Interleukin-2-inducible T Cell Kinase (Itk) Network Edge Dependence for the Maturation of iNKT Cell*

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Innate natural killer T (iNKT) cells are a unique subset of innate T lymphocytes that are selected by CD1d. They have diverse immune regulatory functions via the rapid production of interferon-γ (IFN-γ) and interleukin-4 (IL-4). In the absence of signaling nodes Itk and Txk, Tec family non-receptor tyrosine kinases, mice exhibit a significant block in iNKT cell development. We now show here that although the Itk node is required for iNKT cell maturation, the kinase domain edge of Itk is not required for continued maturation iNKT cells in the thymus compared with Itk-null mice. This rescue is dependent on the expression of the Txk node. Furthermore, this kinase domain independent edge rescue correlates with the increased expression of the transcription factors T-bet, the IL-2/IL-15 receptor β chain CD122, and suppression of eomesodermin expression. By contrast, α-galactosyl ceramide induced cytokine secretion is dependent on the kinase domain edge of Itk. These findings indicate that the Itk node uses a kinase domain independent edge, a scaffolding function, in the signaling pathway leading to the maturation of iNKT cells. Furthermore, the findings indicate that phosphorylation of substrates by the Itk node is only partially required for maturation of iNKT cells, while functional activation of iNKT cells is dependent on the kinase domain/activity edge of Itk.

Natural killer T cells (NKT cells) are a unique population of innate-like T lymphocytes that play important functions in diverse immune responses. Invariant NKT (iNKT) cells are the dominant subset of NKT cells that express an invariant TCRα chain (Vα14-Jα18). When activated, iNKT cells rapidly produce large amounts of IL-4 and IFNγ, along with a number of other cytokines and chemokines. iNKT cells also regulate other immune cells, such as NK cells and B cells, thus playing multiple roles in immune responses. iNKT cells develop from the DP (CD4+CD8+ double-positive) thymocytes and are selected by the MHC class I-like molecule CD1d expressed on DP cells (1). After selection, the iNKT precursors (CD44lowNK1.1−) further develop through more mature CD44hiNK1.1−iNKT to finally mature CD44hiNK1.1+iNKT cells. A number of signaling molecules, transcription factors and cytokine receptors have been identified to be important for iNKT cell development through different stages. For example, SLAM, SLAM-associated protein (SAP), and Src-family kinase FYN and NFκB have been suggested to form a signaling pathway that controls iNKT cell development at a very early stage (2–11). The cytokine IL-15, the vitamin D receptor, PTEN, SLP76 and transcription factors T-bet and AP-1 are all involved in the final maturation of iNKT cells (12–18).

Itk and Txk are two Tec family non-receptor tyrosine kinases expressed in T cells. Itk-null mice show impaired Th2 cell development, as well as cytokine secretion. In addition, both Itk and Txk regulate the development of naive phenotype CD4+ and CD8+ T cells (19–22). Itk-null mice have also been shown to have reduced iNKT cell number, impaired maturation and cytokine secretion, which is exaggerated in Itk/Txk DKO mice (23, 24). Thus, signals through Itk and Txk nodes are important for T cell development and function.

Signal transduction pathways travel via nodes along the pathway. Each node has at least 2 edges, an input edge and an output edge (25). Multi-domain signaling proteins may contain more than 1 input or output edges (26). Itk has at least 2 input edges, coming from upstream Src family kinases and from PI3 kinase (27). At least 1 output edge has been identified for Itk, tyrosine phosphorylation of PLC-γ1 and subsequent calcium influx, leading to the activation of NFκB, NFAT, and Ras (27). Whether the Itk node has a single edge leading to PLC-γ1 activation, or whether it has multiple edges leading to other signaling pathways is not clear. There is evidence for other edges with which Itk can connect to downstream signaling pathways independent of the edge leading to PLC-γ1 phosphorylation. Itk can interact with the adaptor protein SLP-76 via its SH2 and SH3 domains (28–31). This interaction is not dependent on its kinase activity and could represent additional edges that emanate from the Itk signaling node. Indeed, kinase-deleted or kinase-inactive Itk have been shown to activate the transcription factor serum response factor or induce antigen-induced actin polarization in T cell lines (29, 32, 33). These data suggest that additional edges emanate from the Itk signaling node, independent of the edge leading to tyrosine phosphorylation of PLC-γ1. However, whether such edges are important in vivo is not clear.

We and others (29, 32) have shown that the Itk node has edges that can activate specific pathways in a kinase inde-
meabilization. BrdU was detected using APC-BrdU kit from BD Biosciences.

α-Galactosyl Ceramide Stimulation and Cytokine Secretion Assays—WT, Itk-null, and Tg(Lck-ItkΔKin)/ItkK390R mice were injected intraperitoneally with 2 μg of α-galactosylceramide. After 2 h, blood was collected and serum samples were obtained. IL-4 and IFN-γ level were analyzed using Bioplex (Millipore). For in vitro analysis of cytokine secretion, 96-well plates were preincubated with 10 μg/ml anti-CD3 and 10 μg/ml anti-CD28 at 4°C overnight. Sorted hepatic iNKT cells from WT, Itk-null, and Tg(Lck-ItkΔKin)/ItkK390R mice were stimulated at 2 × 10^6/well in duplicate for 3 days, and cytokines were analyzed using Bioplex.

Data Analysis—Statistical evaluation was conducted for all repetitions of each experiment using Student’s t test with a probability value, p ≤ 0.05, considered statistically significant.

RESULTS

The Kinase Domain Edge of Itk Is Required for Generating Normal Numbers of Thymic iNKT Cells—To determine if the kinase domain edge of Itk is required for the development of iNKT cells, we compared the iNKT population in WT, ItkK390R, Itk/Txk double knock-out (DKO) mice and mice carrying a mutant ITK lacking its kinase domain instead of WT Itk in Itk null or Itk/Txk (DKO) mice (Tg(Lck-ItkΔKin)/ItkK390R and Tg(Lck-ItkΔKin)/Itk/Txk DKO (22)). For simplicity, we refer to these as ItkK390R/ΔKin and DKO/ΔKin, respectively. The expression level of the transgene in iNKT cells is similar to endogenous Itk as determined by quantitative RT-PCR (data not shown). We found that the absence of Itk results in a reduction in the percent of iNKT cells in the thymus, and expression of the kinase-deleted mutant of Itk could rescue this percentage to WT levels (Fig. 1A). By contrast, while the percentage of thymic iNKT cells in WT, ItkK390R, and Itk/Txk DKO was similar to that seen in the ItkK390R mice (i.e. reduced compared with WT mice), this percentage was not rescued by expression of the kinase-deleted mutant (Fig. 1A).

The numbers of thymic iNKT cells were also significantly reduced in ItkK390R mice compared with WT mice as previously reported (Fig. 1B and Refs. 23, 24). Expression of the kinase domain-deleted mutant in ItkK390R mice did not rescue the numbers of thymic iNKT cells as there was no significant difference between ItkK390R/ΔKin and ItkK390R mice (Fig. 1B). Similarly, the numbers of thymic iNKT cells was significantly reduced in Itk/Txk DKO mice compared with ItkK390R mice (Fig. 1B), and these numbers were not rescued by expression of the kinase domain-deleted mutant (Fig. 1, A and B). Together, these results suggest that the generation of proper numbers of iNKT cells requires the kinase activity of Itk, and the expression of Txk.

Role of Kinase Domain Edge of Itk in iNKT Cell Maturation—Analyzing the three stages of iNKT cell maturation in the thymus, we found that the absence of Itk results in a significant accumulation of iNKT cells at stage 2 (Fig. 2A). By contrast, the percentage of thymic iNKT cells at stage 2 was reduced and the percentage at stage 3 was significantly increased in the ItkK390R/ΔKin transgenic mice compared with ItkK390R mice (Fig. 2A). This suggests that the kinase domain independent
Role of Itk Kinase in iNKT Cell Maturation

FIGURE 1. The kinase activity edge of Itk is required for generating WT numbers of iNKT cells in the thymus. A, flow cytometric analysis of iNKT cells in the thymus from WT, Itk+/−, Itk−/−/ΔKin, and DKO/ΔKin mice. Numbers indicate the percentage of iNKT cells. B, numbers of iNKT cells in the thymus of WT, Itk-null, Itk/Txk DKO, Itk−/−/ΔKin and DKO/ΔKin mice. Data are from four independent experiments. *, p < 0.05, compared with WT iNKT cells; †, p < 0.05, compared with Itk−/− iNKT cells.

edge of Itk is sufficient to allow maturation of iNKT cells through the immature stage 2 to the more mature stage 3. However, because the percentage of thymic stage 3 iNKT cells in the Itk−/−/ΔKin mice is still lower than in the WT mice, the kinase activity of Itk is required for the full maturation of iNKT cells through to stage 2. The percentage of stage 2 iNKT cells in Itk/Txk DKO mice was similar to that seen in the absence of Itk, and expression of the kinase domain-deleted mutant did not rescue the percentage, suggesting that Txk may be involved in the non-kinase domain edge of Itk in iNKT cell maturation (Fig. 2A).

There are two defined subsets of murine iNKT cells, CD4+ or CD4−CD8− (DN). Analysing these two populations, we found that about 50% of thymic iNKT cells in WT mice were DN, which was significantly decreased to around 20% (p < 0.05) in Itk−/− mice, demonstrating that Itk is important in iNKT subset development (Fig. 2B). However, analysis of iNKT cell subsets in Itk−/−/ΔKin mice revealed that the percentage of thymic DN iNKT cells was similar to that in Itk−/− mice, indicating that the kinase activity edge of Itk is important for the generation of these two subsets of iNKT (Fig. 2B). Because the kinase domain deletion mutant partly rescues the maturation of iNKT cells, this also suggests that the effect of Itk on the distribution of iNKT cell subsets may not be due to larger numbers of immature iNKT cells in these mice.

Thymic NK1.1+ iNKT cells have higher proliferative rates than NK1.1+ iNKT cells. Labeling with BrdU would therefore result in higher incorporation in the former population over the same time period. Compared with WT iNKT cells, a much higher percentage of Itk−/− iNKT cells labeled with BrdU (Fig. 2C), and as expected most of the BrdU+ iNKT cells in Itk−/− mice were NK1.1+, and the percentage of BrdU+ Itk−/−/ΔKin iNKT cells was lower than those from Itk−/− mice, consistent with the higher percentage of mature NK1.1+ iNKT cells in the Itk−/−/ΔKin mice (Fig. 2C). These data add further support for a role for Itk in iNKT development and maturation that is partly independent of its kinase activity.

We also examined whether expression of CD1d and the SLAM family receptors, SLAM and Ly 108, on DP thymocytes are affected by Itk. We found that these receptors were expressed at similar levels on DP thymocytes from WT, Itk−/−, and Itk−/−/ΔKin mice, indicating that differential expression of these molecules is not responsible for the reduced iNKT cell numbers in these mice (Fig. 2D).

Itk is suggested to form either intramolecular folded monomers or intermolecular dimer in the cells, which may maintain Itk in the inactive state (36–39). Deletion of the kinase domain may disrupt the conformation of Itk, allowing for easier interactions between Itk and other signaling proteins, and making that edge more active. To test this possibility, we generated mice carrying an Itk mutant, K390R, which is defective in kinase activity instead of the WT Itk (Tg(CD2-ItkK390R)/Itk−/−, Itk−/−/K390R for simplicity). This mutant of Itk has all domains intact, and instead carries a single point mutation in the kinase domain. We have shown that such a mutant likely folds in a similar fashion as the WT kinase (37). Analysis of these mice revealed that the numbers of thymic iNKT cells were similar to that found in Itk−/− and Itk−/−/ΔKin mice, and significantly lower than WT mice (Fig. 3A). More importantly, there was a significant increase in the percentage of more mature stage 3 iNKT cells in the Itk−/−/K390R mice, but no change in the altered CD4/DN ratio of iNKT in the thymus compared with Itk−/− mice (Fig. 3, B and C). These data are very similar to the data from the Itk−/−/ΔKin mice, further supporting the conclusion that maturation of iNKT cells is partly independent of Itk kinase activity edge.

Examination of the maturation status of peripheral iNKT cells reveals that expression of the Itk K390R mutant (as well as the ItkΔ kinase mutant, data not shown) was not able to rescue the defect in maturation or numbers of splenic iNKT cells (Fig. 4A). By contrast, liver iNKT cell maturation is not
affected by the absence of Itk (2, 22), and expression of the Itk K390R mutant does not affect this process (Fig. 4).

The Kinase Domain Edge of Itk Is Required for Cytokine Production of iNKT Cells—in the absence of Itk, iNKT cells are defective in secreting IL-4 and IFN-γ in response to α-GalCer stimulation (23, 24). To determine whether the kinase activity of Itk is important in iNKT cell function, WT, Itk−/− and Tg(Lck-ItkΔKin)/Itk−/− mice were injected with α-GalCer.
and serum samples collected 2 h after injection. Analysis of the serum revealed that Itk−/− mice secreted significantly lower amounts of IL-4 and IFN-γ than WT mice consistent with previous reports (23, 24). Expression of kinase domain-deleted mutant in Itk-null mice did not rescue this defect in cytokine production, indicating that the kinase activity of Itk

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**FIGURE 4.** Kinase activity edge of Itk is required for cytokine secretion by iNKT cells. A, top panel, flow cytometric analysis of splenocytes from WT, Itk-null, and Itk−/−K390R iNKT cells. Bottom panel, numbers of iNKT cells in the spleen. *, p < 0.05 compared with WT iNKT cells. B, liver lymphocytes were gated for iNKT (tetramerα TcRβ) cells and analyzed for the maturation markers CD44 and NK1.1. C, WT, Itk-null, and Itk−/−ΔKin mice were injected intraperitoneal with 2 μg of α-GalCer. After 2 h, serum samples from blood were collected. Cytokine levels were analyzed by Bioplex. D, hepatic iNKT cells (tetramerβ TcRβ) were sorted and stimulated with plate-bound anti-CD3 and -CD28 for 3 days. IL-4 and IFN-γ expression levels were analyzed by Bioplex. *, p < 0.05 compared with WT iNKT cells.
is required for the function of iNKT cells (Fig. 4C). Because Itk-null mice (and the Itk<sup>-/-</sup>/ΔKin mice) have reduced numbers of iNKT cells, we also stimulated purified hepatic iNKT cells in vitro with anti-CD3 and CD28 for 3 days to confirm the reduction in cytokine secretion. We found that Itk<sup>-/-</sup> and Itk<sup>-/-</sup>/ΔKin iNKT cells secreted comparable amounts of IL-4 and IFN-γ, which were significantly lower than that secreted by WT iNKT cells (Fig. 4D). Altogether, these data indicate that the kinase activity edge of Itk is important for IL-4 and IFN-γ production of iNKT cells.

**Kinase Domain Edge Independent Rescue of CD122 and T-bet, and Suppression of Eomesodermin Expression in Developing iNKT Cells**—Our analysis indicates that the population of thymic CD122<sup>+</sup> (IL-2/IL-15 receptor β chain) iNKT cells in Itk deficient was reduced compared with WT mice (Fig. 5A). By contrast, we found that this CD122<sup>+</sup> iNKT cell population was significantly higher in the Itk<sup>-/-</sup>/ΔKin mice than in Itk<sup>-/-</sup> mice, indicating that the increased expression of CD122 may contribute to the increased maturation of iNKT cell in the mice carrying the mutant Itk. These data also suggest that Itk may regulate CD122 expression via a kinase independent edge.

The transcription factor T-bet can regulate the expression of CD122, and we found that Itk<sup>-/-</sup>/ΔKin iNKT cells had significantly increased levels of T-bet mRNA and protein compared with Itk<sup>-/-</sup> iNKT cells (Fig. 5A and B). In addition, expression levels of CXCR3, another target of T-bet (40), was also rescued by the expression the equivalent K390R Itk mutant (Fig. 5D). More dramatically, the expression level of eomesodermin, another transcription factor of T-box family that also regulates CD122, was not detected in WT iNKT cells, but was highly expressed in the Itk<sup>-/-</sup> iNKT cells (Fig. 5B). Pointedly, the expression of the Itk kinase-deleted mutant significantly reduced eomesodermin expression in Itk<sup>-/-</sup> iNKT cells (i.e. iNKT cells that develop in the Itk<sup>-/-</sup>/ΔKin mice), suggesting that kinase domain independent edge signals may affect signaling pathways leading to T-bet and eomesodermin expression in iNKT cells.

Two recent studies have shown that the transcription factor PLZF is important for iNKT cell development at early stage (10, 11), and we found that PLZF mRNA levels were significantly elevated in Itk-null iNKT cells, and this was not normalized by expression of the Itk mutant (Fig. 5B).

**DISCUSSION**

We show here that the Itk node in T cell receptor signaling regulates the maturation of iNKT cells in part via an edge that is kinase-independent. The partial rescue of iNKT cell maturation depends on the continued expression of the related kinase node Tsk, and occurs primarily by signaling the maturation of these cells through the immature stage 2 to the more mature stage 3. This correlates with increased expression of T-bet and CD122, and decreased expression of eomesodermin. Our data suggest that signals emanating from the non-catalytic domains of Itk can act as an edge in the signaling pathway that regulates the expression of these factors, thus modulating iNKT cell development.

Our analysis revealed that the number of thymic iNKT cells cannot be rescued by the expression of the kinase domain mutants of Itk, indicating that the kinase activity edge is critical for transducing signals that lead to WT numbers of these cells. This could be intrinsic, or could be related to the re-
duced numbers of total thymocytes observed in the Itk \(-/-\) and Itk \(-/-\)/\Delta\text{Kin} mice, because the overall numbers of thymocytes, and in particular DP thymocytes, play critical roles in iNKT cell development and numbers (1). Indeed, while there is a slight increase in the percentage of iNKT cells in the thymus of Itk \(-/-\)/\Delta\text{Kin} mice, the total number of thymocytes is not rescued in these mice, and this translates into reduced numbers (although slightly higher) of iNKT cells in these mice.

We also tested whether the kinase-deleted mutant would behave differently from a full-length kinase that has little to no kinase activity. We compared these two mutants as it is possible that the folding of the kinase-deleted mutant may be different from the WT kinase. The structure of full-length Itk is not known, but based on a number of experiments using isolated domains, and other approaches in cells, we and others have proposed one of two models for folding of this protein, either an intramolecular folded monomer, or an intramolecular folded dimer (36, 37, 41). Deletion of the kinase domain in both models could potentially result in enhanced interactions with the SH2 and SH3 domains. However, both the kinase-deleted mutant and the kinase activity point mutant behaved in the same fashion with regards to the generation of WT numbers of iNKT cells, as well as in their development and maturation, suggesting that any potential alterations in the structure of Itk does not explain our data.

The related kinase Txk makes some contributions to the development of iNKT cells because Itk/Txk DKO mice have significant reduction in thymic iNKT cells numbers compared with both WT and in particular Itk \(-/-\) mice (24). We find that the kinase domain independent edge of Itk can drive the maturation of a significant percentage of stage 2 iNKT cells to the more mature stage 3. However, in the absence of Txk, this does not happen. We suggest that the function of the kinase domain independent edge may be dependent on the expression of Txk. Whereas these data suggest a genetic interaction, we have not been able to get enough purified iNKT cells to examine this biochemically. However, these findings suggest that kinase activity edge of Itk may be rescued by Txk, however, this may be less efficient due to the lower levels of expression of Txk in these cells (24). However, in the absence of both Itk and Txk, the kinase domain independent edge cannot drive maturation of these cells. These findings suggest that there may be some cooperation between these two nodes, Itk and Txk, such that expression of a kinase activity edge from another Tec kinase may be able to cooperate with a kinase domain independent edge of Itk in these functions.

Several studies have shown that IL-15 is required for the final maturation of iNKT cells and that the IL-2/IL-15 receptor \(\beta\)-chain (CD122) is important for NKT cell development (13, 14). Thus the defect of final maturation in Itk \(-/-\) iNKT cells may be due to the lower CD122 expression in these cells. In support of this, Felices and Berg (24) have reported that Itk-deficient iNKT cells express lower levels of CD122 than WT iNKT cells. Our finding that CD122 and T-bet expression are independent of the kinase domain edge of Itk suggest that the IL-15 signaling pathway may contribute to the defect of Itk-null iNKT cells in maturing from stage 2 to stage 3.

More dramatically, the significant increase in expression of the T-bet-related transcription factor eomesodermin in Itk \(-/-\) iNKT cells, and its reduction upon expression of the kinase domain-deleted mutant of Itk, suggest that this kinase domain independent edge is critical in suppressing the expression of this transcription factor. It is likely that overexpression of eomesodermin alters iNKT cell maturation, and that the T-bet:eomesodermin ratio is critical in iNKT cell maturation. T-bet and/or eomesodermin have been shown to regulate the expression of CD122, although the exact nature of their contributions is not clear, particularly in iNKT cells (42–44). It is possible that CD122 expression is strictly dependent on T-bet expression in iNKT cells, and not on eomesodermin. Nevertheless, our data show that the kinase domain independent edge of Itk can partially restore this ratio or balance, thus partially restoring CD122 expression in iNKT cells. By contrast, CXCR3 has been demonstrated to be a prominent target of T-bet (40), and we observed a reduction in its expression in the absence of Itk, and prominent rescue upon expression of the K390R Itk mutant, indicating that as seen for CD122, rescue of T-bet expression by the kinase domain independent edge has functional consequences in these cells.

Two recent studies have shown that the transcription factor PLZF is important at an early stage of iNKT cell development, with arrest at stage 1 in PLZF-null, and arrest at stage 2 in PLZF overexpressed transgenic mice (10, 11). This suggests that proper expression of PLZF is critical for normal iNKT cell development and maturation. We found that PLZF mRNA levels were significantly elevated in Itk-null compared with WT iNKT cells, suggesting that elevated expression of PLZF may contribute to the defect in Itk \(-/-\) iNKT cells. Indeed, transgenic overexpression of PLZF results in defects in iNKT cell development, with arrest in maturation in stage 2, similar to that seen in the absence of Itk (45). In addition, iNKT cells from the Itk \(-/-\)/\Delta\text{Kin} have levels of PLZF that is closer to that seen in Itk \(-/-\) iNKT cells, suggesting that the interaction between the kinase domain independent edge and regulation of expression of these transcription factors may modulate the maturation of these cells. Of course, we cannot exclude the possibility that other transcription factors and signaling pathways may also be involved downstream of Itk signals to iNKT cell development.

Perturbation of T cell receptor signaling pathway nodes often result in different effects in iNKT cell maturation and development compared with conventional T cell development (46). Indeed, the absence of the Itk node affects the development of naïve or conventional CD4+ and CD8+ T cell development, while leaving development of non-conventional or innate memory phenotype T cells intact (19–21). These non-conventional or innate memory phenotype T cells have properties of iNKT cells, with the presence of preformed cytokine message, ability to rapidly produce cytokines, as well as the requirement for both SAP and Itk nodes for their development (47). However, while the kinase domain independent edge of Itk cannot rescue conventional T cell development (21, 22), it can partially rescue maturation of iNKT cells (this work). Conventional T cells express low levels of T-bet, eome-
sodermin, and PLZF, while non-conventional or innate memory phenotype T cells express high levels of these transcription factors (compared with naïve or conventional T cells) (19–22, 47). The ability of the kinase domain edge of Itk to regulate these factors also differs between iNKT cells and non-conventional or innate memory phenotype T cells. Taken together, this suggests that while iNKT cells share some characteristics with non-conventional of innate memory phenotype T cells, there are clear differences in signaling networks that control their development.

Upon TcR stimulation, Itk is recruited to the cell membrane through its PH domain binding to PIP3 in the cell membrane, where Itk is phosphorylated and activated, as well as interacts with other signaling proteins, including SLP-76, LAT, GADS, PLC-γ1, and Vav, to assemble the productive signaling complex and subsequently initiate the downstream signaling pathways. Itk interacts with other proteins mainly through its SH2 and SH3 domains and this adaptor function is very important for the downstream signaling pathways. Itk can regulate Vav localization and TCR-induced actin polymerization independent of its kinase activity edge, but requires its PH and SH2 domains (29, 33). In addition, the SH3 domain, but not kinase activity edge of Itk, is required for antigen receptor induced transcription factor SRF activation (32). This kinase domain independent edge can partially rescue antigen receptor induced activation of Erk in Tec kinase-null DT40 cells (32). Itk may therefore utilize this edge with scaffolding function in regulating signaling pathways that contribute to the maturation of iNKT cells. These pathways regulate the expression level of CD122, which in turn may be regulated by the balance of T-bet and eomesodermin expressed in these cells. The related kinase node Txk may play a part in regulating the function of this kinase independent edge. Our data indicate that the Itk kinase domain/activity edge may be targeted to affect iNKT cell function, while leaving some iNKT maturation intact.

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