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IL-15Rα of Radiation-Resistant Cells Is Necessary and Sufficient for Thymic Invariant NKT Cell Survival and Functional Maturation

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The development of invariant NKT (iNKT) cells depends on the thymus. After positive selection by CD4+CD8+CD1d+ cortical thymocytes, iNKT cells proceed from CD44lowNK1.1+ cells. The programming of cytokine production occurs along the three differentiation stages, whereas the acquisition of NK receptors occurs at stage 3. Stage 3 thymic iNKT cells are specifically reduced in Il15ra−/− mice. The mechanism underlying this homeostatic deficiency and whether the IL-15 system affects other thymic iNKT cell developmental events remain elusive. In this study, we demonstrate that increased cell death contributed to the reduction of stage 3 cells in Il15ra−/− mice, as knockout of Bim restored this population. IL-15–dependent upregulation of Bcl-2 in stage 3 cells affected cell survival, as overexpression of hBcl-2 partially restored stage 3 cells in Il15ra−/− mice. Moreover, thymic iNKT cells in Il15ra−/− mice were impaired in functional maturation, including the acquisition of Ly49 and NKG2 receptors and the programming of cytokine production. Finally, IL-15Rα expressed by radiation-resistant cells is necessary and sufficient to support the survival as well as the examined maturation events of thymic iNKT cells. The Journal of Immunology, 2011, 187: 1235–1242.

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Thymus total RNA was prepared using TRIzol (Ambion, Austin, TX). Reverse transcription was performed with SuperScript III (Invitrogen, Carlsbad, CA) and oligo(T). Quantitative real-time PCR was performed using an ABI Prism 7500 sequence detection system (Applied Biosystems, Foster City, CA). The amounts of CXCL9 and CXCL10 transcripts were normalized to the endogenous GAPDH control. Data were analyzed with ABI Prism 7500 SDS software. Primers were: for CXCL9, 5′-TCTTCTT- TCGCCGCACTACCTCC-3′ (sense) and 5′-TTTGTATGGAGATGGCTGCT- CG-3′ (antisense); for CXCL10, 5′-CCAAGTGCCTGCGTTTTC-3′ (sense) and 5′-GCTCGCAGGGATGTATTCAA-3′ (antisense); and for GAPDH, 5′-CATGTCCAGATGCTACACTC-3′ (sense) and 5′-GGCC- TTCACCACATTGATTG-3′ (antisense).

**PMA and ionophore stimulation**

Thymocytes enriched for iNKT cells were stimulated with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and A23187 (1 μM; Sigma-Aldrich) for 90 min. Brefeldin A (5 μg/ml; Sigma-Aldrich) and monensin (10 μM; Sigma-Aldrich) were added for the first 30 min to block cytokine secretion and TCR internalization, respectively. IL-4 and IFN-γ production were examined by intracellular staining.

**Statistical analysis**

All data were analyzed by the unpaired, two-tailed Student’s t test using GraphPad Prism (GraphPad Software, San Diego, CA).

**Results**

The increase of cell death results in the reduction of stage 3 thymic iNKT cells in KO mice

We first examined the expression of IL-15R components in thymic iNKT populations of WT and KO mice. For WT cells, the level of IL-15Rx and IL-15Rβ increased, whereas the level of IL-15Rγ decreased along iNKT development, with the greatest change occurring in stage 3 (Fig. 1). KO cells displayed normal IL-15Rγ expression but impaired upregulation of IL-15Rβ (Fig. 1). These results indicate that IL-15 is required for the upregulation of IL-15Rβ during thymic iNKT development and suggest that stage 3 cells are most sensitive to IL-15 effects due to the highest level of IL-15Rβ expression.

KO mice showed an 85% reduction in stage 3 thymic iNKT cells (Fig. 2A). Several possibilities may account for this deficiency. One possibility is impaired cell retention. Stage 3 iNKT cells are retained in the thymus by the CXCRI3 chemokine system (11). We found that the pattern of CXCR3 expression is comparable between WT and KO thymic iNKT cells (Fig. 2B). The mRNA level of CXCR3 ligands, CXCL9 and CXCL10, were also similar between WT and KO thymus (Fig. 2C). These results imply normal CXCR3-mediated retention of stage 3 iNKT cells in the thymus of KO mice. The other possibility is reduction of thymic iNKT cell proliferation. Using BrdU labeling, we found that the proportion of BrdU+ cells in stage 3 cells is comparable between WT and KO mice, whereas the proportion of BrdU+ cells in stage 1 and stage 2 cells is higher in KO mice than those in WT mice (Fig. 2D, Supplemental Fig. 2A). This alteration in cell proliferation unlikely contributes to the reduction of stage 3 cells in KO mice.

Another possibility is impaired cell survival. Stage 3 cells in KO mice contained more cells with active caspase-3/7 cells compared with those in WT mice ex vivo (Fig. 2E, Supplemental Fig. 2B). This result implies that KO stage 3 cells were prone to death. Bim, a proapoptosis member of the Bcl-2 family, regulates cell death induced by various cytokine withdrawal conditions (22–24). It was reported that IL-15 mediates NK cell survival by reducing the level of Bim (23). To determine whether Bim is involved in IL-15-
mediated survival of thymic iNKT cells, we examined stage 3 thymic iNKT cells in Bim−/−, IL15ra−/−, and Bim−/−Il15ra−/− mice. The number of stage 3 cells in the Bim−/−Il15ra−/− mice increased 3-fold compared with that in Il15ra−/− mice and was similar to that in Bim−/− mice (Fig. 2F). These results indicate that the increase of cell death results in the reduction of stage 3 thymic iNKT cells in KO mice. These results also imply the participation of Bim in the IL-15–regulated survival/death of stage 3 cells in vivo.

**Overexpression of Bcl-2 partially restores stage 3 thymic iNKT cells in KO mice**

The balance among prosurvival and proapoptotic Bcl-2 family proteins is a critical determinant of cell survival/death (22–24). Because knockout of Bim restored stage 3 thymic iNKT cells in KO mice, we examined the expression of prosurvival proteins Mcl-1, Bcl-xL, and Bcl-2. The level of Mcl-1 and Bcl-xL was similar between WT and KO cells at each differentiation stage (Fig. 3A, left and middle panels), whereas the level of Bcl-2 was upregulated in stage 3 cells of WT mice but not in those of KO mice (Fig. 3A, right panel). We then examined the role of the Bcl-2 level in IL-15–mediated cell survival by overexpressing hBcl-2. Human bcl-2 Tg was bred into WT and KO mice. The level of Tg product was comparable between WT and KO thymic iNKT cells (Fig. 3B). Overexpression of hBcl-2 did not affect the number of stage 3 cells in the WT background, but increased this population by 2-fold in the KO background (Fig. 3C). Taken together, these results indicate that IL-15–dependent upregulation of Bcl-2 contributes to the homeostasis of stage 3 thymic iNKT cells.

**Thymic iNKT cells in KO mice show impaired acquisition of NKRs**

Thymic iNKT cells acquire the expression of NKRs at stage 3 (3, 9, 12, 13). We examined whether the IL-15 system participates in this maturation process and found that the proportion of cells expressing Ly49A, Ly49C/I, Ly49G2, CD94, NKG2A, or NKG2D was significantly reduced in stage 3 thymic iNKT cells of KO mice (Fig. 4). This result indicates an essential role of the IL-15 system in the acquisition of NKRs by stage 3 thymic iNKT cells.

**Thymic iNKT cells in KO mice show altered programming for cytokine production**

The IL-4 productivity is decreased whereas the IFN-γ productivity is increased progressively during thymic iNKT cell development (10, 12). We examined the role of IL-15Rα in the programming of IL-4 and IFN-γ production by stimulating WT and KO iNKT
subsets with PMA and ionophore, which directly activate protein kinase C and Ca²⁺ mobilization that is downstream of TCR. The proportion of IFN-γ producers increased from 25 to 70% along thymic iNKT cell differentiation in WT mice (Fig. 5A, left, Supplemental Fig. 3A). Despite following a similar ascending trend, the proportion of IFN-γ producers in stage 2 and stage 3 KO cells were significantly smaller compared with the WT counterparts (Fig. 5A, left, Supplemental Fig. 3A). In contrast, the proportion of IL-4 producers descended from 65% in stages 1 and 2 to 35% in stage 3 in WT mice (Fig. 5A, right, Supplemental Fig. 3A), but it was maintained at 65% in all three stages in KO mice (Fig. 5A, right, Supplemental Fig. 3A). These results indicate that the IL-15 system affects the programming of IFN-γ and IL-4 production during thymic iNKT development.

We next examined the expression of T-bet and GATA-3, which are the key transcription factors for Ifnγ and Il4, respectively. The proportion of T-bet⁺ cells as well as the level of T-bet increased along thymic iNKT cell differentiation in WT mice (Fig. 5B, Supplemental Fig. 3B). Thymic iNKT cells of KO mice showed a similar trend of T-bet expression but with reduced magnitude. The level of T-bet was reduced by ~25% in KO stage 3 cells (Fig. 5B). Nearly all thymic iNKT cells in WT and KO mice expressed GATA-3, whose level decreased progressively along differentiation (Fig. 5C, Supplemental Fig. 3C). However, the reduction was less pronounced in KO cells. The level of GATA-3 was 1.6-fold higher in stage 3 KO cells compared with the WT counterpart (Fig. 5C, right). The levels of T-bet and GATA-3 correlated with INF-γ and IL-4 production in thymic iNKT subsets, respectively (Fig. 5). These data suggest that the IL-15 system regulates programming of IFN-γ versus IL-4 production by adjusting T-bet and GATA-3 expression during thymic iNKT cell development.

**IL-15Ra of radiation-resistant thymic cells is necessary and sufficient to support the homeostasis of thymic iNKT cells**

Several types of cells in the thymus express IL-15Ra, including thymic epithelial cells (TECs), dendritic cells, and thymocytes. A recent study found that IL-15Ra expressed by the recipient’s radiation-resistant cells, but not by BM-derived cells, supports homeostasis of thymic iNKT cells in a conventional radiation BM chimera (15). However, it is unclear whether IL-15Ra of radiation-resistant cells in the thymus, presumably the TECs, fulfills this function. We thus specifically assessed the role of IL-15Ra of radiation-resistant thymic iNKT cells in the homeostasis of stage 3 thymic iNKT cells using two thymus-grafting systems. In the first system, adult WT and KO mice were thymectomized, engrafted with WT or KO neonatal thymus, lethally irradiated, and reconstituted with CD45.1+ congenic WT or KO BM. The iNKT cells derived from CD45.1⁺ donor BM in the thymus grafts were analyzed for their homeostasis. A total of eight groups of chimera mice were generated. We found that the numbers of stage 1 and stage 2 thymic iNKT cells were similar among all eight types of chimera (data not shown). The KO–WT–WT chimera mice had similar numbers of stage 3 thymic iNKT cells as the WT–WT–WT chimera. However, the KO–KO–KO chimera had significantly smaller numbers of stage 3 thymic iNKT cells compared with the WT–WT–WT chimera (Fig. 6A, Supplemental Fig. 4A). In contrast, the WT–KO–KO chimera had similar numbers of stage 3 thymic iNKT cells as the KO–WT–WT chimera (Fig. 6A, Supplemental Fig. 4A). In the second system, eGFP⁻ WT or KO neonatal thymus was transplanted into WT nude mice and analyzed for recipient-
With regard to the acquisition of NKRs by stage 3 thymic iNKT cells, we found that the KO→WT chimera was similar to the WT→WT chimera, whereas the WT→KO chimera was similar to the KO→KO chimera (Fig. 7C). Concerning programming of IFN-γ/IL-4 production in thymic iNKT cells.

**Discussion**

iNKT cells undergo a series of differentiation steps in the thymus to develop from NK1.1+IL-15Rb+sIL-4high/IL-15Rb+high/IL-4lowIFN-γlow/mice (stage 1) cells to NK1.1+IL-15Rb+high/IL-4+IFN-γhigh/mice (stage 3) cells (1, 2). IL15ra−/− and IL15ra−/− mice particularly lose the most mature stage 3 thymic iNKT cells. In this study, we examined how the IL-15 system affects homeostasis of stage 3 cells, and whether this system affects other developmental events. We found that the IL-15 system not only supports the survival of stage 3 thymic iNKT cells, but also influences the acquisition of NKRs. These results suggest that the IL-15 system may play a critical role in the maturation and survival of thymic iNKT cells.
iNKT cells but also regulates the acquisition of NKRAs and programming of cytokine production. We also demonstrate that IL-15Rα expressed by radiation-resistant cells, but not by BM-derived cells, is necessary and sufficient to support these developmental events of thymic iNKT cells.

The IL-15 system likely maintains thymic iNKT cell homeostasis through supporting the survival of stage 3 cells via balancing the prosurvival and proapoptosis molecules of the Bcl-2 family. We found comparable levels of Mcl-1 and Bcl-xL between WT and KO thymic iNKT cells (Fig. 3A), which indicates that the IL-15 system exerts little effect on Mcl-1 and Bcl-xL expression in these cells under steady-state conditions in vivo. We did examine one Il15ra<sup>−/−</sup> hMcl-1 Tg mouse and found no rescue of the stage 3 thymic iNKT cells (data not shown). This preliminary observation is consistent with the idea that Mcl-1 is not involved in the IL-15–regulated stage 3 thymic iNKT homeostasis. In contrast, we found that Bcl-2 upregulation in stage 3 thymic iNKT cells occurred in WT but not in KO mice (Fig. 3A), and that overexpression of hBcl-2 increased stage 3 cells by 2-fold in KO mice (Fig. 3C). These results indicate that the IL-15–dependent Bcl-2 upregulation contributes to the homeostasis of stage 3 thymic iNKT cells.

Bim is critical for the IL-15–regulated thymic iNKT homeostasis in vivo, as removal of Bim restored stage 3 thymic iNKT cell number in KO mice to the same level as in Bim<sup>−/−</sup> mice (Fig. 2F). Bim function is regulated by its level of expression (25–28), by intracellular localization (29), and by controlled association with prosurvival Bcl-2 family members (30). Given the partial rescue of stage 3 cells by hBcl-2 Tg and the reduced Bcl-2 but normal Mcl-1 and Bcl-xL levels in the stage 3 cells of KO mice, we speculate that the IL-15 system modulates the balance between the proapoptotic Bim and the prosurvival Bcl-2 family members in the following non-mutually exclusive ways. In addition to upregulating Bcl-2 expression, IL-15 may affect either the amounts of Bim or the association of Bim with prosurvival Bcl-2 family members, including Mcl-1 and Bcl-xL. We are currently investigating these possibilities.

Regulation of T-bet level through the IL-15 system likely affects thymic iNKT cell development. T-bet is a key transcription factor for iNKT cell maturation (13, 31). Re-expression of T-bet in T-bet<sup>−/−</sup> iNKT cells was sufficient to promote iNKT cell
maturation via induction of multiple genes, including those participating in cell migration (e.g., CXCR3), survival (e.g., IL-15Rβ), and effector functions (e.g., IFN-γ) (13, 32). Although the expression of T-bet increased along thymic iNKT differentiation in WT and KO mice (Fig. 5B), the proportion of T-bet+ cells in stage 3 cells and the level of T-bet in stages 2 and 3 cells of KO mice were smaller compared with the WT counterparts. These data indicate that the IL-15 system positively regulates T-bet levels in thymic iNKT cells during development. The reduction of IL-15Rβ expression (Fig. 1 and Ref. 15) and IFN-γ production (Fig. 5A) of stage 3 cells in KO mice likely resulted from the reduction of T-bet expression. However, the level of CXCR3 expressed by KO stage 3 cells appeared normal. It is possible that the level of T-bet in KO cells was still sufficient for normal CXCR3 expression, or that factors other than IL-15 regulate the expression of CXCR3 and compensate for the T-bet deficiency.

Approximately 65% of stage 1 and stage 2 cells in either WT or KO mice produced IL-4 upon PMA/ionophore stimulation. However, the IL-4 producers were reduced to 35% in stage 3 cells of WT mice, but remained unchanged in stage 3 cells of KO mice (Fig. 5A). GATA-3 is a master regulator of IL-4 gene expression in conventional T cells (33). GATA-3−deficient splenic iNKT cells have normal IFN-γ production but fail to produce IL-4 in response to PMA/ionophore stimulation (34). The level of GATA-3 mRNA in thymic iNKT cells stays relatively constant through the three development stages (31), but the level of GATA-3 protein expression during iNKT development had not been reported. In this study, we found that nearly all thymic iNKT cells in WT and KO mice express GATA-3 (Fig. 5C). In WT mice, the GATA-3 level decreases along development with an obvious reduction at stage 3, which correlates with the decrease of IL-4 producers in stage 3 cells. In KO mice, the proportion of IL-4 producers remained the same in all three stages, despite a decrease in the level of GATA-3 in stage 3 cells (Fig. 5A, 5C). These observations indicate that the IL-15 system participates in the regulation of GATA-3 level during thymic iNKT cell development, which likely underlies the regulation of IL-4 production. Although the level of GATA-3 decreased in stage 3 cells of KO mice, it may still be sufficient to support 65% of cells to produce IL-4. It is also possible that the IL-15 system controls other factors to regulate IL-4 production.

Although various types of cells express IL-15Rα, it carries out different functions in regulating different lymphocytes. The generation of NK cells requires the expression of IL-15Rα by both parenchymal and hematopoietic cells. However, the optimal acquisition of Ly49R by NK cells only requires IL-15Rα expression by hematopoietic cells (35). The generation of hepatic iNKT cells depends more on IL-15Rα of parenchymal than of hematopoietic cells. In contrast, IL-15Rα of dendritic cells is sufficient for the upregulation of Bcl-2 and for the functional maturation and homeostatic proliferation of hepatic iNKT cells (15). In this study, we demonstrate that IL-15Rα of thymic radiation-resistant cells, presumably TECs, is necessary and sufficient for the survival and development of stage 3 thymic iNKT cells. Although iNKT cells and other BM-derived cells also express IL-15Rα (Fig. 1A and data not shown), they failed to support the examined developmental events of thymic iNKT cells (Figs. 6A, 7A). Stage 3 iNKT cells are long-term residents in the thymus due to retention through CXCR3. Medullary TECs constitutively express a CXCR3 ligand, CXCL10 (11). We speculate that stage 3 thymic iNKT cells are attracted to medullary TECs and interact directly with TECs to receive IL-15 transpresented by TECs.

In summary, this study reveals the essential role of IL-15Rα expressed by radiation-resistant cells in the survival, NKR-acquisition, and programming of IFN-γ/IL-4 production during the development of thymic iNKT cells.

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References


