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Transgenic Expression of Ly-49A in Thymocytes Alters Repertoire Selection

Mary Pauza,* Kathleen M. Smith,† Heather Neal,* Christina Reilly,* Lewis L. Lanier,† and David Lo2*

A T cell-specific Ly-49A transgene inhibits TCR-mediated activation in the presence of H-2Dd. Expression of this transgene by developing thymocytes impairs negative selection evidenced by a failure to delete potentially autoreactive T cells and development of a graft-vs-host-disease-like syndrome. In mice carrying both the Ly-49A and a class II-restricted TCR transgene, positive selection was lost, but only when H-2Dd was present on thymic epithelium. These results are consistent with models suggesting that thymic selection is dependent on the perceived intensity of TCR signaling. More interestingly, these results show that Ly-49A does not simply provide a strict on/off switch for T cell responses. Since Ly-49A may shift the signaling threshold of TCR-induced triggering, inducible expression of Ly-49A may regulate peripheral memory/activated T cells by raising the threshold for T cell reactivation. The Journal of Immunology, 2000, 164: 884–892.

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election of the self-MHC-restricted TCR repertoire is known to require recognition of restricting determinants on the selecting MHC molecules along with peptides loaded within the MHC pocket. In studies on positive selection of the TCR repertoire, much attention has been given to the notion that positive selection is dependent on an array of low-affinity agonist ligands (1). This hypothesis suggests that proper signals for positive selection of self-MHC-restricted TCRs fall into an arbitrary “low to intermediate affinity” range. Thus, T cells with receptor binding to self-peptides/MHC complexes on thymic epithelium below a minimum threshold are doomed to “death by neglect,” whereas those binding with much higher affinity are summarily deleted.

The response of T cells to agonist ligands can be modified by a number of factors, including adhesion molecule-mediated enhancement of binding to APC (2), coreceptor CD4 (3) or CD8 (4) binding to MHC, or IL-2 mediated augmentation of low-affinity responses (5). Inhibition of T cell responses can also occur through the triggering of molecules such as CTLA4 (6). The activation of NK cells is also regulated by inhibitory receptors that bind MHC class I ligands (reviewed in Ref. 7). In mice, the Ly-49 family of NK cell receptors are encoded by ~9 polymorphic genes on chromosome 6, and these molecules are expressed on overlapping subsets of NK cells (8–10). Many of the Ly-49 receptors contain immunoreceptor tyrosine-based inhibition motifs (ITIM)3 in their cytoplasmic domains that are responsible for recruitment of intracellular tyrosine phosphatases and inhibition of NK cell function (11–13). Recent studies have also found that peripheral mature T cells are inducible for expression of the NK inhibitory receptors such as Ly–49A, and it is possible that expression of these molecules in activated T cells may influence subsequent immune responses (14–17). Ly–49A expression by T cells is not found during early stages in T cell development, presumably because its activation might alter signals important in triggering specific developmental decisions (18).

In the present work, we have studied the consequences of altering TCR signaling with an Ly–49A transgene. When activated by its target ligand H-2Dd, the Ly–49A molecule inhibits T cell function, in part, through modulation of signaling (11–13). Because these events are dependent on the presence of the Ly–49A ligand, T cell function appears to be normal in the absence of the ligand, allowing for proper assessment of the selected TCR repertoire. Our results support the conventional affinity model of repertoire selection in which the intensity of TCR signaling determines the outcome of positive selection. In addition, the alterations in thymic selection induced by the Ly–49A transgene suggest that T cell expression of Ly–49A can influence both peripheral activation and the TCR repertoire.

Materials and Methods

Generation of the Ly–49A transgene construct

The CD4 transgene cassette is construct “i” from Sawada et al. (19), which was modified to remove the SaI site in the promoter leaving two SaI sites flanking the CD2 cDNA insert. The human CD2 cDNA was excised from the cassette using SalI. The plasmid containing the mouse Ly–49A cDNA (20) was obtained from Dr. Wayne Yokoyama (Washington University, St. Louis, MO). The entire Ly–49A coding sequence was PCR amplified using primers that included SaI sites on their 5’ ends. The PCR product was cloned into a TA vector (Invitrogen, San Diego), sequence verified, then subcloned into the CD4 promoter cassette. Transgene integration was assessed using a 1.15-kb Pol–BglII fragment from the first intron of the CD4 transgene cassette and PCR using primers from the CD4 cassette vector and the Ly–49A insert (CD4 cassette vector sense 5’-CAGATTCCCAAC CAACAAGAG-3’ and Ly–49A antisense 5’-CCATGTTTTTCTGT CCATGACG-3’).
Mice

C57BL/6, B10.A(3R), B10.D2, and B10.HTG mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and The Scripps Research Institute (TSRI) rodent breeding colony. To make transgenic mice, fertilized embryos from C57BL/6 mice were microinjected with Ly-49A transgene DNA according to standard procedures. Founder mice were screened by Southern blot analysis using tail DNA. Ly-49A-transgenic mice were bred to B10.D2 mice until homozygosity was achieved at the MHC as determined by microsatellite analysis. The backcross of TCR-SFE mice (21) to B10.D2, generating TCR-SFE × B10.D2-transgenic mice has been described previously (22); these were used to generate SFE × Ly-49A double-transgenic mice. The SFE TCR is restricted to I-E^d and is specific for the hemagglutinin peptide 110–119 (SFYQFEILFPR) of influenza A/PR/8/34 (H1N1) virus, which binds to phosphatidylserine that becomes translocated to the outer cell membrane shortly after apoptosis initiation. Dual staining with Annexin V, which binds to phosphatidylserine that becomes translocated to the outer cell membrane shortly after apoptosis initiation. Dual staining with Annexin V and propidium iodide (PI), following the manufacturer’s instructions, allows for discrimination between apoptotic (Annexin V^-/PI^+) and necrotic cell death (Annexin V^+/PI^+). Immunoﬂuorescence analysis was performed using a FACs calibur flow cytometer with CellQuest version 3.2 software (Becton Dickinson, San Jose, CA).

Flow cytometric analysis

Mononuclear single-cell suspensions were stained with combinations of anti-CD4-PE, anti-CD8-allophycocyanin, anti-Ly-49A (YEI/48)-FITC, anti-CD62L-FITC, V_53-FITC, V_58-FITC, or V_8-8FITC (PharMingen). Peripheral blood and splenic mononuclear cells were prepared using Lympholyte-M (Accurate Chemical and Scientific, Westbury, NY) before staining. Determination of apoptotic cell death was performed using FITC-conjugated Annexin V (Chontec, Palo Alto, CA), which binds to phosphatidylserine that becomes translocated to the outer cell membrane shortly after apoptosis initiation. Dual staining with Annexin V and propidium iodide (PI), following the manufacturer’s instructions, allows for discrimination between apoptotic (Annexin V^-/PI^+) and necrotic cell death (Annexin V^+/PI^+). Immunoﬂuorescence analysis was performed using a FACs calibur flow cytometer with CellQuest version 3.2 software (Becton Dickinson, San Jose, CA).

In vitro stimulation of CD4 T cells with staphyloccocal enterotoxin B (SEB)

Spleens from male C57BL/6-transgenic or -nontransgenic mice from each lineage were harvested and pooled. Dispersed cells were treated with ammonium chloride solution, washed, and incubated with the following mixture of Abs at 10 µg/ml for 25 min at 4°C: Anti-I-A^b/E^b (2G9), CD11b (Mac-1, M1/70), CD45R (B220, RA3-6B2), TER-119, Ly-6G (Gr-1, RB6-8C5), and CD8 (53-6.7; PharMingen). After washing, cells were incubated with anti-mouse IgG and anti-rat IgG beads (Dynal, Great Neck, NY) for 25 min, and unwanted cells were removed with a magnet. The remaining cells were 65–75% CD4^+ T cells. APC were prepared from C57BL/6 or B10.A(3R) mice, but depleted using anti-CD4 (GK1.5), anti-CD8 (53-6.7; PharMingen). After washing, cells were gated on CD4 single positive populations, the absolute number of apoptotic cells (Annexin V^-/PI^+) was determined.

Histology

Tissues from Ly-49A (B10.D2) and wild-type B10.D2 mice were paraffin embedded, sectioned, and stained with hematoxylin and eosin. Photographs were taken at x 200 (original magnification).

Results

Selection of forbidden clones in Ly-49A-transgenic mice

We generated an Ly-49A transgene driven by a modified CD4 promoter that lacks the sequences suppressing expression in CD8 T cells (19). As a result, in mice harboring the transgene, Ly-49A was expressed early in T cell development, and expression continued throughout T cell differentiation and emigration to the periphery in both CD4 and CD8 T cells (Fig. 1A). In mice expressing the specific ligand H-2D^d, cell surface expression of the transgene was decreased, as described for NK cells in other similar transgenic models (23, 24), but was still present on nearly all T cells (Fig. 1A). The Ly-49A molecule is believed to inhibit NK responses by activating an intracellular phosphatase (SHP-1) when triggered by its ligand (11–13). In similar fashion, Ly-49A activation also inhibited T cell responses, as responses of T cells from Ly-49A-transgenic mice (on the ligand-negative C57BL/6 strain) to the superantigen SEB were inhibited when the APC expressed the Ly-49A ligand (Fig. 1B). This inhibitory effect is also consistent with previous reports with another Ly-49A transgene (25).

The triggering of the transgenic Ly-49A and the presumed modulation of TCR signaling during thymocyte development would predict two related effects on repertoire selection. First, although the diverse preselection repertoire may retain an intrinsic affinity for MHC molecules (26, 27) and preselection thymocytes may have a low stimulation threshold (28), modulation of TCR signaling in the thymocytes during positive selection would reduce most low-affinity signaling events below the minimum threshold to trigger continued development. As a result, many cells expressing receptors that would normally have sufficient affinity to be positively selected would be lost instead through death by neglect. If the proportion of low to moderate affinity self-MHC-restricted receptors is limited, then we should detect an increased steady-state proportion of cells undergoing apoptosis. Indeed, when thymocytes of Ly-49A-transgenic mice backcrossed to the ligand-positive strain B10.D2 were analyzed, we found a decrease in the proportion and number of CD4 single positive cells (Fig. 2A). When gated on CD4 single positive populations, the absolute number of HSA-blow cells (mature CD4 thymocytes) was 4.6 x 10^6 and 0.4 x 10^6 from D^b and D^d thymuses, respectively. Similarly, 1.6 x 10^6 and 0.9 x 10^6 CD8 single positive HSA-blow thymocytes were recovered from D^b and D^d thymuses, respectively. The minimal effect on CD8 thymocytes may be due to competition between Ly-49A and CD8 for binding to H-2D^d. Alternatively, CD8 thymocytes may be less sensitive to Ly-49A-mediated inhibition than CD4 thymocytes. The current studies cannot distinguish these possibilities. When stained with Annexin V, the percentage of apoptotic cells (Annexin V^-) was much higher in B10.D2 backcrossed mice expressing the Ly–49A ligand H-2D^d as compared with transgenic mice backcrossed to B10.HTG expressing the same class II I-A^b but different class I H-2D^d instead of H-2D^d (Fig. 2B).

A second prediction is that although autoreactive receptors with high affinity for self-MHC would normally be deleted, Ly-49A modulation of the TCR signal may instead cause many of the autoreactive receptors to fall in the range for positive selection alone. Positive selection of high-affinity autoreactive receptors would not be a problem in the periphery as long as continued expression of the Ly-49A transgene modulates signaling.
As a first indicator of alterations in thymic-negative selection, we studied the representation of T cells expressing the superantigen-reactive TCR Vβ5 (Fig. 2C). In the presence of the endogenous retroviral superantigens mtv-8 and 9 and the class II I-E molecule, Vβ5 cells were deleted in the thymus (29). In C57BL/6 mice, the percentage of TCR Vβ5 cells reflected the nondeleted numbers, whereas normal B10.D2 mice showed the I-E/superantigen-mediated deletion of Vβ5 cells. Interestingly, Ly-49A-transgenic mice on the B10.D2 background showed a failure in superantigen-mediated deletion. This was evident in thymic single positive subsets, but also in the peripheral lymph nodes and spleen (Fig. 2C and data not shown).

Although the Ly-49A transgene may modulate TCR signaling, the presence of autoreactive receptors may still allow for an initial triggering of the T cells, followed by Ly-49A-mediated suppression of the responding cells. Although this might prevent widespread persistent T cell activation and autoimmune disease, it does appear that a high frequency of the peripheral T cells in the Ly-49A-transgenic mice show evidence for prior activation. The peripheral T cells continued to express the transgene Ly-49A, although at significantly lower levels in the B10.D2 mice (Fig. 1A), indicative of their prior engagement with the ligand. Also in both spleen and lymph node, a very high proportion of the peripheral CD4 and CD8 T cells are negative for the lymph node addressin CD62L (Fig. 2D). However, since the thymus output may be impaired (suggested by the increased intrathymic apoptosis), some or most of this peripheral T cell activation may also be due to Ag-independent expansion to compensate for the lymphopenia.

For the reasons discussed, memory or activation markers might not be reliable indicators of autoreactive T cells. Therefore, to reveal the presence of any functional high-affinity autoreactive cells, lymphocyte proliferation assays were done using stimulators presenting self-MHC class II molecules in the absence of the Ly-49A ligand. In this situation, purified CD4 T cells from Ly-49A-transgenic mice were stimulated with APC from B10.D2 or B10.HTG mice (Fig. 2E). Proliferative responses of lymph node CD4 T cells were very low against B10.D2. Interestingly, CD4 T cells showed significant proliferation against the B10.HTG stimulators, although not as high as to the positive control C57BL/6 stimulators. Similar results were obtained with CD8 T cells from spleen (data not shown). Thus, triggering of the transgene Ly-49A during positive selection in the thymus may permit positive selection of a significant proportion of T cells that would otherwise be deleted due to autoreactivity.

Autoimmune inflammatory syndrome in Ly-49A-transgenic mice: autograft-vs-host-disease?

The high-affinity autoreactive T cells selected by the thymus in the presence of the Ly-49A transgene appear to be generally held in check by the triggering of the Ly-49A in the periphery. However, various mechanisms (persistent TCR activation, decreased activation thresholds, or other unknown mechanism) might cause the autoreactive cells to escape regulation by the Ly-49A transgene. Moreover, as shown above, the level of expression of the transgene can be significantly decreased in the presence of the ligand (Fig. 1A), and, in some T cells, transgene Ly-49A expression might be entirely lost. In any case, autoreactive T cells might escape regulation and produce autoimmune disease. Interestingly, in some lines of Ly-49A-transgenic mice, a significant proportion of mice develop a chronic wasting disease associated with skin keratitis and mixed infiltrates in several tissues. Penetrance is less than complete, for reasons that remain unclear, but it may be related to variable expression of Ly-49A. This occurred only in mice with the Ly-49A transgene on the B10.D2 background, and was never seen in backcrosses onto the B10.HTG background (i.e., lacking the Ly-49 ligand). The syndrome was variably found in mice from two different viral pathogen-free mouse colonies, but the affected individuals generally showed symptoms by 2 wk of age. Finally, in one line of Ly-49A-transgenic mice (line 6343) backcrossed to the RAG-1 knockout, the syndrome was also absent, suggesting that the pathology was dependent on the presence of T or B lymphocytes (data not shown).

The histology of the most severely affected mice showed significant mononuclear infiltrates in the portal tracts of the liver and perivascular/peribronchial spaces of the lung (Fig. 3). Occasional granulocytes, including both neutrophils and eosinophils, could
also be seen. Skin also showed diffuse dermal mononuclear infiltrates, and intestinal epithelium showed scattered submucosal accumulations of mononuclear cells. Finally, the spleen showed extramedullary hematopoiesis and loss of white pulp definition, in part due to replacement by lymphoblasts (data not shown). Interestingly, this pattern of infiltrates is similar to the histopathological picture seen in graft-vs-host disease (30–32), consistent with the notion that the disease may represent the activation of autoreactive T cells.

**Failure in T cell-positive selection in TCR-SFE × Ly-49A-transgenic mice**

One disadvantage of studies on polyclonal T lymphocyte populations is that the effects on Ag-specific receptors cannot be observed in detail. The studies above predict the shifting of receptor thresholds for both positive and negative selection. To examine these effects with a known receptor, we crossed the Ly-49A transgene to TCR-SFE-transgenic mice that express a receptor specific for an
influenza hemagglutinin peptide presented on I-E<sup>d</sup> (21). The expression of the Ly-49A transgene was unaffected by the TCR transgene; therefore, expression was evident in all thymocyte subsets (Fig. 4A). As predicted, the skewing of single positive subsets toward CD4 is lost in the presence of the Ly-49A transgene (Fig. 4B), suggesting that the positive selection signals normally present in B10.D2 thymus fail to influence thymocyte development. This loss of positive selection was also evident in the periphery, shown as the absence of CD4 skewing and reduced total numbers of peripheral T cells (Table I).

**FIGURE 3.** Histology of autoimmune inflammatory syndrome in Ly-49A-transgenic mice. Infiltrates (indicated by arrows) in tissues of Ly-49A-transgenic mice from the 6343 (A and D) and 6295 (B, E, and G) lines compared with nontransgenic B10.D2 control (C, F, and H). Transgenic mice showed periportal infiltrates in the liver (A and B), peribronchial and interstitial infiltrates in the lung (D and E), and infiltrates and edema in the intestinal villi (G). Photographs were taken at ×200 original magnification (D–F) or ×400 original magnification (A, B, C, G, and H).
If the defect in skewing and CD4 T cell production was indeed due to alterations in selection in the thymus, it should be possible to identify the step at which the defect was induced. To identify the cell in the thymus responsible for the presentation of the ligand and the alteration in T cell subsets, we generated bone marrow chimera using TCR-SFE × Ly-49A double-transgenic mice on the B10.D2 background as a stem cell donor. Lethally irradiated recipients. Skewing toward CD4 was restored in B10.D2 recipient (Fig. 4C). Thus, the defect in CD4 T cell production appeared to be due to positive selection on thymic cortical epithelium and was independent of T cell or APC expression of H-2Dd.

Discussion

Modulation of TCR signaling in Ly-49A-transgenic mice

In these studies, we assessed the effects of Ly-49A activation on T cell selection in the thymus. The alterations in positive and negative selection observed are consistent with a general shift in the sensitivity of the signal transduction mechanisms associated with TCR triggering. That is, the Ly-49A transgene appears to provide us with a reversible (+/-) ligand adjustment in the “gain” setting of the TCR complex. However, as simple and useful as this effect appears, we must take into account the complexity of TCR-mediated signaling and phosphatase action.

The modulation of TCR signaling in our studies is likely to be dependent in part on Ly-49A recruitment of the tyrosine phosphatase SHP-1 (11–13). SHP-1 has many substrates, but studies have suggested that its ability to regulate ZAP-70 may be the most relevant to the suppression of TCR-mediated activation (33, 34). However, before SHP-1 can be recruited, Ly-49A must first be phosphorylated on its intracytoplasmic ITIM (35), and it is not clear whether this occurs simultaneously with TCR activation, or whether it can only follow an initial TCR trigger with activation of the associated signaling kinases. The exact sequence of events may have an important influence on the outcome during thymic selection, especially with respect to how the affinity of the specific ligand-TCR interaction is perceived by the T cell. In this regard, it is important to note that in studies on NK cells, the ligation of Ly-49A and recruitment of SHP-1 inhibits early signaling events, including polyphosphoinositide turnover and tyrosine phosphorylation (13). Thus, although TCR-mediated signaling may involve different signaling kinases from those required in NK activation, SHP-1 action may act early in TCR responses as well.

It is not yet clear how the affinity of the ligand/TCR binding is conveyed to the T cell, whether it is through the amount of peak intracellular kinase activity, the kinetics of TCR recycling, or some other mechanism. In addition, it has been shown recently that TCR signaling involves both the assembly of supramolecular activation complexes at the interface with the APC (36) and the reorganization of kinase-rich raft microdomains in response to costimulation through CD28 (37). Since positive selection in the thymus is likely costimulation (CD28) independent, it is not clear how these complex structures would be affected by Ly-49A triggering. Because SHP-1 can inhibit both cytokine receptor signaling and chemokine responsiveness (38, 39), the ability of SHP-1 activation to modulate TCR signaling might not be through a simple reduction in a single parameter. In fact the effect of Ly-49A signaling may be through its action on other signaling pathways distinct from those triggered directly by the T cell Ag receptor. However, despite the potential complications listed here, we were still able to demonstrate predictable shifts in the dose response of T cells in several situations, including thymic positive and negative selection and peripheral T cell responses to self and superantigen.

Does control over the levels of phosphorylation of signaling kinases cause directly proportional effects on signal strength? If activation of SHP-1 is indeed able to affect the dose response of lymphocyte signaling, then a similar (but reciprocal) effect should be evident in mice lacking SHP-1, as in mice carrying the viable motheaten (me”) mutation. That is, T cells from motheaten mice
would be predicted to be hyperresponsive to weak stimuli. Consistent with predictions, both thymocytes and mature T cells from motheaten mice showed increased proliferative responses to TCR activation (40, 41), although Fas-mediated apoptosis also appeared to be defective (42). In the case of B cells, autoreactive cells carrying the motheaten mutation have been shown to be far more sensitive to negative selection by low levels of Ag (43). The consequences with regard to TCR repertoire selection have only recently been examined and show a role for SHP-1 in regulating both positive and negative selection (44). Thus, assuming that Ly-49A acts primarily through SHP-1 recruitment, then it appears that control over transgene Ly-49A activation can effectively serve as a rheostat to regulate the signal intensity generated through lymphocyte Ag receptors.

This effect may have important consequences in normal peripheral T cells with inducible expression of Ly-49 molecules or killer inhibitory receptors (KIR) on human cells (14–17). Activation of naive T cells (negative for Ly-49) would be dependent primarily on the strength of the APC and initial local concentration of target ligand. By contrast, in the activated or memory cell population, cells that have induced expression of Ly-49 molecules would now have acquired a new level of regulation. In effect, Ly-49 activation would raise the threshold for reactivation of these cells and potentially counteract any other mechanisms lowering their signaling threshold. Although the mechanisms regulating inducible Ly-49 or KIR expression on T cells are not yet well defined, manipulation of this phenomenon may be very helpful in modifying ongoing immune responses in vivo.

The affinity model for TCR repertoire selection

Selection of the self-MHC-restricted TCR repertoire in the thymus has to solve a number of potentially incompatible problems simultaneously. First, the repertoire has to be selected with a preference for the recognition of self-MHC molecules as restricting elements. To bind more effectively to the Ag-presenting molecules in the face of great peptide diversity, the receptor repertoire is selected on the basis of effective interaction with exposed surfaces on the MHC molecule. Many of these surfaces include amino acid residues that are polymorphic; therefore, recognition of peptide Ag plus MHC tends to be very much dependent on recognition not only of peptide and MHC class-specific determinants, but also the polymorphic determinants (“restriction determinants”), a phenomenon now described as MHC restriction.

Second, given the random nature of TCR gene rearrangement, the selection of T cells with effective binding to self-MHC molecules necessarily includes a great range of affinities. Among the receptors finally chosen for export, affinities for self-MHC cannot be so high as to trigger T cell responses to the array of normal self-peptides, but still must allow for the potential binding with high affinity to self-MHC-presenting target Ags. In addition, dur-
it requires, the positive selection of receptors with high affinity for self-MHC (“high-affinity autoreactive receptors” in Fig. 5, bottom graph). When the modulating phosphatase is removed by presenting the TCR ligand in the absence of the Ly-49A ligand, the autoreactivity is revealed.

In summary, the results show that by manipulating TCR-mediated signaling, we can alter the fates of specific T cell clones during thymic selection. Specifically, in our Ly-49A-transgenic mouse model, the modulation of TCR signaling causes an increased rate of thymocyte loss due to impaired positive selection. At the same time, many high-affinity autoreactive cells fail to be deleted, and instead proceed through normal positive selection and export to the periphery.

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