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Cutting Edge: Ly9 (CD229), a SLAM Family Receptor, Negatively Regulates the Development of Thymic Innate Memory-like CD8+ T and Invariant NKT Cells

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Signaling lymphocytic activation molecule family receptors and the specific adapter signaling lymphocytic activation molecule–associated protein modulate the development of innate-like lymphocytes. In this study, we show that the thymus of Ly9-deficient mice contains an expanded population of CD8 single-positive cells with the characteristic phenotype of innate memory-like CD8+ T cells. Moreover, the proportion of these innate CD8+ T cells increased dramatically postinfection with mouse CMV. Gene expression profiling of Ly9-deficient mice thymi showed a significant upregulation of IL-4 and promyelocytic leukemia zinc finger. Analyses of Ly9+/− Il4ra−/− double-deficient mice revealed that IL-4 was needed to generate the thymic innate CD8+ T cell subset. Furthermore, increased numbers of invariant NKT cells were detected in Ly9-deficient thymi. In wild-type mice, IL-4 levels induced by β-galactosylceramide injection could be inhibited by a mAb against Ly9. Thus, Ly9 plays a unique role as an inhibitory cell surface receptor regulating the size of the thymic innate CD8+ T cell pool and the development of invariant NKT cells. The Journal of Immunology, 2013, 190: 21–25.

Innate-like T cells are derived from double-positive (DP) thymocytes and are selected by nonclassical MHC class I molecules. These cells have several characteristics that distinguish them from conventional T cells, including a highly restricted TCR repertoire and the rapid generation of effector functions after TCR stimulation without previous exposure to Ag. CD1d-restricted invariant NKT (iNKT) cells, MHC class IÎ±–restricted CD8 T cells (H2–M3-restricted), and MHC class IÎ±–restricted innate-like CD8+ T cells are among these T cell types with innate features. The latter population represents a subset of thymic single-positive (SP) CD8 cells characterized by the constitutive expression of high levels of activation markers, which are indicative of a memory-like phenotype (1). Disruption of either the Il4 or the Cd1d genes in BALB/c mice leads to a reduction in thymic CD8 SP T cells, demonstrating that the cytokine IL-4 secreted by iNKT cells is needed for their development (2, 3). Consistently, recent studies have shown that IL-4 produced by promyelocytic leukemia zinc finger (PLZF)+ iNKT cells drives CD8+ T cells to acquire an innate-like phenotype (reviewed in Ref. 4).

The homotypic interaction between the SLAM family (SLAMF) receptors (SLAMF1 and SLAMF6), expressed on cortical DP thymocytes and iNKT cell precursors, and the downstream signaling SLAM-associated protein (SAP) adaptor are essential to the positive selection required for iNKT cell development (5–7). Recently, it has been demonstrated that the innate CD8+ thymocytes driven by iNKT cells need SAP to properly develop (8). However, nothing is known about the specific contribution of other SLAM family members to the development of innate-like T lymphocytes.

Ly9 (SLAMF3, CD229) is a homophilic cell surface receptor present on all thymocytes and is highly expressed on innate-like lymphocytes such as iNKT cells (9, 10). Our findings show that in contrast to SLAMF1 and SLAMF6, Ly9 plays a negative rather than a positive role in the signaling pathways required for innate-like lymphocyte development in the thymus.

Materials and Methods

Mice
Ly9−/− mice (129 × B6), provided by Dr. D.J. McKean (11), were backcrossed for 12 generations to BALB/c or C57Bl/6 backgrounds. IL-4–deficient mice (BALB/c-Il4ra−/−) and Il4ra−/− obtained from The Jackson Laboratory were crossed with Ly9−/− mice to generate Ly9−/−×IL-4Rα double-
deficient mice (Ly9−/−Il6r−/−). All mice were maintained in a pathogen-free facility. Experiments were conducted on animals 8–12 wk old and in compliance with institutional guidelines as well as with national laws and policies.

**Flow cytometry**

Cells were stained with fluorochrome-labeled Abs using standard methods. R-PE-labeled CD1dα-galactosylceramide (αGalCer) tetramer (Proimmune) was used to detect iNKT cells. For intracellular staining with anti-PLZF, comesotermin (Eomes) or IFN-γ cells were made permeable with an intracellular staining buffer (eBioscience). Data were acquired using a FACSCanto II (BD Biosciences) flow cytometer. Anti-mouse mAbs were obtained from BD Pharmingen, ImmunoTools, and eBioscience. The mouse anti-mouse Ly9 mAb (clone Ly9.7.144, IgG1 isotype) was generated in our laboratory.

**Gene expression analysis**

RNA was reverse-transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), whereas PCR reactions were assembled with TaqMan Universal PCR Master Mix (Applied Biosystems). Samples were loaded onto TaqMan low-density arrays and run in duplicate on a 7900HT Fast real-time PCR system (Applied Biosystems). Relative quantification was based on the comparative cycle threshold method using GAPDH as an endogenous control.

**Lymphocyte and iNKT cell activation**

IFN-γ intracellular levels were measured by FACS in CD8 SP thymocytes treated ex vivo with PMA (100 ng/mL), ionomycin (500 ng/mL; Sigma-Aldrich), and GolgStop (BD Biosciences) for 4 h. Isolated thymocytes and splenocytes (10³/well) were also stimulated with different doses of the iNKT cell ligand αGalCer (KRN7000; Enzo Life Sciences) for 48 h. Mice were i.p. injected with 250 µg Ly9.7.144 mAb or mouse IgG1 control 48 h before administering 6 µg αGalCer. IL-4, IFN-γ (BioLegend), and IL-17 (R&D Systems) production was detected by ELISA.

**Murine CMV infections**

Female mice were i.p. injected with 1–2 × 10⁵ PFU tissue culture–propagated murine CMV (MCMV; Smith strain) in 500 µL DMEM. IFN-γ levels were measured by ELISA (BioLegend). Mice were sacrificed at day 4 postinfection and thymic populations were analyzed by FACS.

**Statistical analysis**

Comparisons between groups were performed with an unpaired two-tailed Student t test with a p value <0.05 as the cutoff for statistical significance.

**Results and Discussion**

Ly9 shapes the size of the innate CD8⁺ T pool

Ly9-deficient (Ly9−/−) mice on a BALB/c background developed normally but showed a striking difference in the distribution of thymocyte subsets in comparison with wild-type (wt) mice (Fig. 1A). An expanded population of CD8 SP thymocytes having no apparent defect in the development of the remaining thymic subsets was detected (Fig. 1B). This increased percentage of CD8 SP T cells, also apparent in cell numbers (Fig. 1C), was specific to the thymus, as it could not be observed in the blood or spleen (data not shown). A similar expanded population of thymic CD8⁺ T cells has also been shown to be present in several gene-deficient mice, including the Kruppel-like factor 2−, IL-2-inducible T cell kinase−, CREB-binding protein−, inhibitor of DNA binding 3 (Id3)−, and NF-κB1−deficient mice (reviewed in Ref. 1). Analysis of the CD8 SP T cell compartment in Ly9−/− mice confirmed that the increased CD8 SP population displayed the phenotypic characteristics of CD8⁺ memory-like T cells, as judged by expression of CD122 and CD124 (Fig. 1D). Interestingly, these CD8 SP cells corresponded to a subset that expressed intermediate amounts of CD3, not only readily apparent in the Ly9−/− mice but also present in wt BALB/c mice (Supplemental Fig. 1A, 1B). In contrast, no differences in CD3 expression levels were detected on splenic CD8⁺ T cells (Supplemental Fig. 1B). Further analysis of the CD3⁺CD8 SP thymocytes showed that most cells display the phenotype CD122highCD124highCD24lowCD62LhighCD69low, providing further evidence that these cells correspond to innate-like CD8⁺ T cells (Supplemental Fig. 1C). Additionally, we observed that the expression levels of CD122 and CD124 were higher in the CD3⁺CD8 SP thymocytes from the Ly9-deficient mice compared with those of the wt mice (Supplemental Fig. 1C). The observation that CD8⁺ innate-like T cells expressed intermediate amounts of CD3 has not been noted in previous studies. Consistent with this phenotype, the Ly9-deficient thymocyte population contained a >3-fold higher number of CD8 SP cells expressing Eomes, a transcription factor characteristic of innate-like CD8⁺ T cells (Fig. 1E). This cell subset was further characterized by their negative expression of the transcription factor T-bet (Supplemental Fig. 1D). Notably, the expansion of the innate-like CD8⁺ T cells in Ly9-deficient mice could not be found in age-matched Ly9-deficient mice on a C57BL/6 background (Supplemental Fig. 1E). Taken together, the data show that the innate CD8⁺ T pool in the thymus is regulated by the homophilic receptor Ly9.

MCVM infection induces a predominant pool of thymic innate-like CD8⁺ T cells in vivo

Although thymic innate memory CD8⁺ T cells are unlikely to play a significant role in secondary infections, they may do so...
during early phases of primary infections. The presence of elevated numbers of innate-like CD8+ T cells in the Ly9−/− mice gave us the opportunity to test the behavior of these cells in response to a viral infection. Although iNKT cells have been found to participate in the initial response to MCMV infection, the role of memory-like CD8+ T cells during viral infections has not been examined (12). Thus, we determined the kinetics of memory-like CD8+ T cells in the course of a primary MCMV infection. The infection induced a rapid and large increase in the proportion of CD3intCD8+CD124+CD122+ cells, which was significantly higher in Ly9−/− versus Ly9+/+ mice (Fig. 2A, 2B). Moreover, in Ly9−/− mice these cells also expressed higher levels of CD124 compared with those in wt mice (Fig. 2B). An atrophy of the thymus induced by the MCMV infection, also reported by others, was observed (13). Further experiments are required to test the differential maturation and migration of thymic subsets induced by MCMV infection.

A time course analysis revealed that Ly9−/− mice responded with higher levels of IFN-γ compared with wt mice (Fig. 2C). Analysis of the viral load in different organs 96 h postinfection showed slightly lower virus in the Ly9−/− mice compared with their wt counterparts. However, these differences were not statistically significant (data not shown). Further studies will be needed to analyze the specific impact of these cells on controlling viral replication, in particular during the neonatal and early life stages before conventional memory networks are established (1). To our knowledge, this is the first report to show that viruses are capable of inducing an important increase in the proportion of thymic innate CD8+ cells and demonstrates that these cells may function as viral sensors in an innate cell-like manner.

Ly9 deficiency causes the expansion of PLZF+ iNKT cells

To gain insight into the mechanism of the perturbed T cell development observed in the Ly9-deficient mice, we performed gene expression profile analysis. The expression of 44 genes was compared between the thymi from Ly9-deficient and wt BALB/c mice (Supplemental Table I). We observed a significant upregulation of a subset of genes associated with effector/memory CD8+ T cells and iNKT cells, such as PLZF and IL4 (Fig. 3A). These two genes are specifically expressed by thymic iNKT cells and both are essential to their development and effector functions (4). Remarkably, the lack of Ly9 induced the upregulation of several genes encoding the chemokine receptors Cxcr3 and Cxcr5, which are known markers of effector/memory CD8+ T cells. The most significant increase found was that of Cxcr3, which has similarly been reported to increase in the thymic innate-like CD8+ cells of Kruppel-like factor 2−/− deficient mice (14). Interestingly,

![Figure 2](http://www.jimmunol.org/)

FIGURE 2. MCMV infection augments the ratio of the innate-like CD8+ T cells in the thymus. (A) Frequencies of CD3intCD8 SP cells among thymocytes from Ly9+/+ and Ly9−/− mice at the indicated time points after infection. (B) Contour plot of CD124 and CD122 expression in gated CD3+/−CD8 SP cells (left panels), cumulative data of percentage of CD3+/−CD8+CD124+CD122+ cells (middle panel), and mean fluorescence intensity of CD124 expression in gated CD3+/−CD8 SP cells from Ly9+/+ and Ly9−/− mice 96 h after infection (right panel). (C) Analysis of IFN-γ plasma levels from Ly9+/+ and Ly9−/− mice (n = 6/group) at various time points after viral infection. Data are representative of at least three independent experiments.

![Figure 3](http://www.jimmunol.org/)

FIGURE 3. Ly9 is critical for thymic iNKT cell numbers, a major source of IL-4 required for innate-like CD8+ T cell expansion in Ly9−/− mice. (A) Genes differentially expressed between Ly9−/− and Ly9+/+ thymocytes. (B) Contour plots of CD4 and CD8 expression in gated CD3+ intermediate thymocytes from Ly9+/+, Ly9−/−, and Ly9−/−IL4ra−/−. (C) Absolute CD3+/−CD8 SP cell numbers and (D) expression of Eomes and IFN-γ in CD8 SP thymocytes (black bar/line, Ly9+/+; red bar/line, Ly9−/−; and blue bar/line, Ly9−/−IL4ra−/−). (E) Representative data of PLZF and CD3 expression (left panel) and cumulative data of percentage of PLZF+ cells from thymocytes of Ly9+/+ and Ly9−/− mice (right panel). (F) Representative iNKT staining (left panel), frequency (middle panel), and absolute iNKT cell numbers (right panel) from Ly9+/+ and Ly9−/− thymi. Data are representative of two (A), three (B–D), or at least four (E, F) independent experiments.
thymic iNKT cells express Cxcr3 that retains these cells in the thymus (15). Moreover, an increase in Cxcr3 has also been reported in the CD8 SP T cells of Id3-deficient thymocytes (16).

The expansion of a population of innate CD8 SP T cells in several deficient mice (Klf2, Itk, and Id3) has been attributed to an IL-4–dependent mechanism. These studies demonstrate that the acquisition of the innate T CD8+ phenotype was not an intrinsic defect in these cells, but rather stemmed from an expanded population of IL-4–producing iNKT cells. Thus, to further understand the requirements for the expansion of innate-like CD8+ T cells in Ly9+/- mice, we crossed Ly9+/- mice with others deficient in the IL-4 receptor. This completely prevented the expansion of CD3+CD8 SP cells (Fig. 3B, 3C). Moreover, the increased levels of Eomes found in the Ly9-deficient mice were undetectable in the Ly9+/IL-4ra/- mice (Fig. 3D). Consistent with this result, the percentage of CD8 SP T cells producing IFN-γ after ex vivo activation with PMA and ionomycin was dramatically reduced in the double-deficient cells (Fig. 3D). All of these data clearly demonstrate that in a Ly9 deficiency setting IL-4 is an essential requirement for the generation of an expanded memory-like or innate-like CD8+ population. We also observed a 3- to 6-fold increase in the percentage of thymic PLZF+ and CD1d tetramer+ iNKT cells and a 6-fold increase in the numbers of iNKT cells in Ly9+/- mice (Fig. 3E, 3F), indicating that expansion of the CD8 SP cells was unlikely due to an increased functional capacity of the iNKT cells, but rather resulted from an increase in the number of these cells. In contrast, we only observed a slight increase in the number of iNKT cells in the Ly9+/-C57BL/6 mice, which may be unable to support an expansion of thymic innate CD8+ T cells (Supplemental Fig. 1F). The number of PLZF+ γδ T cells, a subset also involved in thymic IL-4 secretion, was not significantly altered (data not shown). Additionally, no significant alteration was found in the expression of SAP, SLAMF1, or SLAMF6 in Ly9-deficient mice (Supplemental Table I and data not shown). Thus, in contrast to the positive regulators SLAMF1 and SLAMF6, Ly9 (SLAMF3) is a negative regulator of iNKT cell development in the thymus.

**Ly9 deletion enhances cytokine production by stimulated iNKT cells**

Given the important roles played by iNKT cells and IL-4 in the development of innate-like CD8+ T cells, we next sought to determine the response of Ly9-deficient mice to αGalCer, a specific iNKT cell agonist. Compared with wt mice, Ly9-deficient mice presented an enhanced production of IL-4 2 h after in vivo injection with αGalCer (Fig. 4A). Consistent with our in vivo results, the ex vivo activation of Ly9+/- thymocytes with αGalCer also induced significantly higher levels of IL-4 as compared with thymocytes of wt mice (Fig. 4B). In contrast, no significant difference in the in vitro production of IL-4 after αGalCer activation could be observed in splenocytes, where only a moderate increase in the iNKT cell numbers was detected in the Ly9-deficient mice and no alteration in activation markers was observed (Supplemental Fig. 1G). Thus, these results suggest that the increased levels of IL-4 largely resulted from elevated numbers of iNKT cells in Ly9-deficient mice and were likely unrelated to the hyperreactivity of these cells.

Importantly, we demonstrate in this study that IL-4 production could be modulated by the in vivo treatment with an anti-Ly9 mAb. The injection of wt mice with anti-Ly9 mAb (Ly9.7.144) was able to induce a significant decrease in the production of IL-4 but not in IL-17 or IFN-γ after αGalCer administration (Fig. 4C). No effect of this mAb was observed in Ly9-deficient mice, supporting its specificity. This decrease in IL-4 production was most likely due to the agonistic effect of this mAb. This noncytotoxic mAb did not significantly affect the numbers of iNKT cells or the levels of PLZF mRNA expression levels after anti-Ly9 or a matching isotype treatment for 48 h previous to αGalCer administration. Data are representative of at least two independent experiments.
velopment and effector functions. Nevertheless, this work demonstrates that Ly9 is a potential target for therapeutic intervention in diseases where iNKT cell numbers play a relevant role, including cancer, autoimmune and inflammatory diseases, and infections.

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Disclosures
The authors have no financial conflicts of interest.

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