Brief Definitive Report

Developmentally Regulated Extinction of Ly-49 Receptor Expression Permits Maturation and Selection of NK1.1$^+$ T Cells

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Summary

Clonally distributed inhibitory receptors negatively regulate natural killer (NK) cell function via specific interactions with allelic forms of major histocompatibility complex (MHC) class I molecules. In the mouse, the Ly-49 family of inhibitory receptors is found not only on NK cells but also on a minor (NK1.1$^+$) T cell subset. Using Ly-49 transgenic mice, we show here that the development of NK1.1$^+$ T cells, in contrast to NK or conventional T cells, is impaired when their Ly-49 receptors engage self-MHC class I molecules. Impaired NK1.1$^+$ T cell development in transgenic mice is associated with a failure to select the appropriate CD1-reactive T cell receptor repertoire. In normal mice, NK1.1$^+$ T cell maturation is accompanied by extinction of Ly-49 receptor expression. Collectively, our data imply that developmentally regulated extinction of inhibitory MHC-specific receptors is required for normal NK1.1$^+$ T cell maturation and selection.

Key words: NK1.1$^+$ T cells • Ly-49 • development • repertoire selection • Ly-49A transgenic mice

Natural killer (NK)1.1$^+$ T cells (1–4) are an unusual subset of murine T cells that express a highly restricted TCR-α/β repertoire comprised of an invariant Vα14-Jα281 chain and a predominant Vβ domain (Vβ8.2). Successful development of NK1.1$^+$ T cells requires that their canonical TCR interact with CD1, a nonclassical, MHC-like molecule encoded outside of the MHC gene complex (5–8). The developmental origin of NK1.1$^+$ T cells is controversial; nevertheless, it is widely accepted that they originate in the thymus and migrate to peripheral tissues such as spleen and liver (4). Indeed, direct evidence for such a migration pattern has recently been obtained by in vivo adoptive transfer studies (9).

In contrast to conventional T cells, NK1.1$^+$ T cells express several phenotypic markers usually associated with NK cells such as NK1.1, CD122, and Ly-49. Ly-49 is a multigene family (comprised of at least nine members, A–I) encoding homodimeric C-type lectin-like receptors that interact with specific alleles of MHC class I proteins (10). In NK cells, it has been clearly shown that Ly-49–MHC class I interaction negatively regulates effector functions such as cytotoxicity and bone marrow graft rejection (11, 12). Although conventional TCR-α/β cells in the mouse do not normally express Ly-49 receptors, studies of Ly-49A transgenic mice have demonstrated that proliferative responses of mature transgenic T cells to alloantigens can be specifically inhibited by appropriate Ly-49–MHC class I interactions (13), thus raising the possibility that signaling via the TCR is susceptible to a Ly-49–dependent regulatory mechanism. This hypothesis is further supported by recent data indicating that both cytokine secretion and the cytotoxicity of NK1.1$^+$ T cells are specifically inhibited upon Ly-49–MHC class I interaction (14).

The potential ability of Ly-49 receptors to counteract signaling by the TCR could also be of physiological relevance for NK1.1$^+$ T cell development. In this report, we have tested this hypothesis by comparing putatively immature (thymic) and mature (liver) NK1.1$^+$ T cells in normal as well as Ly-49A transgenic mice. Our data indicate that developmentally regulated extinction of Ly-49 receptor expression is required to allow appropriate TCR repertoire selection and subsequent maturation of NK1.1$^+$ T cells.

Materials and Methods

Mice. C57BL/6 mice were obtained from Harlan Olac (Bicester, UK). Mice deficient for the transporter associated with antigen processing (TAP1$^-$) on a C57BL/6 background (15) were obtained from The Jackson Laboratories (Bar Harbor, ME). The production of Ly-49A transgenic mice has been described previously (13). These mice were established on a (CBA/J × C57BL/6) F2 background and subsequently backcrossed to either C57BL/6 (H-2^b) or B10.D2 (H-2^d). Transgenic and littermate mice (fifth backcross) were analyzed in parallel.

Cells. Liver mononuclear cells were prepared as previously described (16). Thymocytes were depleted of heat stable antigen

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$(HSA)^+$ cells by treatment with rat IgM mAb B2A2 plus rabbit complement. Viable recovered cells (~1% of input) were purified on a lympholyte-M gradient (Cedarlane Labs., Hornby, Ontario, Canada).

Flow Microfluorometry. HSA^{low} thymocytes or liver mononuclear cells were first incubated with unlabeled mAb 24G2 (anti–Fc receptor) to block nonspecific binding, and were then triple stained with combinations of the following mAb conjugates: anti-Ly49A-FITC (A1); anti–Ly-49C/I-FITC (5E6); anti-Ly-49G2-FITC (4D11); anti-Vα2-FITC (B20.1); anti-Vα3.2-FITC (RR3-16); anti-Vα8-FITC (B21.14); anti-Vα11-FITC (RR8-1); anti-Vβ8.2-FITC (F23.2); anti-NK1.1-PE (PK136); anti–TCR-α/β–biotin (H57-597); and anti-Ly49C-biotin (4LO-3311). The latter two mAbs were revealed with avidin-tricolor. All mAbs were obtained from PharMingen (San Diego, CA), with the exception of 4LO-3311 (17). Samples were passed on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA) gated to exclude nonviable cells on the basis of light scatter. Data were analyzed by LYSIS II software.

Results and Discussion

Although $NK1.1^+$ T cells are present in small numbers in other tissues, they have been primarily studied in thymus and liver, where they account for ~25% of mature (HSA^{low}) TCR-α/β cells in C57BL/6 mice (Fig. 1 *A*). Interestingly, analysis of expression of Ly-49A, Ly-49C/I, and Ly-49G2 (the only inhibitory Ly-49 family members for which mAbs are available) in these organs revealed that the proportion of $NK1.1^+$ T cells expressing each of the Ly-49 genes was substantially (two- to fivefold) higher in thymus than in liver (Fig. 1 *B*). Conventional (i.e., $NK1.1^-$) TCR-α/β cells did not express Ly-49 receptors to any significant degree (data not shown).

The reduced frequency of $Ly\text{-}49^+$ $NK1.1^+$ T cells in liver as compared to thymus of normal mice could reflect a requirement for loss of Ly-49 expression during $NK1.1^+$ T cell maturation. To test this hypothesis we used a transgenic mouse strain (13) in which all thymic and liver $NK1.1^+$ T cells express Ly-49A, as compared to the minor (10–20%) $Ly\text{-}49A^+$ subset of $NK1.1^+$ T cells in nontransgenic littermates (Fig. 2 *A*). Moreover, endogenous and transgenic Ly-49A were expressed at similar levels on $NK1.1^+$ T cells (Fig. 2 *A*). As previously described (13), the transgene is also expressed on all NK and T cells. In C57BL/6 ($H\text{-}2^b$) Ly-49A transgenic mice (where there is no ligand for Ly-49A), $NK1.1^+$ T cells as well as T and NK cells develop normally in both thymus and liver as compared to nontransgenic littermates (Fig. 2 *B*).

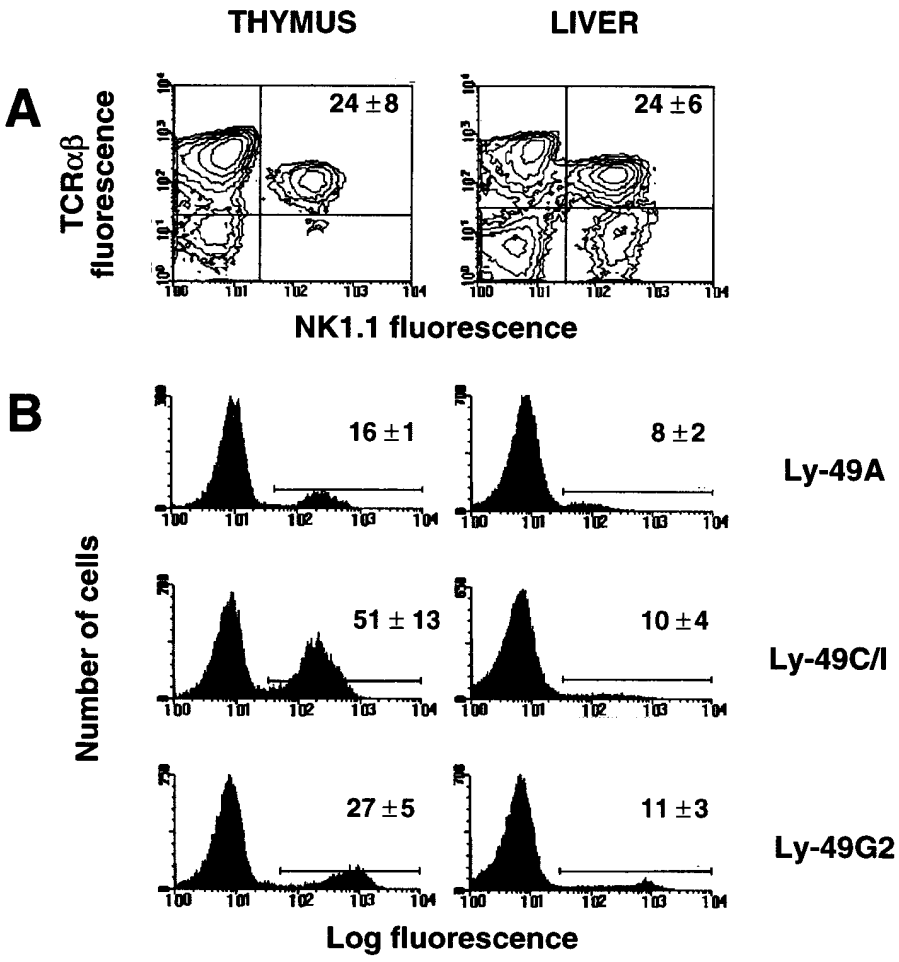

Figure 1. Differential expression of Ly-49 receptors by $NK1.1^+$ T cells in thymus and liver. Thymocytes (HSA^{low} fraction) and liver mononuclear cells from normal C57BL/6 mice were triple stained with mAbs against NK1.1 and TCR-α/β, and Ly-49A, Ly-49C/I, or Ly-49G2. The cytograms in *A* depict NK1.1 versus TCR-α/β fluorescence. The mean percentage (± SD) of $NK1.1^+$ T cells (*upper right quadrant*) is indicated. The histograms in *B* represent Ly-49A, Ly-49C/I, or Ly-49G2 staining gated on $NK1.1^+$ TCR-α/β^+ cells. Numbers represent the mean percentage of positive cells (± SD) in the indicated gates (5–10 experiments).

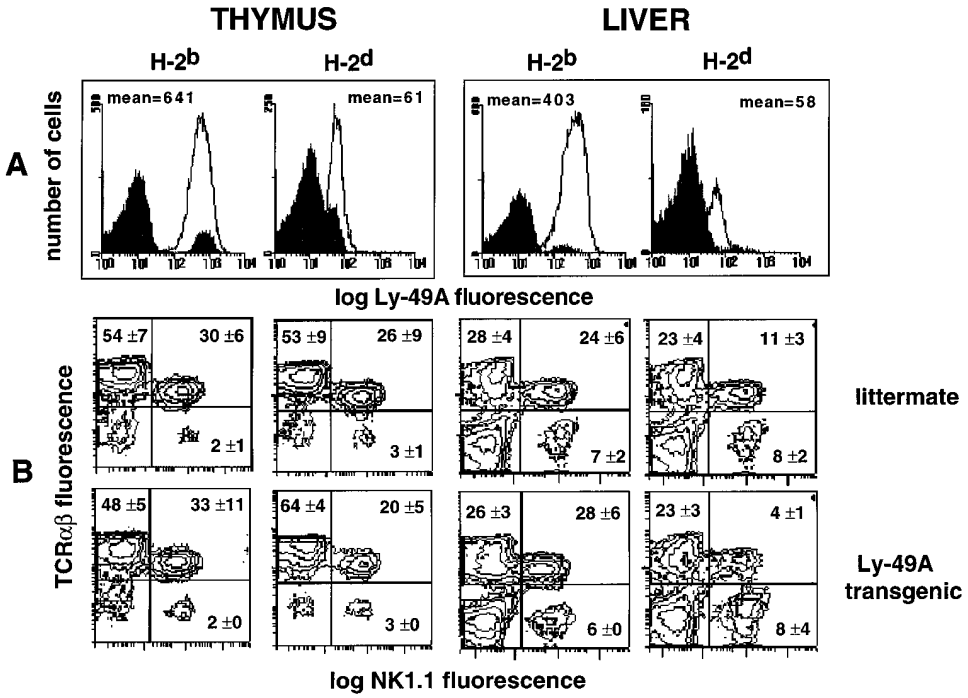

Figure 2. Selectively impaired NK1.1$^+$ T cell development in liver of H-2^d Ly-49A transgenic mice. Thymocytes (HSA^{low} fraction) or liver mononuclear cells from Ly-49A transgenic mice or control littermates on a H-2^b (C57BL/6) or H-2^d (B10.D2) background were analyzed by three-color flow microfluorometry for expression of NK1.1, TCR-α/β, and Ly-49A. In *A*, Ly-49A fluorescence profiles gated on NK1.1$^+$ TCR-α/β$^+$ cells from normal (*closed histograms*) or transgenic (*open histograms*) mice are superimposed. The mean Ly-49A fluorescence intensity of the transgenic NK1.1$^+$ T cells is indicated in each case. The cytograms in *B* depict NK1.1 versus TCR-α/β fluorescence. Numbers represent the mean percentage (± SD) of T cells (*upper left*), NK1.1$^+$ T cells (*upper right*), and NK cells (*lower right*) in transgenic and littermate mice on an H-2^b (n = 4) or H-2^d (n = 7) background. The absolute numbers of HSA^{low} thymocytes (1–2 × 10^6) and liver mononuclear cells (4–5 × 10^6) did not differ significantly among the different groups of mice. The lower proportion of liver NK1.1$^+$ T cells in H-2^d versus H-2^b littermate mice reflects differences between the C57BL/6 and C57BL/10 genetic backgrounds (data not shown).

To ascertain whether NK1.1$^+$ T cell development is affected by Ly-49 engagement, we analyzed Ly-49A transgenic mice on a B10.D2 (H-2^d) background where the ligand for Ly-49A (H-2D^d) is expressed. As shown in Fig. 2 *A*, the cell surface level of Ly-49A was downmodulated to a similar extent in NK1.1$^+$ T cells of transgenic and wild-type H-2^d (as compared to H-2^b) mice. Similar results have been observed for NK cells in these and other Ly-49A transgenic mice (18). Importantly, both the frequency and absolute number of liver NK1.1$^+$ T cells was significantly (threefold) reduced in H-2^d Ly-49A transgenic mice as compared to nontransgenic littermates (Fig. 2 *B*). This effect was specific for the NK1.1$^+$ T cell lineage since both NK cells and conventional (NK1.1$^-$) T cells developed normally in the liver of H-2^d mice despite expression of the Ly-49A transgene (Fig. 2 *B*). In the thymus, only a modest reduction of NK1.1$^+$ T cells was observed in H-2^d Ly-49A transgenic mice as compared to littermates (Fig. 2 *B*).

If Ly-49 engagement in fact modifies signaling via the TCR, then the impaired development of NK1.1$^+$ T cells in H-2^d Ly-49A transgenic mice might reflect a failure to select the highly restricted CD1-specific TCR repertoire. To address this issue, we compared TCR Vα and Vβ use in these mice with nontransgenic littermates. In normal mice, NK1.1$^+$ T cells in thymus and liver exhibit a TCR repertoire that is highly skewed to Vα14 and Vβ8.2 (1–4). Since no reliable mAb to Vα14 is currently available, we assessed Vα14 skewing in NK1.1$^+$ T cells using a pool of four anti-Vα mAbs that together detect 20–25% of the normal TCR Vα repertoire. As shown in Fig. 3, both thymic and liver NK1.1$^+$ T cells from control H-2^d littermates had a very high proportion of Vβ8.2$^+$ cells and very few cells staining with the Vα mAb panel, as would be expected for cells expressing the canonical Vα14/Vβ8.2 TCR. Remarkably, however, liver NK1.1$^+$ T cells from H-2^d Ly-49A transgenic mice exhibited much lower levels of Vβ8.2$^+$ cells and much higher levels of cells expressing the Vα panel. Indeed, the Vα and Vβ repertoires of liver NK1.1$^+$ T cells in these mice were not significantly different from those of conventional (NK1.1$^-$) liver T cells (Fig. 3 *B*), indicating that the small proportion of NK1.1$^+$ T cells that was still able to develop despite the presence of a self-MHC–reactive Ly-49 receptor did not express the canonical Vα14/Vβ8.2 TCR.

In the thymus of H-2^d Ly-49A transgenic mice, the skewing towards Vβ8.2 and Vα14 in NK1.1$^+$ T cells was reduced compared with littermate controls, but still highly significant when compared with conventional (NK1.1$^-$) T cells (Fig. 3 *B*). Thus, most thymic NK1.1$^+$ T cells bearing the self-MHC–reactive Ly-49 receptor still expressed the canonical TCR, whereas a minority did not. Perturbations in the TCR Vα and Vβ repertoires of NK1.1$^+$ T cells were strictly dependent upon Ly-49A–ligand interactions and not related to expression of the transgene per se, since NK1.1$^+$ T cells from the liver and thymus of H-2^b Ly-49A transgenic mice were indistinguishable from their nontransgenic littermates in terms of Vβ8.2 and Vα staining (Fig. 3 *B*).

Taken together, our data with Ly-49A transgenic mice provide direct evidence that the development of NK1.1$^+$ T cells expressing self–MHC class–I-reactive Ly-49 receptors is selectively impaired. Moreover, impaired development of NK1.1$^+$ T cells is clearly correlated with a failure

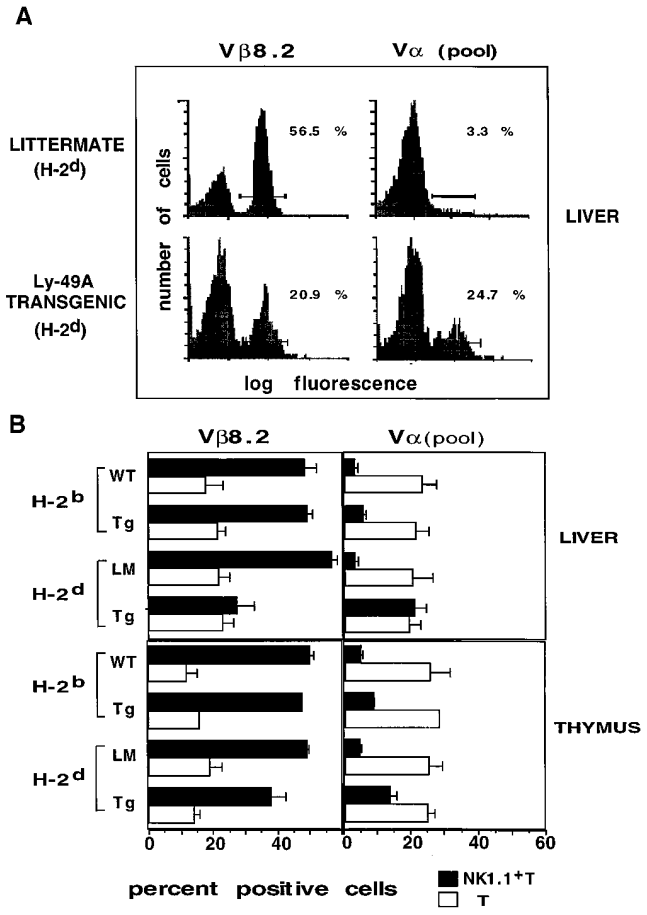

Figure 3. Altered TCR repertoire selection in NK1.1$^+$ T cells from H-2^d Ly-49A transgenic mice. NK1.1$^+$ TCR-α/β^+ cells from thymus or liver of Ly-49A transgenic (*Tg*) mice and their littermate (*LM*) or wild-type (*WT*) controls on either an H-2^b or H-2^d genetic background (gated as in Fig. 2) were analyzed in the third color for expression of Vβ8.2 or Vα (pool of Vα2, Vα3.2, Vα8, and Vα11). Histograms in *A* show representative Vβ8.2 and Vα staining patterns gated on liver NK1.1$^+$ T cells from H-2^d mice. Data in *B* represent the mean percentage (± SD) of Vβ8.2$^+$ or Vα$^+$ cells among NK1.1$^+$ T cells (*black bars*) or conventional T cells (*gray bars*) in the indicated groups of mice (3–6 mice per group). Where no error bars are indicated, the results represent the mean of two mice.

to select the CD1-reactive canonical TCR, strongly arguing that TCR signaling is perturbed by Ly-49 engagement in this lineage. The molecular mechanism responsible for impaired TCR signaling by NK1.1$^+$ T cells in Ly-49A transgenic mice is currently unknown; however, by analogy with signaling events in NK cells (19–21) and conventional T cells (22, 23), it is tempting to speculate that dephosphorylation of TCR-associated protein kinases (such as ZAP-70) in NK1.1$^+$ T cells by Ly-49–associated phosphatases (such as SHP-1) leads to reduced TCR signaling upon CD1 engagement, and hence to the lack of further maturation.

Comparison of our data on normal and Ly-49A transgenic mice leads to a novel model for the regulation of Ly-49 receptor expression during NK1.1$^+$ T cell development. According to this scenario, immature NK1.1$^+$ T cells expressing self-reactive Ly-49 receptors initially develop in the thymus. However subsequent maturation of these cells (and/or their export to peripheral tissues such as the liver) requires loss of Ly-49 expression to avoid interference with TCR signaling if Ly-49 receptors are engaged. Two distinct mechanisms could account for loss of Ly-49 receptor expression during NK1.1$^+$ T cell maturation. One possibility would be that Ly-49 expression is simply extinguished in a developmentally regulated and lineage-specific fashion. Alternatively, selection against NK1.1$^+$ T cells expressing self-MHC–reactive Ly-49 receptors may occur.

Direct evidence that Ly-49 receptors are switched off during normal NK1.1$^+$ T cell development was obtained in TAP1$^-$ mice (15, 24), which express CD1 (the positively selecting ligand for the canonical TCR on NK1.1$^+$ T cells), but not MHC class I (the ligand for Ly-49 inhibitory receptors). Consistent with earlier studies (25, 26), the frequency of NK1.1$^+$ T cells in thymus and liver of TAP1$^-$ mice (36 and 28%, respectively) was comparable with that in wild-type controls. More importantly, the proportion of NK1.1$^+$ T cells expressing Ly-49A, Ly-49C, and Ly-49G2 was reduced fivefold in liver (as compared with thymus) of TAP1$^-$ mice (Fig. 4), similar to what had been observed for wild-type mice (Fig. 1 *B*). Since all potential selecting ligands for Ly-49 receptors are presumably absent in TAP1$^-$ mice, these data argue definitively that extinction of Ly-49 receptor expression must be occurring during normal NK1.1$^+$ T cell development.

In conclusion, our data point to a novel lineage-specific role and regulation of Ly-49 receptor expression during normal NK1.1$^+$ T cell development. Although immature NK1.1$^+$ T cells expressing self–MHC class I–reactive Ly-49 receptors extinguish Ly-49 expression in the course of their maturation, NK cells do not. This dichotomy may reflect a fundamental difference not only in the development, but also in the function, of these two lymphocyte lineages. Thus, mature NK cells are generally believed to require expression of at least one self-reactive Ly-49 inhibitory receptor to avoid potential autoreactivity and to facilitate surveillance of tissues rendered MHC class I–deficient by infection or transformation (27). On the other hand, mature

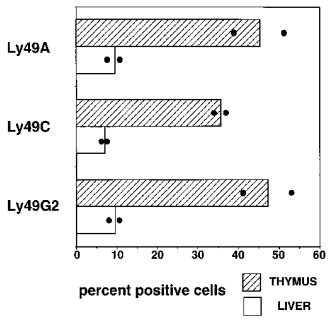

Figure 4. Differential Ly-49 receptor expression by NK1.1$^+$ T cells in thymus and liver occurs independently of MHC class I. Thymocytes (HSA^{low} fraction) and liver mononuclear cells from TAP1$^-$ mice were triple stained with mAbs against NK1.1 and TCR-α/β, and Ly-49A, Ly-49C, or Ly-49 G2. Data are expressed as the mean percentage of Ly-49$^+$ cells among NK1.1$^+$ TCR-α/β^+ cells (2 mice per group).

$NK1.1^+$ T cells, which recognize CD1 (probably in association with a glycolipid; reference 28) via their canonical TCR, may need to extinguish the expression of self-reactive Ly-49 inhibitory receptors so that they can be activated optimally by foreign antigens in any normal (i.e., MHC class I–expressing) tissue.

Finally, in a more philosophical vein, one might ask why Ly-49 receptors are expressed at all in the $NK1.1^+$ T cell lineage if their subsequent extinction is required for correct $NK1.1^+$ T cell maturation. One rather trivial explanation would be that transient expression of Ly-49 receptors in the $NK1.1^+$ T cell lineage represents an "evolutionary accident" resulting from a (hypothetical) close developmental relationship to NK cells. Alternatively, Ly-49 receptor engagement may play some positive role early in $NK1.1^+$ T cell development, as suggested by the fact that some Ly-49 receptor family members (such as Ly-49D) have activating properties (29). The third (and perhaps most interesting) possibility could be that the presence of inhibitory Ly-49 receptors on $NK1.1^+$ T cells may be required at a particular stage of development to establish a correct threshold for activating signals delivered by the TCR or other (as yet unidentified) receptors. In the latter context, it is interesting that inefficient development of "NK1.1-like" T cells expressing the canonical CD1-reactive TCR can occur in the absence of Ly-49 (and NK1.1) expression in the thymus of mice rendered deficient for the common cytokine γ chain (30). However, these cells are not found in peripheral tissues such as the liver, consistent with the possibility that early Ly-49 expression may be necessary for efficient and/or complete $NK1.1^+$ T cell development.

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