the advent of CD1d tetramers, it became clear that not all NKT cells express NK1.1 and, conversely, that some NK1.1$^+$ T cells were not type I NKT cells. In 2002, a breakthrough in our understanding of NKT-cell development came when three groups[6,10,12] independently identified immature NK1.1$^-$ and mature NK1.1$^+$ stages of NKT-cell development. An unexpected finding was that the most recent thymic emigrant NKT cells in the periphery did not express NK1.1 (REFS 6,10), which implied that maturation to the NK1.1$^+$ stage could take place in the thymus or in the periphery. These studies also showed that intrathymic NK1.1$^-$ NKT cells could progress to the NK1.1$^+$ stage following transfer into recipient mice, but not vice versa[6,10,12], which confirms the precursor status of some, if not all, NK1.1$^-$ NKT cells. The immature NK1.1$^-$ stage appears to include a post-selection expansion phase, because many of these cells have a rapid turnover, in contrast to the much slower turnover of NK1.1$^+$ NKT cells in the thymus[10]. The NK1.1$^-$ to NK1.1$^+$ stage of NKT-cell maturation is also accompanied by the upregulation of expression of several other cell-surface receptors, including Ly49C/I, Ly49G2, Ly6C, CD69 and CD122 (REFS 12,13,53), and as discussed below, many developmental deficiencies arise during this transition.

Collectively, these findings established a basic pathway of NKT-cell development that now serves as the framework on which subsequent studies have been building. It is therefore useful to define at least two key control points in NKT-cell development. Control point 1 governs NKT-cell selection, in which CD1d-restricted positive selection of immature thymocytes causes the NKT-cell lineage to branch from the conventional T-cell development pathway, giving rise to immature NK1.1$^-$ NKT cells. Control point 2 governs NKT-cell maturation, in which immature CD1d tetramer$^+$ NKT cells undergo a series of phenotypic and functional changes, which is highlighted and broadly defined by the upregulation of NK1.1 expression (FIG. 3).

Immature NK1.1$^-$ NKT-cell populations in the thymus can be subdivided on the basis of CD24, CD44 and DX5 expression, thereby identifying at least four phenotypically distinct stages within the broad NK1.1$^-$ immature phase: NK1.1$^-$CD24$^+$CD44^{low}DX5^{Low} stage 1 cells; NK1.1$^-$CD24^{low}CD44^{low}DX5^{low} stage 2 cells; NK1.1$^-$CD24^{low}CD44^{hi}DX5^{low} stage 3 cells; and NK1.1$^-$CD24^{low}CD44^{hi}DX5^{hi} stage 4 cells. Stage 1 (CD24$^+$) cells are present at a very low frequency (approximately 1 in 10^6 thymocytes) and, in contrast to other NK1.1$^-$ NKT cells, they are small and apparently non-dividing, suggesting that the extensive post-selection expansion follows this stage[11]. The precursor–progeny relationship between stages 1 to 4 has not been formally demonstrated, but the pathway fits well with ontogeny data from independent groups[10–12]. The implication is that cells at stage 4 are the immediate precursors of the mature NK1.1$^+$ NKT cells (a transition regulated at control point 2), although this too is yet to be formally demonstrated.

Despite their immature status, NK1.1$^-$ NKT cells in the thymus are functionally competent when activated through their TCR, although their cytokine profile is distinct from that of mature cells. Thymic NK1.1$^-$ NKT cells produce higher levels of interleukin-4 (IL-4) than mature NKT cells, but comparatively low levels of interferon-γ (IFNγ). This gives a phenotype that is biased towards the T helper 2 (T_H2)-cell phenotype, whereas NK1.1$^+$ NKT cells produce high levels of IL-4 and IFNγ, reminiscent of a T_H0-cell phenotype[6,10,12]. Among NK1.1$^-$ cells, the ratio of IL-4 to IFNγ decreases with maturity from stage 2 to stage 4 (REFS 10,12). Several other cytokines, chemokines and cytotoxicity molecules also appear to be differentially regulated as NKT cells mature, with some cytokines (such as IL-10, IL-13 and IL-21) expressed at higher levels by NK1.1$^-$ NKT cells, whereas others (such as some chemokines, chemokine receptors and cytotoxicity effector molecules including perforin, granzyme B and FAS ligand) are expressed at higher levels by NK1.1$^+$ NKT cells[53]. The significance of cytokine production by thymic NKT cells (immature and mature) is currently unclear, because there is no evidence that these cells normally become activated, and it remains to be determined whether they have a distinct physiological function.

NKT-cell development defined by CD4 and CD8 expression. In mice, CD1d-tetramer$^+$ NKT cells are either double negative (DN; CD4$^-$CD8$^-$) or CD4$^+$ NKT cells, whereas in humans CD4$^+$, DN and CD8$^+$ NKT-cell subsets are all present. The significance of the ongoing CD4 expression that is observed for many NKT cells is ambiguous as their TCR is not MHC class II restricted, and there is no evidence of any interaction between CD4 expressed by NKT cells and MHC class II molecules on antigen-presenting cells. A recent report demonstrated that CD4 can directly interact with CD1d and that this ligation of CD4 on NKT cells enhances NKT-cell activation[54]. However, NKT cells develop normally in the absence of CD4 (and MHC class II)[55], so the biological significance of CD4 expression by NKT cells remains unclear, as does the presence of a CD8$^+$ NKT-cell subset in humans.

Although CD4 and CD8 molecules have long been used to map the developmental pathway of conventional T cells, the pattern of these markers during NKT-cell development has been more difficult to determine. It is clear that the NKT-cell lineage in mice branches from the conventional pathway at the CD4$^+$CD8$^+$ DP thymocyte stage[13,14,46], but finding a very small number of NKT-cell precursors within the enormous number of 'mainstream' DP thymocytes has been challenging. Surprisingly, even the earliest NKT cells detected during ontogeny (stage 1 CD24$^+$ cells at day 3 after birth) were nearly all CD4$^+$CD8$^-$ rather than DP, which is consistent with an earlier study[6]. Some DP NKT cells were transiently observed within the CD24$^+$ population at day 14 after birth, but surprisingly, not at earlier time points[11]. The appearance of DP CD24$^+$ NKT cells later than CD4$^+$CD8$^-$CD24$^+$ NKT cells makes their place in the developmental progression somewhat uncertain. Logic would suggest that the earliest NKT cells are probably DP but that this phenotype is too transient and/or the cells too rare to be reliably detected with CD1d

T helper 1/ T helper 2/ T helper 0 cells ($T_H1/T_H2/T_H0$ cells). A classification of CD4$^+$ T cells on the basis of the patterns of cytokines that they secrete. T_H1 cells secrete interferon-γ (IFNγ) and associated pro-inflammatory cytokines that promote cell-mediated immunity. T_H2 cells secrete interleukin-4 (IL-4) and associated cytokines that promote humoral immunity. T_H0 cells have a 'hybrid' phenotype, being able to secrete both IFNγ and IL-4.

tetramers. Therefore, the current state of knowledge suggests that, once selected from the conventional DP thymocyte pool, NKT cells rapidly downregulate expression of the CD8 co-receptor but maintain CD4 expression, giving only a transient (at best) subset of DP NKT cells. Although it is technically very challenging to isolate NKT cells in early neonates, the question of whether the earliest detectable NKT cells are DP remains uncertain and is worthy of further investigation.

Divergence of CD4⁺ and CD4⁻ NKT-cell subsets. Although the role of CD4 is not known, mature CD4⁺ and CD4⁻ NKT cells seem to be functionally distinct subsets. Human CD4⁺ NKT cells produce a T_H0-type cytokine profile (IL-4 and IFNγ), whereas CD4⁻ NKT cells have a T_H1-type cytokine production bias (IFNγ)[56,57]. This distinction in cytokine production has not been reported in mice, however, CD4⁻ NKT cells from the liver clearly provide more potent protection against tumours than

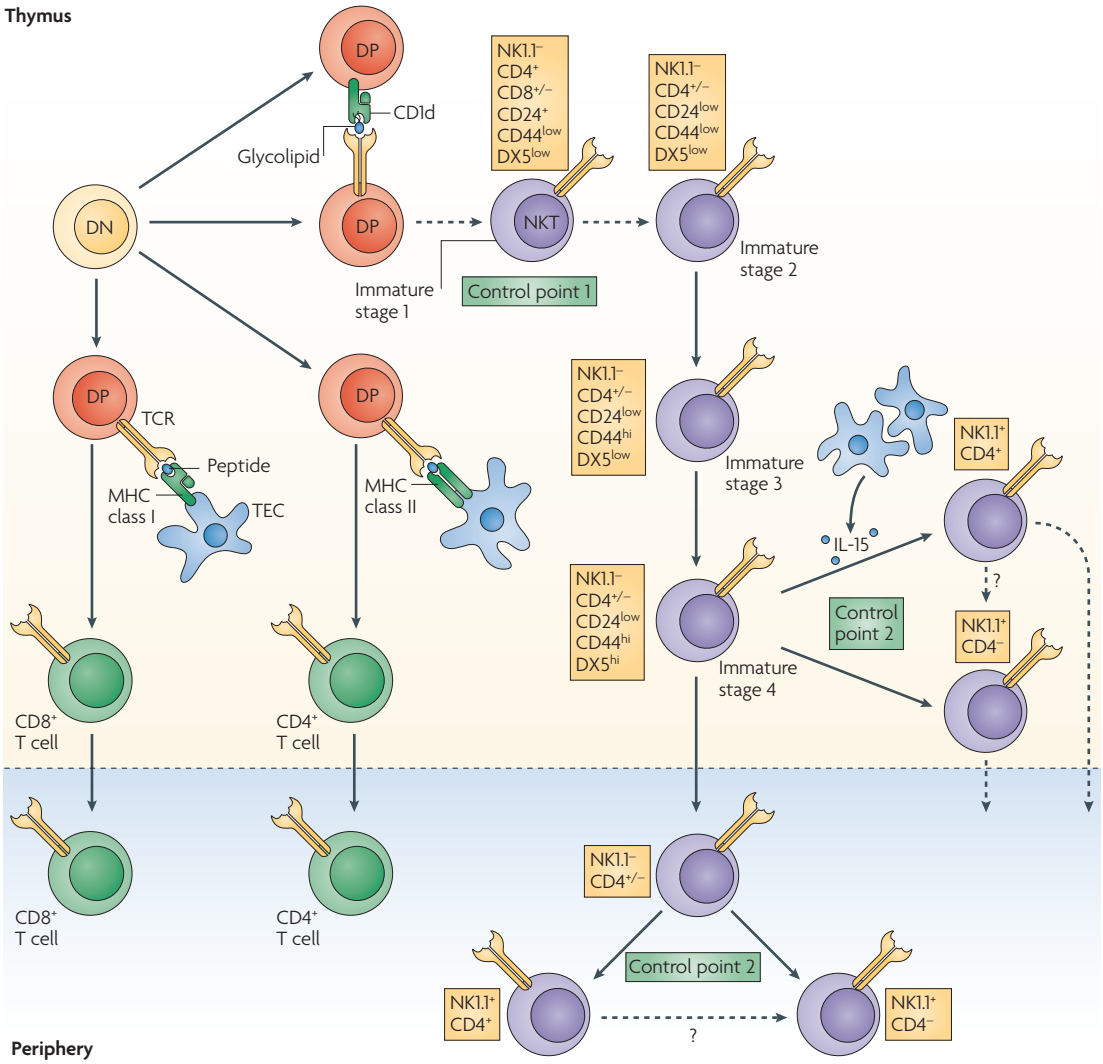

Figure 3 | **NKT-cell development.** Natural killer T (NKT) cells (depicted in purple) arise in the thymus from a common precursor pool of CD4⁺CD8⁺ double positive (DP) thymocytes that have undergone random T-cell receptor (TCR) gene rearrangement and expression. Expression of a TCR that binds with appropriate avidity to self-peptide–MHC class II or I molecules on thymic epithelial cells (TECs) leads to the positive selection of conventional CD4⁺ or CD8⁺ T cells, respectively. Thymocytes that express a TCR that interacts with CD1d bound to self glycolipid, expressed by other DP thymocytes, enter the NKT-cell lineage. Once selected, NKT-cell precursors undergo a series of differentiation steps that ultimately results in the NKT-cell pool. We have termed the initial selection event as control point 1. Immature NKT cells in the thymus are NK1.1 negative, and at least four distinct immature stages have been defined through differential expression of CD24, CD44 and DX5. 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. We have termed this maturation step control point 2. Both control points can take place in the thymus, but surprisingly, most NKT cells that emigrate from the thymus do so as immature cells and undergo control point 2 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⁻ subsets. Although they are functionally distinct, the developmental relationship between these subsets is not fully understood. DN, double negative; IL-15, interleukin-15.

Table 1 | **A comparison of mouse and human natural killer T cells**

Feature	Mouse	Human	References
TCRα	Vα14-Jα18	Vα24-Jα18*	1,16,43,44
TCRβ	Vβ8.2, Vβ7, Vβ2	Vβ11	1,16,43,44
Thymus dependent	Yes	Probably, but difficult to test	1,6–8
CD1d dependent	Yes	Yes	1,20,104
α-GalCer reactive	Yes	Yes	105
iGb3 reactive	Yes	Yes	27–33
Selected by iGb3	Controversial	Controversial	27–29,34–38
Maturation step (control point 2)	Yes	Yes	6,10,12,61–63
Immature phenotype	NK1.1 (CD161)^{low}; CD4$^+$ (or DN?)	CD161^{low}; CD4$^+$	6,10,12,61–63
Mature phenotype	NK1.1 (CD161)$^+$; CD4$^+$ or DN	CD161$^+$; CD4$^+$, DN or CD8$^+$	6,10,12,61–63
Frequency in thymus	~0.2–0.5%	~0.001–0.01%	62,63,106–108
Frequency in blood	~0.2–0.5%	~0.001–1%	109,110
Frequency in spleen	~1%	ND	106–108
Frequency in liver	~20–40%	~1%	106–108

*There are some rare examples of other Vα chains paired with Jα18 (REF. 41). α-GalCer, α-galactosylceramide; DN, double negative; iGb3, isoglobotrihexosylceramide; ND, not determined; TCRα, T-cell receptor α-chain.

CD4$^+$ NKT cells[58]. The developmental relationship between NKT-cell subsets defined on the basis of CD4 and CD8 expression is also poorly understood, although CD4$^-$ NKT cells do appear to develop later than CD4$^+$ NKT cells[6,11,14]. This suggests that CD4$^-$ NKT cells are derived from CD4$^+$ NKT cells but little is known about the precise timing of this branch-point because CD4$^-$ NKT cells can be found with both an immature (NK1.1$^-$) and mature (NK1.1$^+$) phenotype[6,11], although in adult mice, most DN NKT cells are mature.

Long-term intrathymic NKT-cell residents. A puzzling issue associated with NKT-cell maturation relates to the fact that NKT cells mainly leave the thymus at the NK1.1$^-$ stage[6,10]. Given this, why are most NKT cells in the thymus NK1.1$^+$, and what is their fate? Recent findings have shown that most of these cells can reside in the mouse thymus for months[59]. The significance of these intrathymic long-term residents is unknown. A general examination of conventional thymocyte subsets in *Cd1d*-knockout mice (which lack NKT cells) showed no abnormalities in thymocyte number, in subsets defined by CD4 and CD8 expression, or in the negative selection of defined superantigen-specific thymocyte subsets[59]. It is possible, however, that the role of mature intrathymic NKT cells might be more subtle. For example, a recent report suggested that thymic NKT cells are required for the intrathymic deletion of alloreactive T cells in a donor-cell chimerism transplantation model[60]. Although this is not definitive evidence, the possibility that NKT cells might be important for the selection and/or generation of other T-cell subsets is intriguing and worthy of further investigation. Even with the retention of many NK1.1$^+$ NKT cells in the thymus, it remains unclear why mainly immature NKT cells are selected for export to the periphery when so many thymic NKT cells are fully mature.

NKT-cell developmental pathway in humans. NKT cells also develop in the human thymus, although their frequency is much lower than in mice (TABLE 1). This has made the study of human NKT-cell development more difficult, with one study suggesting that they were present in the human embryonic thymus but, at best, were very rare in the neonatal thymus[61]. Although the frequency of thymic NKT cells is probably highest during embryogenesis, two studies[62,63] have succeeded in partially characterizing NKT-cell development from postnatal thymi and have identified some interesting parallels with mice. First, it is important to note that mature human NKT-cell subsets from blood include CD4$^+$, DN and CD8$^+$ subsets that can be further divided on the basis of their expression of CD161 (which is equivalent to the NK1.1 maturation marker in mice). Highlighting the similarities with mouse NKT-cell development, immature human thymic NKT cells are CD4$^+$CD161^{low} (phenotypically similar to immature mouse NKT cells)[62,63] and appear to migrate from the thymus at this immature CD161^{low} stage. However, in contrast to mice, only a minority of human thymic NKT cells are mature (CD161^{hi}), which suggests that, in humans, control point 2 mainly occurs in the periphery. Additionally, CD4$^-$ NKT cells (including DN and CD8$^+$ cells) are relatively infrequent in the human thymus, cord blood and neonatal peripheral blood, yet they accumulate in the blood with age[62,63]. It is unclear whether the CD4$^-$ NKT-cell subsets are directly derived from the CD4$^+$ NKT cells exported from the thymus, or whether there is a disproportionate peripheral expansion of peripheral CD4$^-$ NKT cells that results in them becoming a major subset in adults.

The existence of CD8$^+$ NKT cells (typically expressing CD8αα homodimers)[56,64,65] adds another potential step to human NKT-cell development. Interestingly, forced (transgenic) expression of CD8α by all T cells abrogates NKT-cell development in mice, which prompted the

Superantigen
A microbial protein that binds to MHC class II molecules, as well as a particular set of T-cell receptor Vβ chains, thereby leading to widespread TCR Vβ-dependent, but antigen-specificity-independent, T-cell activation.

suggestion that downregulation of CD8 expression was necessary to avoid negative selection[55]. As for CD4 expression, the purpose of CD8 expression by human NKT cells is unknown, and there is no evidence that CD8 binds to CD1d. Studies directly comparing human CD8$^+$ NKT cells to other NKT-cell subsets are rare, but there is some evidence that CD8$^+$ cells are functionally distinct[66–68], which suggests that it is important to separately examine CD4$^+$, DN and CD8$^+$ NKT cells for functional studies, where possible.

Factors that regulate NKT-cell development

The fact that NKT cells are developmentally and functionally distinct from conventional T cells suggests that they are regulated by different signalling pathways. In line with this, there are many examples whereby the absence of expression of particular molecules differentially affects NKT-cell and conventional T-cell development at different stages. These are summarized in TABLE 2, and the most well-studied factors are listed below.

CD1d. There is no doubt that the interaction with CD1d is essential for NKT-cell positive selection (control point 1) in the thymus. However, the complete deficiency of NKT cells in *Cd1d*-knockout mice made it more difficult to assess whether this molecule had any further role in NKT-cell maturation (control point 2). Targeted expression of CD1d to cortical thymocytes allowed selection of functionally competent NKT cells that migrate to the periphery in near-normal numbers, but less of these cells expressed the NK1.1 maturation marker[23], a finding that was also observed when CD1d was targeted to cortical thymocytes and peripheral T cells[69]. These reports suggest that control point 2 is also dependent on the expression of CD1d, possibly requiring CD1d expression by cells other than T cells. A recent study directly investigated the impact of CD1d on control point 2 by transferring immature NK1.1$^-$ NKT cells (isolated from wild-type mice) into CD1d-deficient mice[70]. The result was incomplete NKT-cell maturation, both within and outside the thymus, with most NKT cells remaining NK1.1 negative. Together, these studies indicate that CD1d is not only required for initial NKT-cell selection events (control point 1) but is also required for subsequent NKT-cell maturation (control point 2), which suggests that ongoing TCR ligation with CD1d is a part of this process. It is not known whether specific glycolipid antigens are also involved in this step.

IL-15. NKT cells upregulate the expression of the IL-2/IL-15 receptor β-chain (CD122) as they progress from the NK1.1$^-$ to the NK1.1$^+$ stage (control point 2) in the thymus[71], indicating an important role for one or both of these cytokines in NKT-cell maturation. It is now clear that IL-15, but not IL-2, is required for progression through control point 2 (REF. 71). This builds on earlier studies showing that mice deficient in either CD122 or IL-15 are deficient in NK1.1$^+$ T cells[72,73]. IL-15 also seems to be crucial for normal NKT-cell turnover and homeostasis, as discussed later[71,74]. A lack of IL-15-induced signalling may also be responsible for the NKT-cell deficiencies observed in some other mutant mice, including IFN-regulatory factor 1 (*Irf1*)-knockout mice, which have impaired IL-15 production and lack NK1.1$^+$ NKT cells[75]. The transcription factor T-bet is another key factor in the NK1.1$^-$ to NK1.1$^+$ maturation step[76] that may also be linked to IL-15 because T-bet promotes the upregulation of CD122 expression as NKT cells mature. It is important to recognize, however, that T-bet may also modulate the expression of other molecules at this stage[77], and so may have a more complex role.

GM-CSF. The influence of granulocyte/macrophage colony-stimulating factor (GM-CSF) on the development of NKT cells has been controversial. An early report suggested that GM-CSF-deficient mice had reduced NKT-cell numbers[4]. However, a more recent study[78] examined NKT cells in these mice and showed that although GM-CSF was not required for the establishment of normal NKT-cell numbers in the thymus or the periphery, it does regulate the ability of NKT cells to secrete cytokines. Cytokines were present in the cytoplasm of NKT cells from GM-CSF-deficient mice, but cytokine secretion could only be restored by addition of exogenous GM-CSF while NKT cells were developing. Because secretion could not be restored in mature peripheral NKT cells, this suggested that GM-CSF regulates a developmental checkpoint rather than cytokine secretion itself. This is an unanticipated role for GM-CSF and an unexpected developmental constraint — to our knowledge, there are no similar examples of this type of developmental defect in the published literature. Further investigation of NKT cells from GM-CSF-deficient mice should provide important new insights into how NKT cells are licensed to become potent cytokine secretors.

Lymphotoxin. The number of peripheral NKT cells is reduced in lymphotoxin (LT)-deficient mice, but there is no consensus on the specific roles of LTα homotrimers and $LT\alpha_1\beta_2$ heterotrimers during NKT-cell development. A recent study[79] reported that peripheral, but not thymic, NKT-cell numbers were reduced in LTβ-deficient and in LTβ receptor (LTβR)-deficient mice. This defect appeared to reflect the role of LTβR-expressing stromal cells in the regulation of the export of NKT cells from the thymus. The implication that there was no direct effect on intrathymic NKT-cell differentiation is at odds with an earlier study that reported a reduced number of NKT cells in the thymus (and periphery) of LTβ-deficient mice[80]. Interestingly, both studies found that LTα-deficient mice have reduced NKT-cell numbers in the thymus and periphery[79,80], which suggests that LTα homotrimers are important for intrathymic NKT-cell development. A possible caveat to this interpretation is that mice that lack either of the tumour-necrosis factor receptors (TNFRI and TNFRII), which act as receptors for LTα homotrimers, reportedly have normal NKT-cell development[81]. However, mice that lack both TNFRI and TNFRII have not been studied, which means that normal thymic NKT-cell numbers in mice that lack just one of these receptors might simply be due to receptor redundancy, or even to the activity of a third TNF

Table 2 | **Factors influencing stages in natural-killer-T-cell development**

Factor	Stage	Comment	Refs
Antigen processing and presentation			
CD1d	CP1, CP2	Responsible for glycolipid presentation to developing NKT cells	1,23,70
TCR Jα18	CP1	Defining element of the invariant NKT-cell TCRα	1,111
Saposins	CP1	Required for CD1d loading and unloading in lysosomes	103,112
NPC1	CP1	Unclear role in NKT-cell development, but potentially involved in endosomal–lysosomal lipid transport	113
NPC2	CP1	Possibly involved in lysosomal CD1d loading, but this is controversial	35,102
MTP	CP1	Thought to be involved in CD1d loading in the ER, but possibly also in lysosomes	100,101
AP3	CP1	Involved in CD1d trafficking to lysosome	114,115
HEXB	CP1	Required for iGb3 production in lysosomes, mutations in *Hexb* are associated with Sandhoff disease	27,34,36
CTSL	CP1	Possibly required for processing of prosaposin into saposins	25,116
Cytokines			
IL-15	CP2	Involved in NKT-cell maturation and homeostasis	71–74
LTα	Unclear	Controversial; may affect CP1 through TNF-family receptors	79–82
LTβR	Unclear	Unclear; may affect thymic emigration	79–82
GM-CSF	CS	Involved in licensing NKT cells to secrete cytokines, but not required for cytokine protein production	78
γ_c	CP2	Main effect on NKT cells is possibly through its role as a component of IL-15R	117
Signal transduction			
FYN	CP1	Associates with the TCR and SAP/SLAM-family members	46,87,88,98
SAP	CP1	Required for signalling through SLAM; probably signals via FYN, PKCθ and NF-κB1	89–93
SLAM	CP1	Signals through SAP; possibly partly responsible for NKT-cell defect in NOD mice	96
NF-κB1	CP1, CP2	Part of the classical NF-κB pathway, downstream of the TCR and SLAM–SAP–FYN pathways	52,85,86
IκBα	CP1, CP2	Dominant-negative IκBα resists degradation and therefore prevents NF-κB1 activation; two different mouse lines were characterized, one affected CP1, the other affected CP2	52,84,85
NF-κB2	CP1?	Part of the alternative NF-κB pathway; influences NKT-cell development extrinsically via thymic stromal cells	85
RelB	CP1	Part of the alternative NF-κB pathway; influences NKT-cell development extrinsically via thymic stromal cells	83,85
NIK	CP1	Signalling element upstream of RelB, and affects NKT-cell development extrinsically; *Nik*-mutant mice (aly mice) are hypomorphs and do not cause complete NKT-cell deficiency	83
IKK2	CP1	Signalling element upstream of NF-κB1 in the classical NF-κB pathway	84
DOK2	CP1	Signalling element downstream of SAP and FYN, phosphorylates RASGAP; may inhibit TCR signalling via MAPKs	118
GATA3	Unclear	*Gata3*$^{-/-}$ mice have normal thymic NKT-cell numbers, but CD4$^+$ NKT cells are selectively reduced; defect in peripheral NKT-cell survival	119
PKCθ	CP1? CP2?	Downstream of the TCR, and upstream of the classical NF-κB pathway; also downstream of SAP and FYN; *Pkcq*$^{-/-}$ mice have fewer thymic NKT cells but normal number of peripheral NKT cells	84,86
BCL-10	Unclear	Signalling element downstream of the TCR and upstream of the classical NF-κB pathway; *Bcl10*$^{-/-}$ mice have normal thymic NKT-cell numbers but fewer in the periphery	84
AP1	CP2	In *Batf*-transgenic mice, AP1 complex in inhibited, revealing a role for AP1 in NKT-cell maturation	120
T-bet	CP2	Regulates the transition through CP2, involving several phenotypic changes, including IL-15R upregulation	76,77
ETS1	CP2?	May regulate T-bet expression, but effect on CP1 versus CP2 not directly tested	121
MEF	CP2?	Affects both NK- and NKT-cell development, but only NK1.1$^+$ T cells were examined	122
RUNX1	CP1	Essential for NKT-cell selection or subsequent expansion	14
RORγ	CP1	Promotes DP cell survival and continued TCRα gene rearrangement; prevents Vα14-Jα18 rearrangement	14
IRF1	CP2?	Probably influences NKT-cell development through IL-15	75
ITK	CP2	*Itk*$^{-/-}$ mice have a partial block during NKT-cell maturation	12
Others			
CD137	Unclear	*CD137*$^{-/-}$ mice have fewer NK and NKT cells and with reduced function, but only NK1.1$^+$ or DX5$^+$ T cells examined	123
XIAP	Unclear	XLP that is not caused by mutations in *SAP*, yet human XIAP patients still exhibit severe NKT-cell deficiency; mechanism is unclear; XIAP mice have normal NKT-cell development	124

aly, alymphoplasia; AP1, activator protein 1; AP3, adaptor protein 3; BATF, basic leucine zipper transcription factor; BCL-10, B-cell lymphoma 10; γ_c, common cytokine-receptor γ-chain; CP, control point; CS, cytokine secretion; CTSL, cathepsin L; DOK2, docking protein 2; DP, double positive; ER, endoplasmic reticulum; GATA3, GATA-binding protein 3; GM-CSF, granulocyte/macrophage colony-stimulating factor; HEXB, β-hexosaminidase B; IκBα, inhibitor of NF-κBα; iGB3, isoglobotrihexosylceramide; IKK2, IκB kinase 2; IL-15, interleukin-15; IL-15R, IL-15 receptor; IRF1, interferon-regulatory factor 1; ITK, IL-2-inducible T-cell kinase; LTα, lymphotoxin-α; LTβR, LTβ receptor; MAPK, mitogen-activated protein kinase; MEF, myeloid ELF1 (E74-like factor 1)-like factor; MTP, microsomal triglyceride transfer protein; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; NKT, natural killer T; NOD, non-obese diabetic; NPC1, Niemann-pick type C1 protein; PKCθ, protein kinase Cθ; RASGAP, RAS GTPase-activating protein; RORγ, retinoic-acid-receptor-related orphan receptor-γ; RUNX1, runt-related transcription factor 1; SAP, SLAM-associated protein; SLAM, signalling lymphocytic activation molecule; TCR, T-cell receptor; TNF, tumour-necrosis factor; XIAP, X-linked inhibitor-of-apoptosis protein; XLP, X-linked lymphoproliferative syndrome.

family receptor, known as herpesvirus-entry mediator (HVEM), with which LTα homotrimers can potentially interact. Adding to the uncertainty surrounding the role of LT, another study reported (although the data were not shown) normal numbers of NK1.1$^+$αβTCR$^+$ cells in the thymus of *Lta*$^{-/-}$*Tnf*$^{-/-}$ mice (which lack LTα and LTβ signalling)[82], but they did not investigate NKT cells in the periphery and did not use CD1d tetramers to definitively identify NKT cells.

NF-κB-family members. Despite being dispensable for conventional T-cell differentiation, several studies have shown that members of the nuclear factor-κB (NF-κB) family have key roles in thymic NKT-cell development[52,83–86]. Interestingly, the importance of NF-κB appears to be both NKT-cell intrinsic (via the 'classical' NF-κB pathway) and extrinsic (via the 'alternative' NF-κB pathway, in thymic stromal cells). The effect on NKT-cell development of mutations in the molecules of the classical NF-κB pathway varies according to which components are targeted. For example, mice with a T-cell-targeted deletion of IKK2 (inhibitor of NF-κB kinase 2)[84], and mice expressing an IκBα (inhibitor of NF-κBα) dominant-negative transgene[52], had almost no NKT cells (~20-fold reduction), which implies a fundamental defect at a very early stage (control point 1). However, NF-κB1-deficient mice[52,85], and a different IκBα dominant-negative transgenic mouse line[85], had a less severe deficiency (fourfold to fivefold reduction) that mainly affected mature NK1.1$^+$ NKT cells, which implies a defect at control point 2.

Defects in the alternative NF-κB pathway also inhibit NKT-cell development, and again, the effect of mutations in the molecules involved in this pathway varies according to which components are targeted. Mice with a RelB deficiency in stromal cells had severely reduced NK1.1$^-$ and NK1.1$^+$ NKT-cell numbers (~20-fold reduction)[83,85], corresponding with a block at control point 1. However, mice with an NF-κB2 deficiency, and alymphoplasia (aly) mice (which have a mutation in NF-κB-inducing kinase (*Nik*)), have a less severe reduction in NKT-cell numbers (~twofold to fourfold reduction). The observation that the residual NKT cells in aly mice had a normal ratio of mature (NK1.1$^+$) to immature (NK1.1$^-$) NKT cells prompted the authors to suggest that the mutation affected a late stage in NKT-cell development[83]. Considering the low numbers of immature and mature NKT cells in aly mice, an alternative explanation might be that, similar to RelB, the aly mutation (which is known to be leaky) inhibits control point 1, but some NKT cells escape and continue to differentiate. There is some evidence that reduced IL-15 production by thymic stromal cells in RelB-deficient mice may be the basis for the NKT-cell deficiency[85], but this seems unlikely to be the only contributing factor because, although IL-15 is important for control point 2, it does not appear to regulate control point 1 (REF. 71).

The SLAM–SAP–FYN pathway. A possible role for TCR signalling leading to NF-κB activation during NKT-cell development is consistent with earlier observations that NKT-cell development is also selectively inhibited in mice lacking the SRC-family kinase FYN[46,87,88] and protein kinase Cθ (PKCθ), which are both potential intermediates in the TCR–NF-κB pathway[84,86]. However, the fact that mutations in the genes encoding other intermediates in the TCR–NF-κB pathway (BCL-10, CARMA1, MALT1) did not inhibit intrathymic NKT-cell development prompted speculation that FYN, PKCθ and NF-κB may regulate NKT-cell development through a TCR-independent pathway[84]. A major breakthrough came in 2005, when three studies showed that NKT-cell development is completely abrogated in mice lacking signalling lymphocytic activation molecule (SLAM)-associated protein (SAP)[89–91] and furthermore, that NKT-cell numbers are greatly diminished in individuals suffering from X-linked lymphoproliferative syndrome (XLP) caused by mutations in *SAP*. These studies, suggested that XLP might have an NKT-cell component, but also importantly, they shed light on the intracellular pathways that control NKT-cell development. As its name suggests, SAP binds to the SLAM family of receptors (including SLAM, 2B4, CD84, Ly9 and NTB-A) and, when SLAM-family molecules are engaged at the cell surface, SAP recruits FYN (reviewed in REFS 92,93). The activated SLAM–SAP–FYN complex initiates at least two signalling cascades (FIG. 4), one that links to NF-κB activation through PKCθ and the other that leads to dampening of TCR signalling through the inhibition of mitogen-activated protein kinase (MAPK). The suppressive pathway may allow NKT-cell positive selection, rather than negative selection, upon recognition of agonist ligands such as iGb3, whereas the SLAM–SAP–FYN–NF-κB pathway appears to provide unique signals that drive precursors towards the NKT-cell lineage[92].

The relative contribution of signalling from different SAP-binding SLAM receptors remains to be determined. So far, only Ly9-deficient mice[94] have been carefully examined for NKT-cell development and these showed no obvious deficiency. Whether NKT-cell development is normal in SLAM-deficient mice is unclear. These mice are capable of rapid IL-4 production in response to CD3-specific antibody *in vivo*[95], which is a hallmark of NKT-cell activity, but more direct studies of NKT cells in these mice are necessary. Intriguingly, a recent report suggested that the *Slam* locus is responsible for impaired NKT-cell development in non-obese diabetic mice (NOD mice)[96,97], and showed that DP thymocytes from NOD mice have markedly lower levels of SLAM expression compared with C57BL/6 mice, which suggests an important role for SLAM in NKT-cell development. Regardless of which SLAM-family surface molecules are involved, there is clearly a key role for the SAP–FYN pathway at a very early stage in NKT-cell development. Interestingly, one report showed that expression of a Vα14-Jα18 TCR transgene could restore NKT-cell numbers in FYN-deficient mice, suggesting that FYN regulates NKT-cell development at a stage prior to NKT-cell selection[98]. This evidence suggested that NKT cells develop from distinct precursors rather than through a selective event (control point 1) after random TCR rearrangement and expression at the

Nuclear factor-κB
(NF-κB). A family of transcription factors (including NF-κB1 (p50), NF-κB2 (p52), cRel, RelA and RelB) that regulate a range of cellular processes, including cell survival, proliferation, differentiation and cytokine production.

Alymphoplasia
(aly). A mouse phenotype that is characterized by the absence of lymph nodes and Peyer's patches. It is caused by a spontaneous mutation in the gene that encodes nuclear-factor-κB-inducing kinase (NIK).

X-linked lymphoproliferative syndrome
(XLP). Individuals with XLP have complicated immune dysfunctions, often triggered by infection with Epstein–Barr virus. Many patients develop fatal B-cell lymphoproliferation. The gene that encodes SLAM-associated protein (SAP) is mutated in these patients.

Non-obese diabetic mice
(NOD mice). A strain of mice that normally develops idiopathic autoimmune diabetes that very closely resembles type 1 diabetes in humans. These mice have a developmental and functional deficiency in the NKT-cell compartment.

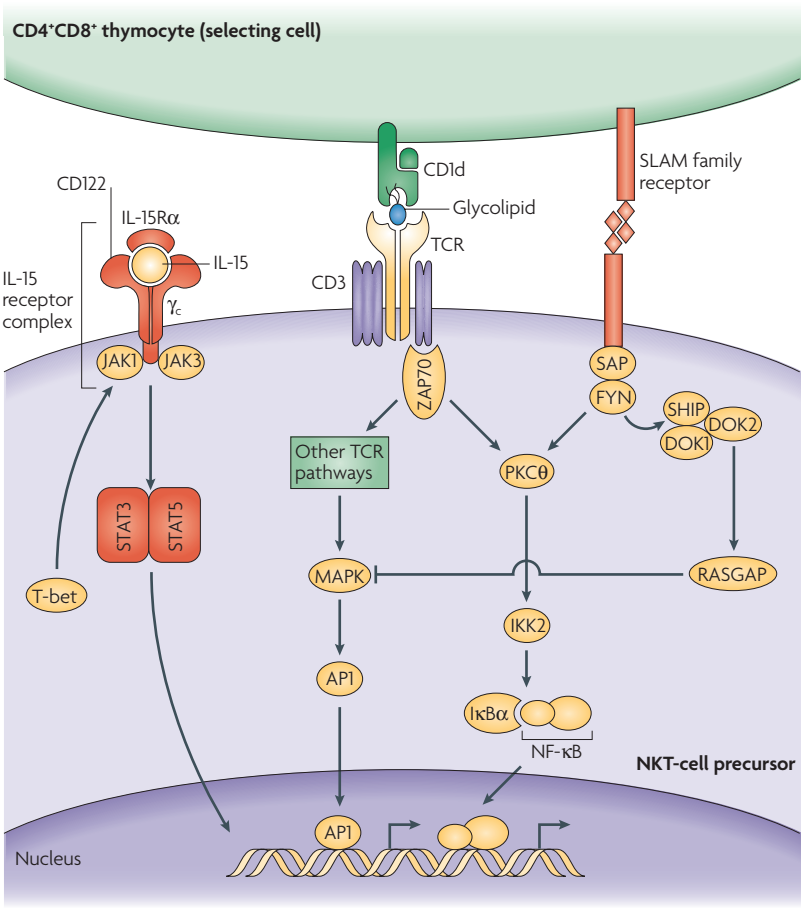

Figure 4 | **Intracellular signalling pathways that regulate NKT-cell development.** Natural killer T (NKT)-cell selection and maturation requires a range of signalling events that are not essential for conventional T-cell development, in addition to factors that are common to both T-cell and NKT-cell development. The unique signalling requirements of NKT cells are not fully understood, but the three main intrinsic pathways that link most of the known mutants that affect NKT-cell development are depicted. These include the SLAM–SAP–FYN pathway, the T-cell receptor (TCR)-signalling cascade (particularly the classical nuclear factor-κB (NF-κB) pathway) and the interleukin-15 (IL-15) pathway. AP1, activating protein 1; γ_c, common cytokine-receptor γ-chain; DOK, docking protein; IκBα, inhibitor of NF-κBα; IKK2, IκB kinase 2; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PKCθ, protein kinase Cθ; RASGAP, RAS GTPase-activating protein; SAP, SLAM-associated protein; SHIP, SRC-homology-2-domain-containing inositol-5-phosphatase; SLAM, signalling lymphocytic activation molecule; STAT, signal transducer and activator of transcription; ZAP70, ζ-chain associated protein kinase of 70kDa.

DP stage. Although this seems to be contrary to the well-established 'selective' model of NKT-cell development, it has been suggested that the results may be complicated by altered (transgenic) TCR expression, combined with possible leakiness in the *Fyn*-knockout model[25]. The most probable role for the SLAM–SAP–FYN complex may be during the selection process itself (control point 1), in which interactions between SLAM-family members expressed by DP thymocytes and NKT-cell precursors could initiate the unique differentiation pathway that leads to development of the NKT-cell lineage. Although the detailed steps in these signalling pathways remain to be fully explored, this model would bring together a seemingly diverse range of mutations in molecules that specifically affect NKT-cell development[25,92].

Homeostasis of the NKT-cell pool

In broad terms, the regulation of peripheral numbers of NKT cells seems to be similar to that of memory T cells. Both of these subsets exhibit a basal level of slow proliferation (0–2 divisions per week) that is dependent on IL-15 and to a lesser extent on IL-7, with little or no requirement for 'tickling' (low-avidity stimulation) of the TCR by the selecting ligands. NKT cells from human peripheral blood also turn over slowly in response to IL-15 (REF. 62), although there is a bias towards the accumulation of $CD4^-$ NKT cells that is not seen in mice[62,63]. Whether the long-term survival of mature NKT cells also relies on IL-15 has not been directly tested. It is clear, however, that mature NKT cells can survive for many weeks in the absence of CD1d[70], which suggests that although TCR–CD1d interactions are crucial for the development and activation of NKT cells, they are less important for homeostatic regulation.

Only one study has directly addressed whether NKT cells occupy an independently regulated niche. Matsuda *et al.*[71] adoptively transferred NKT cells together with conventional $CD4^+$ T cells into mice that were broadly immunodeficient (RAG-deficient mice) or deficient only for NKT cells (Jα18-deficient mice). The $CD4^+$ T cells and, to a lesser extent, NKT cells both divided more rapidly in RAG-deficient recipients than in wild-type mice, but neither showed increased proliferation in Jα18-deficient recipients. The increased proliferative response by NKT cells in RAG-deficient but not Jα18-deficient mice suggests that they are not regulated independently of conventional T cells, but the possibility of an NKT-cell niche cannot be fully ruled out. Whereas the study by Matsuda *et al.*[71] was the most rigorous examination of this issue to date, large numbers of NKT cells were transferred ('near-normal' numbers could be achieved after only 3–4 divisions) for relatively short periods of time (1 or 2 weeks). It is possible that homeostatic pressures may only become evident when smaller numbers of NKT cells are transferred and examined over a longer time course.

The most important challenges facing the field?

The past 5 years has seen dramatic progress in our understanding of NKT-cell development. This has provided a robust framework on which to build as we investigate the specific factors that regulate development of individual NKT-cell stages and subsets, and this should ultimately give us the ability to manipulate the process of NKT-cell development. Nevertheless, many important questions remain.

What are the endogenous CD1d-restricted glycolipid antigens that are responsible for NKT-cell selection (and self recognition) in mice and humans? The extent to which iGb3 fulfils this role is controversial, and an increasing number of studies suggest that other antigens are likely to be involved. Is there a level of redundancy in NKT-cell selecting antigens? Do these antigens shape the limited TCRβ diversity within the NKT-cell compartment, and do different TCRβs facilitate recognition of different self antigens? Are the antigens involved in NKT-cell selection

in the thymus similar to the self antigens that drive NKT-cell activation in the periphery?

What is the developmental and functional significance of the different NKT-cell subsets? It is increasingly clear that there are many different subsets of NKT cells in mice and humans, yet at this point in time, few have been well characterized. To fully understand the complexity of the NKT-cell pool, there is a pressing need to investigate the developmental and functional significance of NKT cells that differ in cell-surface phenotype and/or location.

Finally, what underlies the enormous variability in NKT-cell levels within the human population? Given that NKT-cell numbers have a direct effect on various disease states, one of the most important challenges we face in addressing the problem is to understand why these numbers are so variable. The relative contribution of genes versus environment is not clear, which means that studies of families and defined social groups will probably be very informative. This is an important first step towards the manipulation of NKT-cell frequency as a future therapeutic benefit to patients.

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Competing interests statement
The authors declare no competing financial interests.

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