Ly49D-Mediated ITAM Signaling in Immature Thymocytes Impairs Development by Bypassing the Pre-TCR Checkpoint

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Ly49D-Mediated ITAM Signaling in Immature Thymocytes Impairs Development by Bypassing the Pre-TCR Checkpoint

Estelle Merck,1 Rosemary K. Lees,1 Roger B. Voyle,1 Werner Held, and H. Robson MacDonald

Activating and inhibitory NK receptors regulate the development and effector functions of NK cells via their ITAM and ITIM motifs, which recruit protein tyrosine kinases and phosphatases, respectively. In the T cell lineage, inhibitory Ly49 receptors are expressed by a subset of activated T cells and by CD1d-restricted NKT cells, but virtually no expression of activating Ly49 receptors is observed. Using mice transgenic for the activating receptor Ly49D and its associated ITAM signaling DAP12 chain, we show in this article that Ly49D-mediated ITAM signaling in immature thymocytes impairs development due to a block in maturation from the double negative (DN) to double positive (DP) stages. A large proportion of Ly49D/DAP12 transgenic thymocytes were able to bypass the pre-TCR checkpoint at the DN3 stage, leading to the appearance of unusual populations of DN4 and DP cells that lacked expression of intracellular (ic) TCR-β-chain. High levels of CD5 were expressed on ic TCR-β- DN and DP thymocytes from Ly49D/DAP12 transgenic mice, further suggesting that Ly49D-mediated ITAM signaling mimics physiological ITAM signaling via the pre-TCR. We also observed unusual ic TCR-β single positive thymocytes with an immature CD24high phenotype that were not found in the periphery. Importantly, thymocyte development was completely rescued by expression of an Ly49A transgene in Ly49D/DAP12 transgenic mice, indicating that Ly49A-mediated ITIM signaling can fully counteract ITAM signaling via Ly49D/DAP12. Collectively, our data indicate that inappropriate ITAM signaling by activating NK receptors on immature thymocytes can subvert T cell development by bypassing the pre-TCR checkpoint.

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entiation of NKT cells (12) and that forced expression of the Ly49D–DAP12 complex and its ligand H2-Dd in Tg mice blocks NKT cell differentiation at an early precursor stage (13). In the current study, we show that thymic cellularity is greatly reduced in Ly49D/DAP12 × H-2Dd Tg mice due to an impaired transition from the DN to DP stages. Moreover we demonstrate that Ly49D/DAP12 signaling starting in DN3 thymocytes can bypass pre-TCR signaling and allow some thymocytes to progress to the DN4, DP, and even SP stages in the absence of expression of intracellular (ic) TCRβ protein. This abnormal differentiation pathway is abortive, however, as these unusual TCRβ−CD4+ and CD8+ T cells are not found in the periphery.

Materials and Methods

Mice
C57BL/6 wild-type (WT) and RAG1−/− (C57BL/6 background) mice were obtained from Harlan Laboratories and The Jackson Laboratory, respectively. B6 mice Tg for Ly49D (hereafter referred to as Ly49D TG) (6), B6 mice double Tg for Ly49D and DAP12 line no. 14 (hereafter referred to as Ly49D/DAP12 TG) (6, 13), B6 mice Tg for Ly49A (14), B6 mice Tg for Ly49D/DAP12 (JF17.12) (21), C57BL/6 mice double Tg for Ly49D and DAP12 line no. 14 (hereafter referred to as Ly49D–DAP12 Tg) (6, 13), B6 mice Tg for Ly49A (14), and B6 mice Tg for Ly49D/DAP12 line no. 14 (hereafter referred to as Ly49D–DAP12 Tg) (6, 13) were provided by Dr. J. Flaherty (University of California, San Francisco, CA). All mice were bred and housed under specific pathogen-free conditions. Experiments were performed according to Swiss guidelines and authorized by the Cantonal Veterinary Office (Lausanne, Switzerland).

Abs and flow cytometry
The following mAb conjugates were purchased from BD Biosciences (San Jose, CA): CD3ε-PE (500A2), CD4-Alexa Fluor 700 (RM4.5), Ly49D–FITC (4E5), NK1.1-PerCP-Cy5.5 (PK136) mAbs, CD4-PECy7 (RMA-5), CD4-allophycocyanin-εFcy7 800 (RM4-5), CD8ε-PerCP-Cy5 (53.6.7), CD8ε-PerCyc7 (53.6.7), CD11b-PE-Cy7 (M170), CD24-PE (M1/39), CD45-PE, anti-TCRε–PE-Cy5, and anti-icCD3ε (4E5), CD3ε-gd–PE requires saturation of the cell surface molecules by anti-ic-CD3ε–PE (GL3), CD11b, Ter119, together with anti-CD117–PerCP-Cy5.5, TRC8–PE-Cy7 (53.6.7), 5-FITC, respectively. Samples were analyzed on an LSRII SORP flow cytometer (BD Biosciences), and the ratio 405 nm/530 nm was analyzed.

Measurement of calcium flux
DN-enriched thymocytes (17) were resuspended in DMEM 2% FCS and incubated with 2 μM Indo-1 AM (Molecular Probes) for 1 h at 37˚C. After washing, the cells were labeled with CD25-PE (PC61.5; Caltag) and anti-Ly49A. Once the baseline fluorescence ratio was established, the calcium flux response to anti-Ly49D (4E5; BD Biosciences) and anti-C3d (500A2 or 7D6; produced in our laboratory) mAb stimulation (10 μg/ml) was determined. For stimulation with the clone 7D6, biotinylated anti-C3d mAb cross-linking was required and achieved with 5 μg/ml avidin (Molecular Probes). In some experiments, anti-Ly49A (JF17.12; produced in our laboratory) mAb was added at the same time as anti-Ly49D. Indo-1 was excited by UV laser on an LSRII SORP flow cytometer (BD Biosciences), and the ratio 405 nm/530 nm was analyzed.

Cell cycle analysis
Simultaneous cell cycle analysis and surface(ic staining were performed on DN-enriched cells (17). Cells were first preincubated with anti-CD3ε (500A2) mAb protein and then surface labeled with a mixture of allophycocyanin/Alexa 647-conjugated mAbs to B220, CD4, CD8α, TCRβ, TCRγδ, CD11b, Ter119, together with anti-CD117–PerCP-Cy5.5, anti-CD44–Alexa Fluor 700, and anti-CD25–allophycocyanin-Alexa 75 mAbs. DN-enriched cells were then fixed and permeabilized using BD Perm&Fix kit (BD Biosciences) and subsequently stained with anti-CD3ε–PE, anti-TCRβ–PE-Cy5, and anti-Ki67–FITC (B56; BD Biosciences) for 1 h followed by Hoechst 33342 (Invitrogen) at 20 μg/ml for 5 min. After washing, cells were analyzed on an LSRII Flow Cytometer (BD Biosciences). Doublets were eliminated using the DDM unit.

Results

Impaired thymus development in Ly49D/DAP12 Tg mice in the presence of H-2Dd ligand
The activating NKR Ly49D is normally expressed on NK cells but not on T cells or NKT cells (19, 20). In a mouse pre-B cell line, efficient surface expression of Ly49D can be achieved by cotransfection of Ly49D with the adapter signaling molecule DAP12 (21). Because T cells do not normally express DAP12 (13, 22, 23), we made Tg mice expressing Ly49D either alone or together with DAP12. We previously published that Ly49D was expressed on peripheral T cells at levels comparable with endogenous Ly49D expression levels in NK cells in the Ly49D/DAP12 Tg mice but poorly expressed in the Ly49D Tg mice (6).

To test whether signaling via the activating NKR Ly49D can impact on T cell development, Ly49D/DAP12 Tg mice and control Ly49D Tg mice were crossed with mice expressing H-2Dd, a known ligand for Ly49D (24). As shown in Fig. 1A, the absolute number of thymocytes was dramatically decreased (~5-fold) in Ly49D/DAP12 Tg mice in the presence, but not in the absence, of ligand. This reduction was most pronounced for the CD4+ CD8+ (DP) thymocyte subset, whereas the CD4+ CD8− (DN) subset was not reduced in number (Fig. 1A, 1B). In contrast, no significant effect on thymus development was observed in Ly49D Tg mice lacking DAP12 in the presence of H-2Dd ligand (Fig. 1A, 1B) indicating that the ITAM signaling DAP12 chain is required for the impaired transition from the DN to DP stages.

DN subsets and Ly49D expression in Ly49D/DAP12 Tg mice
To characterize further the block in thymus development in Ly49D/DAP12 × H-2Dd Tg mice, we analyzed DN thymocyte subsets defined by CD44 and CD25 expression. As shown in Fig. 2A, the small proportion of DN1 (CD44+ CD25−) and DN2 (CD44+ CD25+) subsets were not affected in Ly49D/DAP12 × H-2Dd Tg mice expressing H-2Dd. In contrast, the proportion of DN3 (CD44− CD25−) cells was decreased and DN4 (CD44− CD25+) cells were actually increased when Ly49D was ligated by H-2Dd. These effects correlated with the developmental timing of expression of the Ly49D/DAP12 transgenes, as surface expression of Ly49D was first detected in a subset of DN3 cells and became progressively more pronounced for the CD4+ CD8− DN4 and DP subsets (Fig. 2B).

Western blot
Thymocytes were lysed in 1% Nonidet P-40 lysis buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, 2 mM EDTA) containing the protease inhibitor mixture Complete Mini (Roche). Proteins were electrophoresed under denaturing conditions on 14% polyacrylamide gels and then blotted onto Immobilon-P (Millipore) membrane. Membrane was blocked with 5% milk in PBS 0.5% Tween 20 and then incubated overnight with rabbit anti-mouse DAP12 anti-serum [generous gift from Dr. Naoko Aoki (18)] and subsequently with ECL-anti-rabbit–HRP (GE Healthcare). Proteins were detected using ECL reagents (Amersham).

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Ly49D/DAP12-mediated signaling can bypass the pre-TCR checkpoint

During normal T cell development, the TCRβ locus is rearranged and expressed at the DN3 stage. TCRβ protein then associates with CD3 components and the pTα-chain to form a pre-TCR, which is necessary to drive further T cell development through the subsequent DN4 and DP stages. Because expression of the Ly49D/DAP12 transgenes coincided with expression of the pre-TCR in DN3 thymocytes (Fig. 2), we investigated whether Ly49D/DAP12 signaling could interfere with this critical developmental step. As shown in Fig. 3A, a large proportion of thymocytes in Ly49D/DAP12 Tg mice progressed to the DN4 and DP stages in the absence of ic TCRβ expression when H-2Dβ ligand was present. These unusual ic TCRβ- thymocytes were nevertheless T lineage cells because they all expressed ic CD3ε (Fig. 3B). Furthermore, ic TCRβ- thymocytes were not γδ lineage cells that had been aberrantly redirected to the DP stage, as they did not express surface or ic TCRγδ protein (data not shown). Taken together, these data suggest that signaling through the activating NKR Ly49D at the DN3 stage of development can bypass pre-TCR signaling and allow thymocytes to progress to the DN4 and DP stages in the absence of expression of ic TCRβ protein.

To prove formally that Ly49D-mediated ITAM signaling can bypass the pre-TCR checkpoint, we crossed Ly49D/DAP12 Tg mice on a RAG1-/- background. RAG1-/- mice are unable to rearrange TCRβ genes, and consequently thymocyte development is completely blocked at the DN3 stage. Notably, the Ly49D/DAP12 Tg promoted development of RAG1-/- thymocytes to the DP stage in the presence of H-2Dβ ligand (Fig. 3C). The absolute number of DP thymocytes induced by Ly49D engagement in RAG1-/- mice was in fact very similar to the absolute number of ic TCRβ- DP thymocytes in Ly49D/DAP12 × H-2Dβ Tg mice on a WT background. These data establish formally that Ly49D signaling can bypass the pre-TCR checkpoint in RAG1-/- mice and further suggest that a significant fraction of DN3 cells undergoing Ly49D/DAP12 signaling in WT mice fail to rearrange and express TCRβ.

Ly49D/DAP12 signaling mimics pre-TCR signaling as measured by CD5 expression

CD5 is a phosphatase that negatively regulates TCR signaling. Studies from several groups using TCR Tg mice have clearly established that CD5 expression levels on mature T cells correlate with the avidity of TCR interactions with self MHC–peptide complexes (25, 26). In this way, TCR signaling during thymus development can presumably be maintained within a window that is compatible with positive selection. CD5 levels are also influenced by pre-TCR signaling, as ic TCRβ- DN3 cells express significantly more CD5 than their ic TCRβ+ counterparts (Fig. 4A and Ref. 25). Therefore, we reasoned that Ly49D signaling, which is mediated via ITAM motifs on the associated DAP12 adapter chain, would affect CD5 levels in a similar fashion as other ITAM-dependent signaling pathways such as those mediated through the TCR and pre-TCR. To test this hypothesis directly, we monitored CD5 expression levels on thymocytes at various developmental stages in Ly49D/DAP12 Tg mice in the presence or absence of

FIGURE 1. Tg coexpression of Ly49D and DAP12 induces ITAM-dependent impairment of thymic development in presence of ligand. A, CD4/CD8 ratio was analyzed in absence (WT B6 background, H-2Dβ) or presence (H2-Dd Tg) of ligand for Ly49D. Non-Tg littermates were used as controls. Thymus size (cell number) is indicated above each contour plot and percentage CD4/CD8 positive cells (±SD n = 3) below. B, Histograms showing absolute numbers of thymic populations in the different Tg mice or littermate controls. SD calculated from three mice. Fold reduction of cell numbers in Ly49D/DAP12 Tg promoted development of RAG1-/- mice is indicated. *p < 0.001, **p < 0.05. C, Ly49A transgene reverses the block in thymocyte development in Ly49D/DAP12 × H-2Dβ Tg mice. CD4/CD8 ratio was analyzed in presence of H2-Dβ ligand in the Tg mice for Ly49A or for Ly49D/DAP12 and in the inbred mice expressing the two receptors (Ly49A × Ly49D/DAP12 × H-2Dβ). Thymus size (cell number) is indicated above each contour plot and percentage CD4/CD8 positive cells (±SD n ≥ 2) below. D, TCRβ transgene cannot rescue thymocyte development in Ly49D/DAP12 × H-2Dβ Tg mice. CD4/CD8 ratio was analyzed for Ly49A/DAP12 × H-2Dβ Tg mice in absence or presence of a TCRβ transgene. Thymus size (cell number) is indicated above each contour plot and percentage CD4/CD8 positive cells (±SD n = 3) below.
H2Dd ligand (Fig. 4A). Notably, CD5 was dramatically upregulated upon Ly49D engagement in DN3 and DN4 thymocyte subsets independently of ic TCRβ expression (Fig. 4B). These data provide suggestive evidence that ITAM signaling through Ly49D/DAP12 mimics (and in fact quantitatively exceeds) physiological ITAM signaling via the pre-TCR in immature thymocytes.

Ly49D/DAP12 signaling induces calcium flux in immature DN thymocytes

ITAM signaling via Ly49D/DAP12 cross-linking has been shown to induce a tyrosine phosphorylation cascade involving the Syk family kinases Syk and ZAP70, which ultimately leads to calcium mobilization in both normal NK cells and Ly49D-transfected JURKAT cells (27). In Ly49D/DAP12 Tg mice, peripheral T cells stimulated by Ly49D engagement became activated as assessed by phenotypic markers, proliferation, cytokine production, and cytotoxicity (6); however, proximal events linked to ITAM signaling were not investigated. To investigate whether downstream signaling events can be induced by Ly49D/DAP12 in immature thymocytes, we measured calcium flux in purified DN or gated CD25+ DN thymocytes from control or Ly49D/DAP12 Tg mice upon ligation of Ly49D with specific mAbs. As shown in Fig. 5A, anti-Ly49D mAbs induced a rapid calcium flux in DN thymocytes from Ly49D/DAP12 Tg mice (but not from control WT mice) that was quantitatively greater than the calcium flux induced by either of two anti-CD3 mAbs that were tested in parallel. Calcium flux could be induced in both small (ic TCRβ-2) and large (ic TCRβ+3) CD25+ DN thymocytes by anti-Ly49D mAbs (Fig. 5B), indicating that the presence of Ly49D/DAP12 and ligand (H2-Dd) on a Rag1-/- background. Collectively, these data confirm that Ly49D ligation can induce downstream markers of ITAM signaling in immature Ly49D/DAP12 Tg thymocytes and provide a quantitative explanation for the observation that Ly49D-mediated ITAM signaling can override pre-TCR signaling during thymus development.
Rescue of thymocyte development in Ly49D/DAP12 × H-2Dd Tg mice by a Ly49A transgene

To analyze further the role of ITAM signaling in the phenotype observed in Ly49D/DAP12 × H-2Dd Tg mice, we crossed these mice with Ly49A Tg mice. These Tg mice express the inhibitory NKR Ly49A, an ITIM-bearing receptor recognizing H-2Dd ligand, whose expression is directed by the same H-2Kb promoter/IgH enhancer cassette used to generate Ly49D/DAP12 Tg mice (14). Ly49D/DAP12 × Ly49A × H-2Dd Tg mice have a thymic cellularity comparable with that of WT or Ly49A × H-2Dd Tg mice and five times greater than that of Ly49D/DAP12 × H-2Dd Tg mice (Fig. 1C). In addition, the CD4/CD8 staining pattern of thymocyte subsets appears normal in Ly49D/DAP12 × Ly49A × H-2Dd Tg mice, and the DN population is not increased in proportion. Notably, the decreased Ly49D expression in the presence of H-2Dd ligand, which was observed on DN and DP thymocytes in Ly49D/DAP12 Tg mice, was completely restored to normal in Ly49A × Ly49D/DAP12 Tg mice (Fig. 2B). Most importantly, calcium flux induced by anti-Ly49D mAbs in CD25+ DN thymocytes from Ly49A × Ly49D/DAP12 Tg mice could be almost completely inhibited by simultaneous addition of mAbs directed against the inhibitory NKR Ly49A, whereas (as expected) no such inhibition was observed in Ly49D/DAP12 Tg mice (Fig. 5B). Taken together, these results show that impaired thymocyte development in Ly49D/DAP12 × H-2Dd Tg mice can be completely rescued by Ly49A-mediated ITIM signaling, which fully counteracts ITAM signaling induced by Ly49D engagement of H-2Dd.

A TCRβ transgene cannot rescue thymocyte development in Ly49D/DAP12 × H-2Dd Tg mice

Given the appearance of unusual populations of ic TCRβ- thymocytes in Ly49D/DAP12 × H-2Dd Tg mice, it remained formally possible that Ly49D/DAP12-mediated signaling could allow the selective survival and/or expression of a rare ic TCRβ- DN population that arises in normal mice. To address this issue, we crossed Ly49D/DAP12 × H-2Dd Tg mice with TCRβ Tg mice that express a Vβ8.1-DJβ2-4-CJβ2 transgene under the control of the same H-2Kb promoter/IgH enhancer cassette. No detectable rescue of thymocyte development occurred in Ly49D/DAP12 × TCRβ × H-2Dd Tg mice (Fig. 1D), despite the fact that no ic TCRβ- thymocytes could be detected beyond the DN3 stage of development (data not shown). Thus, impaired thymocyte development in Ly49D/DAP12 × H-2Dd Tg mice cannot be explained by the selective expansion of a rare normally occurring ic TCRβ- DN subset.

Ly49D/DAP12 signaling is less efficient than the pre-TCR in promoting proliferation from DN to DP stage

A major function of pre-TCR signaling is to induce and sustain proliferation of DN thymocytes as they mature to the DP stage. Indeed, in normal mice, proliferation of DN3 thymocytes increases sharply when ic TCRβ is expressed, and this high level of proliferation is maintained throughout the ic TCRβ- DN4 stage, only to decrease as thymocytes enter the DP stage. We used Hoechst versus Ki67 staining to determine the percentage of ic TCRβ- or ic TCRβ+ DN3 and DN4 cells in the S+G2/M phases of the cell cycle in Ly49D/DAP12 Tg mice in the presence or absence of ligand. Proliferation of ic TCRβ- DN3 cells was actually increased compared with controls in Ly49D/DAP12 Tg mice in the presence of H-2Dd ligand (Table I), consistent with the fact that CD5 is already upregulated at this stage (Fig. 4). However, after TCRβ rearrangement and pre-TCR expression, proliferation of ic TCRβ+ DN3, and especially ic TCRβ+ DN4 thymocytes, was reduced significantly upon Ly49D engagement (Table I). Indeed, the levels of proliferation of ic TCRβ+ DN4 cells were only slightly higher than those of the expanded population of ic TCRβ- DN4 cells in Ly49D/DAP12 × H-2Dd Tg mice, suggesting that pre-TCR signaling has a minimal effect on proliferation in the presence of high levels of ITAM signaling mediated by Ly49D receptor engagement.

Later stages of thymus and peripheral T cell development in Ly49D/DAP12 × H-2Dd Tg mice

Given that DP thymocytes lacking ic TCRβ expression develop in Ly49D/DAP12 Tg mice in the presence of H-2Dd ligand, it was of interest to investigate whether these cells could mature to the CD4 SP or CD8 SP thymic stage and subsequently emigrate to the periphery. In normal adult WT mice, essentially all CD4 SP and ~75% of CD8 SP thymocytes express surface TCRβ as a result of positive selection and maturation from the DP stage. The remaining CD8 SP cells (not expressing surface TCRβ) are precursors of DP thymocytes that express ic TCRβ. As shown in Fig.
A proportion of these peripheral CD4+ and CD8+ T cells had an unusual TCR ε and δ. Nevertheless, these unusual cells are not found in the periphery of Ly49D/DAP12 Tg mice, almost all CD4+ and CD8+ T cells expressed surface TCR ε and δ present versus 1.6% in absence of ligand. Calcium fluxes are represented as the ratio of fluorescence at 405 nm and 530 nm and were recorded before and after addition of mAbs (arrow) eventually followed by cross-linking with avidin (dashed arrow). B, Ly49A binding by specific mAb (clone JR9-318) impairs calcium flux triggered by anti-Ly49D. DN subpopulations were gated by CD25 staining and forward scatter/side scatter as indicated. Bold line represents calcium flux triggered by anti-Ly49D mAb alone; thin line shows response to anti-Ly49A mAb stimulation; and gray line represents response to simultaneous stimulation with anti-Ly49A and anti-Ly49D mAbs. Results are representative of two experiments.

Table I. Cell cycle analysis of DN3 and DN4 subsets

|        | DN3    |     | DN4    |     |
|--------|--------|-----|--------|-----|
|         | ic TCRβ+ | ic TCRβ- | ic TCRβ+ | ic TCRβ- |
| Ly49D/DAP12 Tg | 12 ± 1 | 49 ± 1 | 17 ± 1 | 49 ± 1 |
| Ly49D/DAP12 × H-2Dd Tg | 27 ± 1 | 43 ± 5 | 22 ± 3 | 28 ± 5 |

Cell cycle was analyzed using Ki67 versus Hoechst profile. Numbers represent percentage of cells in S+G2/M ± SD.

FIGURE 5. Ly49D/DAP12 signaling induces calcium flux in immature DN thymocytes that can be inhibited by Ly49A engagement. A, Calcium flux triggered by anti-Ly49D mAb (clone 4E5) or anti-CD3 mAb (clone 500A2 or 7D6) stimulation of DN-enriched thymocytes from non-Tg and Ly49D/DAP12 Tg mice. Calcium fluxes are represented as the ratio of fluorescence at 405 nm and 530 nm and were recorded before and after addition of mAbs (arrow) eventually followed by cross-linking with avidin (dashed arrow). B, Ly49A binding by specific mAb (clone JR9-318) impairs calcium flux triggered by anti-Ly49D. DN subpopulations were gated by CD25 staining and forward scatter/side scatter as indicated. Bold line represents calcium flux triggered by anti-Ly49D mAb alone; thin line shows response to anti-Ly49A mAb stimulation; and gray line represents response to simultaneous stimulation with anti-Ly49A and anti-Ly49D mAbs. Results are representative of two experiments.

64%, a much larger fraction of both CD4 SP and CD8 SP thymocytes failed to express surface TCRβ in Ly49D/DAP12 Tg mice in the presence of H-2Dd ligand than in the absence of H-2Dd ligand. Moreover, approximately half of the surface TCRβ- CD4 SP and CD8 SP thymocytes also failed to express ic TCRβ in the presence of ligand, whereas essentially no cells with this phenotype were detected in the absence of ligand (Fig. 6B). These unusual ic TCRβ- SP thymocytes did however express ic CD3ε (Fig. 6B) but appeared to be immature in that they expressed high levels of CD24 typical of immature CD8 SP and DP subsets (Fig. 6B). In the periphery of Ly49D/DAP12 Tg mice, almost all CD4+ and CD8+ cells expressed surface TCRβ irrespective of the presence or absence of ligand, although the percentages of CD4+ and CD8+ subsets were reduced ~3-fold when ligand was present (Fig. 6C). CD4/CD8 ratios were maintained at normal levels (1.7 ± 0.2 in presence versus 1.6 ± 0.1 in absence of ligand); however, a larger proportion of these peripheral CD4+ and CD8+ T cells had an activated/memory phenotype in the presence of ligand with downregulation of CD62L and upregulation of CD44 (Fig. 6C). Notably, CD25 and CD69, markers linked to recent activation, were not upregulated (data not shown).

Taken together, these data indicate that some “immature” CD4 SP and CD8 SP thymocytes lacking ic TCRβ expression can develop in Ly49D/DAP12 Tg mice in the presence of H-2Dd ligand. Nevertheless, these unusual cells are not found in the periphery suggesting that they either die in situ in the thymus or fail to survive and/or proliferate after export to peripheral tissues.

Discussion

Signals transduced by the pre-TCR and TCR complexes regulate early stages and positive/negative selection of developing T cells, respectively (2). Fine tuning of T cell differentiation is directly determined by the qualitative and quantitative signals delivered by these ITAM-linked complexes. Other ITAM-signaling receptors, such as activating NKR, which signal through FcRγ or DAP12, are known to costimulate TCR activities in peripheral T cells (3, 5). Recently, we have demonstrated that Tg expression of Ly49D/DAP12 on peripheral T cells can even induce ligand-dependent cellular activation and a full range of TCR-independent effector functions (6). These recent data raise the question of the role of ITAM-linked NKR in conventional T cell development and in particular their ability to influence or replace pre-TCR and TCR signaling in the thymus.

In this study, we show that Tg expression of Ly49D/DAP12 in the presence of the corresponding ligand H-2Dd leads to impaired thymic development due to a block in maturation of thymocytes from the DN3 to DP stages. Ly49D-mediated ITAM signaling early during thymus development led to the appearance of a large population of unusual DN4, DP, and SP thymocytes that failed to express ic TCRβ protein, suggesting that they had bypassed the pre-TCR checkpoint. This interpretation was confirmed by the fact that the Ly49D/DAP12 × H-2Dd transgenes partially rescued thymus development in recombination-deficient RAG1−/− mice. This abnormal differentiation pathway was abortive, however, as ic TCRβ− T cells were not found in the periphery of Ly49D/DAP12 × H-2Dd Tg mice.

Several independent lines of evidence indicate that the thymic phenotypes observed in Ly49D/DAP12 × H-2Dd Tg mice depend upon Ly49D-mediated ITAM signaling. First, no phenotype was observed in the absence of H-2Dd (ligand for Ly49D) or in another Ly49D Tg strain that did not coexpress DAP12, the adapter chain
FIGURE 6. Phenotype of mature thymocytes and peripheral T cells in Ly49D/DAP12 Tg mice in presence of H2-Dd. A. Surface expression of TCRβ was analyzed for CD4 SP and CD8 SP thymocytes. Numbers indicate percentage of surface TCRβ+ cells (±SD, n > 3). B. Intracellular TCRβ expression was analyzed versus surface TCRβ, ic CD3ε, or surface CD24 staining on CD4 SP and CD8 SP thymocytes. Percentage of positive cells in each quadrant is shown below. C. Surface expression of TCRβ, CD44, and CD62L were analyzed for gated CD4+ and CD8+ splenocytes. Percentages of positive cells are shown in the corresponding quadrant of the dot plots. Numbers on histograms indicate the percentage of surface TCRβ+ cells within the ic CD3ε+ CD4+ and CD8+ populations.

Sensitized T cells were intracellularly stained and analyzed by flow cytometry to determine TCR signal strength and associated calcium mobilization. These data were used to estimate the avidity of TCR engagement and to determine the impact of TCR engagement on tyrosine kinase signaling through Leu13 and Leu19. The TCR was cross-linked through the use of anti-TCR antibodies. In addition, the thymic phenotype in Ly49D/DAP12 Tg mice in presence of H2-Dd ligand, as Ly49A readily binds H-2Dd tetramers, whereas Ly49D to H-2Dd is generally very weak (30, 31). Ly49A readily binds H-2Dd tetramers, whereas Ly49D to H-2Dd is generally very weak (30, 31).

The ability of Ly49D-mediated ITAM signaling to bypass the pre-TCR checkpoint is not unique. Indeed, it has been known for some time that signaling via CD3ε, CD4, or through an activated Lck transgene (36, 37) is capable of restoring thymic development to the DP stage in various pre-TCR-deficient mouse models. Moreover, other ITAM-independent signaling pathways such as the CD28/B7 costimulatory axis can bypass the pre-TCR checkpoint in Tg overexpression studies (38). Thus, it appears that the pre-TCR is not unique in its ability to promote proliferation and differentiation of immature thymocytes to the DP stage. Nevertheless the pre-TCR, in contrast to alternative pathways, is the only mechanism to ensure that the TCRβ-chain is productively rearranged and will thus be able to subsequently pair with the TCRα-chain to allow positive and negative selection of DP thymocytes.

Although allowing immature thymocytes to bypass the pre-TCR checkpoint, Ly49D signaling did not mimic pre-TCR signaling in all respects. For example, the levels of CD5 expressed by DN3 and DN4 subsets were much higher in Ly49D/DAP12 × H-2Dd Tg mice than in controls, suggesting that ITAM signaling mediated by Ly49D was much stronger than that mediated by the pre-TCR. This interpretation was directly confirmed by analysis of calcium flux induced by mAb ligation of either Ly49D or the pre-TCR (via CD3) in immature thymocytes of Ly49D/DAP12 Tg mice. Indeed, Ly49D engagement induced much higher levels of ic calcium than pre-TCR stimulation in DN3 thymocytes. Previous studies have shown that pre-TCR-mediated calcium flux in DN3 thymocytes ultimately leads to upregulation of transcription factors such as NF-kB and NFAT, which are involved in cell survival and proliferation (39). Notably, Ly49D/DAP12-induced ITAM signaling significantly inhibited the proliferation of DN3 and DN4 thymocytes expressing ic TCRβ, suggesting that excessive ic calcium levels may disrupt the normal differentiation program orchestrated by the pre-TCR. Whatever the explanation, signaling differences between the pre-TCR and Ly49D/DAP12 are not unexpected because (in contrast to Ly49D) pre-TCR signaling is ligand independent (40).

Although the role of Ly49D-mediated ITAM signaling in bypassing the pre-TCR checkpoint in DN3 thymocytes is clear, the impact of this pathway at later DP and SP stages of thymus development is more difficult to interpret. In this context, unusual subsets of ic TCRβ+ CD4 SP and CD8 SP thymocytes are present in Ly49D/DAP12 × H-2Dd Tg mice. These cells express high levels of CD5 (suggesting that they are undergoing continuous ITAM signaling), but they retain an immature CD24high phenotype and are not detected in the periphery. It is possible that these ic TCRβ+ CD24high subsets represent SP thymocytes that are blocked in their maturation because of inappropriate (or incomplete) Ly49D-mediated ITAM signaling that cannot replace TCR signaling during the positive selection process. Alternatively,
these cells could undergo negative selection due to high levels of ITAM signaling that mimic high-affinity TCR interactions with self peptide–MHC complexes. Crossing Ly49D/APAP12 × H-2Dd Tg mice with TCR Tg mice bred on nonselecting or selecting MHC backgrounds might help to resolve this issue.

Peripheral CD4+ and CD8+ T cells in Ly49d/APAP12 × H-2Dd Tg mice all expressed TCRβ but were reduced 3-fold in frequency and absolute number. The remaining cells had an activated/memory phenotype with increased frequencies of CD44high and CD62Llow cells. It is possible that the peripheral T cell pool in Ly49D/APAP12 × H-2Dd Tg mice arises by homeostatic expansion of small numbers of precursors exported by the thymus. In this regard, the “central memory” CD44high CD62Llow phenotype expressed by the majority of CD8+ T cells is consistent with cells undergoing homeostatic proliferation (41).

In conclusion, we have shown that engagement of ectopically expressed Ly49D receptors by H-2Dd ligand early in thymocyte development induces inappropriately high levels of ITAM signaling that can bypass the pre-TCR checkpoint and lead to abortive development of DP thymocytes that do not express a productive TCRβ-chain. Further studies of this model system may shed light on the unique signaling properties of the pre-TCR that allow optimal progression of immature thymocytes through the β-selection checkpoint.

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Disclosures

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References

1. Hayday, A. C., and D. J. Pennington. 2007. Key factors in the organized chaos of early T cell development. Nat. Immunol. 8: 137–144.
2. von Boehmer, H. 2004. Selection of the T-cell repertoire: receptor-controlled checkpoints in T-cell development. Adv. Immunol. 84: 201–238.
3. Mandelboim, O., D. M. Davis, H. T. Reyburn, M. Valés-Go´mez, E. G. Sheu, L. Pazmany, and J. L. Strominger. 1996. Enhancement of class II-restricted T cell responses by costimulatory NK receptors for class I MHC proteins. Science 274: 2097–2100.
4. Phillips, J. H., J. E. Gumperz, P. Parham, and L. L. Lanier. 1995. Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. Science 268: 403–405.
5. Remontoula, N., A. Berezovska, and A. Marie-Caridine. 2008. Cutting edge: selective expression of inhibitory or activating killer cell Ig-like receptors in circulating CD4+ T lymphocytes. J. Immunol. 180: 2767–2771.
6. Merck, E., R. B. Boyle, and H. R. MacDonald. 2009. Ly49D engagement on T lymphocytes induces TCR-independent activation and CD8 effector functions that control tumor growth. J. Immunol. 182: 183–192.
7. Isakov, N. 1998. Role of immunoreceptor tyrosine-based activation motif in signal transmission from antigen and Fc receptors. Adv. Immunol. 69: 183–247.
8. Ravetch J.V., and L. L. Lanier. 2000. Immune inhibitory receptors. Science 290: 84–89.
9. Lanier, L. L. 2005. NK cell recognition. Annu. Rev. Immunol. 23: 225–247.
10. Pauza, M., K. M. Smith, H. Neal, C. Reilly, L. L. Lanier, and D. L. Lo. 2000. Transgenic expression of Ly-49A in thymocytes alters repertoire selection. J. Immunol. 164: 884–892.
11. Fahlen, L., L. Oberg, T. Brännström, N. K. Khoo, U. Lendahl, and C. L. Sentman. 2000. Ly49A expression on T cells alters T cell selection. Int. Immunol. 12: 215–222.
12. Robson MacDonald, H. R. K. Lees, and W. Held. 1998. Developmentally regulated extinction of Ly49 receptor expression permits maturation and selection of NK1.1+ T cells. J. Exp. Med. 187: 2109–2114.
13. Voyle, R. B., F. Beer, R. K. Lees, J. Schitmann, J. Zimmer, W. Held, and H. R. MacDonald. 2003. Ligand-dependent inhibition of CD1d-restricted NKT cell development in mice transgenic for the activating receptor Ly49D. J. Exp. Med. 197: 919–925.
14. Held, W., D. Cado, and D. H. Rautel. 1996. Transgenic expression of the Ly49A natural killer cell receptor confers class I major histocompatibility complex (MHC)-specific inhibition and prevents bone marrow allograft rejection. J. Exp. Med. 184: 2037–2041.
15. Ioamidis, V., J. Zimmer, F. Beermann, and W. Held. 2001. Cre recombinase-mediated inactivation of H-2M transgene expression: evidence for partial missing self-recognition by Ly49A NK cells. J. Immunol. 167: 6286–6282.