Mutation in Fas Ligand Impairs Maturation of Thymocytes Bearing Moderate Affinity T Cell Receptors

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Abstract

Fas ligand, best known as a death-inducer, is also a costimulatory molecule required for maximal proliferation of mature antigen-specific CD4+ and CD8+ T cells. We now extend the role of Fas ligand by showing that it can also influence thymocyte development. T cell maturation in some, but not all, strains of TCR transgenic mice is severely impaired in thymocytes expressing mutant Fas ligand incapable of interacting with Fas. Mutant Fas ligand inhibits neither negative selection nor death by neglect. Instead, it appears to modulate positive selection of thymocytes expressing both class I- and class II-restricted T cell receptors of moderate affinity for their positively selecting ligands. Fas ligand is therefore an inducer of death, a costimulator of peripheral T cell activation, and an accessory molecule in positive selection.

Key words: Fas ligand • T cell development • TNF family • reverse signaling • gld

Introduction

T cell maturation is a carefully orchestrated process by which multipotential cells are sequentially stripped of alternate fates and primarily molded into self-tolerant CD4+ cells, that finally, after traversing transitional CD4+CD8hi and CD44 low CD25lo stages, mature into CD24low single positive (SP) CD4+ or CD8+ T cells (2).

Three distinct checkpoints – β selection, positive selection, and negative selection – ensure development of a T cell repertoire that will best protect against invading pathogens while maintaining self-tolerance. β selection operates through the pre-TCR (TCRβ paired with pre-Fα) and checks that rearrangement has created a functional β chain gene (3, 4). Success at this stage marks the cessation of rearrangement at the TCRβ locus and drives proliferation of thymocytes and entry into the DP compartment. The multi-step process of positive selection rescues thymocytes from programmed cell death (death by neglect) through signals generated when αβTCRs undergo an appropriate interaction with self-MHC/peptide expressed on cortical epithelial cells (for reviews, see references 1 and 2). Positive selection results in CD4/CD8 lineage commitment, the cessation of TCRα gene rearrangement, and self-MHC restriction specificity. Negative selection results in the elimination of thymocytes with overt self-reactivity (for reviews, see references 5 and 6).

While positive and negative selection hinge upon the avidity of a particular TCRαβ for self-MHC/peptide complexes, additional cofactors impact these and other aspects of thymocyte development. The CD8 and CD4 coreceptors clearly influence negative versus positive selection and the acquisition of class I and class II restriction, and the expression of desialylated CD8 molecules by thymocytes increases class I binding affinity (for a review, see reference 7). CD30 and CD28 influence negative selection in vivo, and an in vitro model has provided evidence that CD43 and CD5 both influence negative selection of a subpopulation of thymocytes (for a review, see reference 6). The modulation of positive selection by adhesion and costimulatory molecules is still uncharted, although the apparent requirement for sustained TCR signaling during this checkpoint is compatible with their involvement (8).

One costimulatory molecule with the potential to influence thymic selection is Fas ligand (FasL), a member of the

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The thymus is one of the few organs in the body that co-expresses Fas and FasL. (12, 14, 15), although whether these molecules interact at this site has remained a mystery until recently. This receptor/counter-receptor pair is not required for negative selection of superantigen- or conventional antigen-responsive thymocytes (16–18), except at high antigen concentrations (19). We now show that FasL is required for the proliferative capacity of antigen-reactive peripheral T cells in vivo and in vitro. A similar effect of FasL on thymocyte survival and proliferation has been reported recently. This receptor/counter-receptor pair is not required for negative selection of thymocytes by superantigens (16–18), except at high antigen concentrations (19). We now show that FasL is required for the proliferative capacity of antigen-reactive peripheral T cells in vivo and in vitro. A similar effect of FasL on thymocyte survival and proliferation has been reported recently.

Materials and Methods

Mice. C57BL/6 (B6), B6Smn.C3H-FasL bm1 (B6.gld), B6.MRL-Fas bm3 (B6.Ipr), and BALB/c Pnlm Pep3/bObyj (Ly5.1) (B6.Ly5.1) mice were purchased from The Jackson Laboratory. TCR transgenic (Tg) mice, either generated or backcrossed >10 generations onto a B6 background, include P14 (20), from The Jackson Laboratory, and OT-1 (21), H-Y (22), AND (23), and TEa (24), all bred at the University of Washington. TCR Tg mice were maintained as heterozygotes by crossing TCR Tg with B6 mice and screening PBLs for expression of Vβ5 (OT-1), Vα2 (LCMV and TEa), T3.70 (H-Y), or Vβ3 (AND) by flow cytometry. FasL-deficient OT-1, LCMV, H-Y, AND, and TEa Tg mice were generated by cross/backcross breeding of TCR Tg and B6.gld mice and screening for the gld mutation as described previously (12). Fas-deficient OT-1 mice (OT-1.Ipr) were generated by cross/backcross breeding of OT-1 and B6.Ipr mice, and screening for Vβ5 expression by flow cytometry and for the Ipr mutation by PCR (17). OT-1 and Ipr gld mice on the H-2 b or H-2 k backgrounds were obtained by cross/backcross breeding of OT-1 and B6.Ipr mice, both from The Jackson Laboratory, and screening as above for TCR and gld. Homozygosity for H-2 t was assessed by screening PBLs for expression of H-2 k and absence of H-2 k by flow cytometry. Homozygosity for H-2 bm1 was determined by a PCR protocol provided by K. Hogquist (University of Minnesota, Minneapolis, MN). PCR using tail DNA was performed (35 cycles; 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C) using the forward primer 5'-GGCAGTACGCCCTCGAAGCGCT and the reverse primer 5'-TTATCTGAATTCTTCAGGATCGAG. Reaction products were digested with PstI and run on a 6% polyacrylamide gel to reveal a single band of 190 bp for H-2 k or two bands of 115 and 75 bp for H-2 bm1. AND and AND gld mice on an I-E b background were obtained by cross/backcross breeding of AND with C3H/HeJ or with C3H/HeJ-FasL bm1 mice (both from The Jackson Laboratory) and screening for absence of Vβ3-deleting endogenous superantigens and for Vβ3, I-E, and I-A expression as above. Ly5.1 congenic OT-1 mice (OT-1.Ly5.1) were generated by cross/backcross breeding of OT-1 with B6.Ly5.1 mice and screening for presence of Vβ5 and Ly5.1 and absence of Ly5.2 expression by flow cytometry. All experiments were performed in compliance with the University of Washington Institutional Animal Care and Use Committee.

Flow Cytometry. Single cell suspensions were prepared, stained as described previously (12), and analyzed on a FACScan™ or FACSCalibur™ using CELLQuest software (Becton Dickinson). Dead cells were excluded on the basis of forward and side scatter profiles, and at least 10^4 live-gated events were collected from stained thymus samples. Monoclonal antibodies used to stain cell surface molecules were: FITC-, PE- or allophycocyanin (APC)-labeled anti-CD4 (clone RM4-5), FITC- or biotin-labeled anti-Vα2 TCR (clone B20.2), FITC-labeled anti-CD45.1 (clone A20), FITC-, PE-, or peridinin chlorophyll-α protein (PerCP)-labeled anti-CD8α (clone 53–6,7), PE-labeled anti-Vβ3 TCR (clone KJ25), biotin-labeled anti-CD24 (clone M1/69), anti-CD5 (clone 53–7,3), and anti-H-2 K4 (clone SF1–1.1), all from BD Biosciences, anti-H-2 Y TCR (clone T3.70, provided by Dr. A. Farr, University of Washington), and TriColor (TC)-labeled anti-CD8 (clone CT-CD8α; Caltag Laboratories). Biotin-labeled antibodies were detected with either streptavidin–TC (Caltag) or streptavidin–APC (BD Biosciences).

Surface expression of FasL was detected using the enzymatic amplification staining (EAS) Kit (Flow-Amp Systems) following the manufacturer’s protocol. Thymocytes were incubated with either biotinylated anti-FasL antibody (clone MFL3; BD Biosciences) or biotinylated isotype control hamster IgG (clone A193; BD Biosciences). During the streptavidin–FITC step, cells were simultaneously stained to detect surface CD4 and CD8 expression. Markers showing the percentage of FasL+ cells were set individually on the basis of isotype control staining for each thymic subpopulation in each mouse strain. From 1–6% of isotype control stained cells fell within the FasL+ marker.

Fas Ligand Expression by RT-PCR. Single cell suspensions were prepared from thymic lobes of B6 or OT-1 mice, stained with anti-CD4 and anti-CD8 antibodies and sorted on a FACScan™ cytometer (Becton Dickinson). Total RNA was purified and reverse transcribed using random hexamer primers and AMV reverse transcriptase (Invitrogen). FasL PCR reactions were performed using forward and reverse primers 5'-ATGCGACGCCATGATACCT and 5'-CCCATCTGCTGCAGTGTC. PCR products were run on 1% agarose plus 0.5% Nusieve (FMC Bioproducts), transferred to GeneScreen+ (NEN Life Science Products), and hybridized according to standard protocols. Membranes were probed with the end-labeled oligonucleotide 5'-TGCCCTCTGGTTGGAATTG/3'-TGCAGGCCCATGATACCT and the reverse primer 5'-TTATCTGAATTCTTCAGGATCGAG. Reaction products were digested with PstI and run on a 6% polyacrylamide gel to reveal a single band of 190 bp for H-2 k or two bands of 115 and 75 bp for H-2 bm1. AND and AND gld mice on an I-E b background were obtained by cross/backcross breeding of AND with C3H/HeJ or with C3H/HeJ-FasL bm1 mice (both from The Jackson Laboratory) and screening for absence of Vβ3-deleting endogenous superantigens and for Vβ3, I-E, and I-A expression as above. Ly5.1 congenic OT-1 mice (OT-1.Ly5.1) were generated by cross/backcross breeding of OT-1 with B6.Ly5.1 mice and screening for presence of Vβ5 and Ly5.1 and absence of Ly5.2 expression by flow cytometry. All experiments were performed in compliance with the University of Washington Institutional Animal Care and Use Committee.

Results

Mutation in FasL Alters Thymocyte Maturation in OT-1 TCR Tg Mice. To study the influence of FasL on T cell maturation, we used mice bearing the gld point mutation,
encoding a variant of FasL that is incapable of binding Fas, thereby abolishing both costimulator and death-inducer functions of this bidirectional molecule (11, 26, 27). T cell development was compared between wild type and gld mice either Tg for the (ovalbumin + H-2Kb)-specific OT-1 TCR or expressing a polyclonal TCR repertoire. Fig. 1 A shows notable differences in the CD4/CD8 profiles of wild-type OT-1 (left) and OT-1.gld thymocytes (right). Percentages of cells contained within each of the illustrated gates are shown to the right of the dot plots. Most striking is the reduction of CD8 SP thymocytes in OT-1.gld compared with wild-type OT-1 mice. Both strains have similar numbers and percentages of DN thymocytes, while DP thymocytes are relatively overrepresented in OT-1.gld mice. The reproducibility of these findings is illustrated in Fig. 1 B (left panels). In agreement with previously published work (16, 28, 29), similar analyses of B6 and B6.gld mice expressing a diverse TCR repertoire (Fig. 1 B, right) reveal no such differences. Total thymic cellularity is roughly equivalent between OT-1 and OT-1.gld (see Fig. 4 B, right), and 2–3-fold lower than in B6 and B6.gld mice (unpublished data).

In addition to skewed CD4/CD8 profiles, OT-1.gld thymocytes also display deficient down-regulation of CD24 and up-regulation of TCR expression, both correlated with successful thymocyte maturation (Fig. 1 C). Poor expression of the Tg TCR in OT-1.gld thymocytes is not due to the coexpression of endogenous TCRs, as similar staining patterns were seen using a pan-TCR antibody (unpublished data). Thus, the absence of functional FasL is associated with grossly defective T cell maturation in OT-1 mice, but not in congenic mice expressing a diverse T cell repertoire. OT-1.gld mice do accumulate Tg TCR+ CD8+ T cells in the periphery (unpublished data), which may be driven by homeostatic regulation of peripheral T cell niches.

It is likely that disruption of Fas–FasL interactions is largely responsible for the defect in maturation experienced by thymocytes expressing mutant FasL. T cell development in Fas-deficient OT-1.lpr mice exhibits a pattern similar to

![Figure 1](http://rupress.org/jem/article-pdf/198/2/349/1146984/jem1982349.pdf)

Figure 1. Defects in FasL alter thymocyte maturation in OT-1 TCR Tg mice. (A) CD4/CD8 profiles of thymocytes from OT-1 (left) and OT-1.gld (middle) mice are compared. Gates designated by letters in the dot plots represent thymocyte subpopulations described in the table (right), listing percentages of thymocytes in each compartment. Bold numbers highlight major differences observed. (B) Percentages of thymocytes in the indicated subpopulations are shown for individual animals. Left panels: OT-1 (circles, n = 8) are compared with OT-1.gld (triangles, n = 7) and OT-1.lpr (diamonds, n = 5) mice. Right panels: B6 (circles, n = 4) are compared with B6.gld mice (triangles, n = 4). Means are designated by solid bars. Note changes in y-axis scales for DP thymocytes. (C) Va2 (top) and CD24 (bottom) expression in DP, CD8+CD4dim (transitional cells along the CD8 lineage), and CD8 SP thymocytes is compared between OT-1 (open histograms, thick lines) and OT-1.gld mice (light gray histograms), and in CD8 SP thymocytes between OT-1 and two OT-1.lpr mice (rightmost panels). OT-1.lpr 1 (open histograms) had 3.5% CD8 SP, while OT-1.lpr 2 (dark gray histograms) had 6.5% CD8 SP remaining.
that seen in OT-1.gld mice (Fig. 1 B, left), although the abnormal representation within each subcompartment is less severe in the former relative to the latter mice. Furthermore, thymocytes from OT-1.lpr mice with the most severely depleted CD8 SP compartments tend to display the most abnormal CD24 and TCR expression levels (Fig. 1 C, right, and unpublished data).

**FasL Is Expressed by Both Wild-Type OT-1 and B6 Thymocytes.** To determine whether dysregulated FasL deficiency on thymocyte maturation in B6 versus OT-1 mice, FasL expression in thymocyte subpopulations from these two strains were compared at both the molecular and cellular levels. A semi-quantitative RT-PCR assay demonstrates FasL expression in each thymocyte subpopulation, although in some cases at low levels compared with positive controls, without any appreciable difference between OT-1 and B6 mice (Fig. 2 A).

FasL protein on the cell surface was also revealed using EAS, a flow cytometric technique for amplifying weak surface expression (30). Fig. 2 B shows that FasL is expressed on the surface of both B6 and OT-1 thymocytes, at levels that vary with developmental stage (Fig. 2 B). At the DN stage, 16–31% of B6 and 21–32% of OT-1 thymocytes are FasL⁺; 7–14% of B6 and 20–29% of OT-1 DP thymocytes express FasL surface protein. Beyond this stage, FasL expression diminishes in both strains. Although there is an increased percentage of FasL⁺ DP thymocytes in OT-1 relative to B6 mice, this is not universally correlated with susceptibility to the influence of FasL on T cell development (see below). Thus, expression of FasL at both the RNA and surface protein levels suggests that the maturational defects seen in OT-1.gld thymocytes are not the result of developmentally dysregulated FasL expression in TCR Tg mice.

**The Defect in OT-1.gld Thymocyte Development Is T Cell Autonomous and Cannot Be Rescued by Neighboring Wild-Type Thymocytes.** To localize the influence of FasL on T cell maturation to either the thymic epithelium or BM-derived cells, radiation chimeras were constructed using OT-1 or OT-1.gld BM donors and irradiated B6 or B6.gld hosts. In each of the four types of chimeras analyzed at all time points, the thymocyte subcompartment composition was dependent upon the BM donor type. Representation of donor CD8 SP thymocytes was diminished using OT-1.gld BM, whether the host was of wild-type (Fig. 3 A, open bars) or gld background (gray bars), compared with that of wild-type OT-1 BM in either host (striped and black bars). Therefore, FasL expression by BM-derived cells dictates the pattern of thymocyte maturation, while...
FasL expression by radioreistant thymic epithelial cells does not.

Mixed BM chimeras were generated to determine whether BM-derived cells expressing wild-type FasL can rescue the maturation of neighboring OT-1 gld thymocytes. T cell–depleted BM cells from OT-1 and OT-1 gld donors were mixed 1:1 and used to reconstitute irradiated B6 and B6 gld recipients. In each of three separate experiments analyzed at three time points postreconstitution, maturation of wild-type OT-1 thymocytes failed to correct the defective maturation of OT-1 gld T cells within the same thymus (Fig. 3 B). Thus, T cells expressing mutant FasL fail to mature properly, and their development cannot be rescued in trans.

The OT-1 gld Defect in T Cell Maturation Does Not Result from Defective Negative Selection or Death by Neglect. To test whether FasL influences T cell maturation through cell death by negative selection or lack of selection leading to death by neglect, the distribution of thymocytes was compared on wild-type and gld backgrounds in which thymocytes expressing the OT-1 TCR are positively selected (H-2Kb), negatively selected (H-2d), or not selected (H-2Kbm1). The CD4/CD8 profiles of thymocytes on the H-2d and H-2Kbm1 backgrounds are indistinguishable in OT-1 and OT-1 gld mice (Fig. 4 A). As expected (31), OT-1 thymocytes on the H-2d and H-2Kbm1 backgrounds are depleted for CD8 SP and enriched for DP cells, as are OT-1 gld thymocytes on the H-2k background (Fig. 4 B). The total thymic cellularity in wild-type mice is lower in the negatively selecting H-2d background and higher in the neutral H-2Kbm1 compared with H-2Kbm1 background, as previously shown (31). However, their gld counterparts look no different, indicating that there is no defect in negative or nonselection in the absence of functional FasL. In addition, on the negative-selecting and neutral backgrounds, thymocytes from OT-1 and OT-1 gld mice express similar levels of both TCR and CD24 (unpublished data).

FasL Can Also Influence the Maturation of Class II–restricted T Cells. To examine whether the T cell maturational defects in OT-1 gld mice can be generalized to other TCR Tg lines, the class I–restricted H-Y and P14 TCR transgenes were bred onto the gld background, as were two class II–restricted TCR transgenes, AND and TEa. T cell maturation is unaffected by the gld mutation in H-Y and P14 TCR Tg mice (Fig. 5 A). However, FasL deficiency leads to a decrease in the number of CD4 SP thymocytes and reduced thymic cellularity in cytochrome c–specific AND TCR Tg H-2d mice (Fig. 5 B, left). Wild-type AND CD4 SP thymocytes also attain a more mature phenotype than their gld counterparts, as evidenced by up-regulation of TCR and down-regulation of CD24 expression (Fig. 5 B, right). Thus, FasL influences the size of the mature thymocyte compartment in a class II–restricted TCR Tg mouse strain. These differences were not seen when comparing TEa and TEa gld mice (unpublished data), indicating that this maturational defect is not universal for thymocytes expressing all class II–restricted TCRs.

No correlation exists between FasL expression levels and the degree to which FasL influences thymocyte maturation. FasL expression by AND and P14 thymocytes is indistinguishable (Fig. 5 C), although thymocyte maturation in the former strain is defective on the gld background, while the latter is unaffected (Fig. 5, A and B). Thus, defects in thymocyte maturation due to mutation in FasL cannot be solely attributed to differences in FasL expression among the wild-type TCR Tg lines. It may be significant that the fraction of FasL+ thymocytes in the H-Y TCR Tg line is substantially lower than that in the other Tg strains (see Discussion).

Increasing the Strength of Positive Selection Bypasses the Influence of FasL on T Cell Maturation in AND Mice. To investigate whether the strength of signal delivered through the TCR is an important variable in determining the contribution of FasL to T cell maturation, analyses were extended to wild-type and gld AND TCR Tg mice carrying I-A^b or I-E^k class II MHC molecules that mediate weaker and

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**Figure 3.** The defect in OT-1 gld thymocyte maturation is T cell autonomous and cannot be rescued by neighboring wild-type thymocytes. (A) Single BM chimeras were analyzed 4, 6, and 8 wk after reconstitution; only the latter two time points are shown. Thymocytes were stained for CD4 and CD8 and mean percentages of donor CD8 SP cells were calculated for each chimera type (striped bars = OT-1 → B6; black bars = OT-1 → B6.gld; open bars = OT-1 gld → B6; gray bars = OT-1 gld → B6.gld). Error bars represent the range of two data points for each group. (B) Mixed BM chimeras were analyzed at 4, 6, and 8 wk after reconstitution; results from week 8 are shown. Top panels: CD4/CD8 profiles of thymocytes from the two different donor types (OT-1 or OT-1 gld) within a single B6 gld host. Dot plots are gated on donor type using Ly5 allelic differences to distinguish donors from each other and the host. Percentages of CD8 SP thymocytes contained within each donor component are shown. Data are representative of three experiments; n = 2–4 for each group.
stronger positive selection for this TCR, respectively (32, 33). FasL expression on wild-type and thymocytes on an I-Ek background is similar to that on an I-Ab background (Fig. 5 C). However, the level of CD5 expression is higher in both DP TCRhi and CD4 SP TCRhi subsets from I-Ek–expressing mice (Fig. 6 A). This up-regulation is expected, given that CD5 expression levels on postselection thymocytes reflect the intensity of signals delivered through the TCR during selection (34), and indicates that the signal delivered to the AND TCR is indeed stronger in I-Ek– than in I-Ab–expressing mice.

Analyses of thymocyte subsets in AND and AND, gld mice expressing I-A^b versus I-E^k reveal the impact of this increase in signal strength. I-Ek not only delivers a stronger positively selecting signal to thymocytes bearing the AND TCR, it also mediates some negative selection, thereby lowering thymus cellularity (reference 33; Fig. 6 B, left). On a homozygous or heterozygous I-E^k background, thymocytes from both AND and AND, gld mice develop normally (Fig. 6 C). Their TCR expression levels are identical (Fig. 6 C) and their CD4/CD8 profiles (unpublished data) and CD24 expression levels are similar to each other (Fig. 6 C) and to wild-type AND on the I-A^b background. Thus, increasing the strength of the positive selection signal overrides the effect of FasL costimulation on the course of T cell development.

Discussion

The FasL variant expressed by gld mice renders them susceptible to a peripheral lymphoproliferative disease caused by the inability of mutant FasL to send death signals through the Fas receptor (26, 27). Recent data from this laboratory have demonstrated that mutant FasL is also incapable of mediating a costimulatory signal that drives maximal proliferation in mature, antigen-reactive T cells (11–13). We now demonstrate that mutations in FasL also impact T cell development. Thymocytes from gld mice carrying certain TCR transgenes display aberrant development (Figs. 1 and 5). This developmental perturbation is evidenced by a decrease in mature thymocytes and altered expression of maturation markers such as the TCR and CD24. While there are no gross alterations in thymocyte maturation in B6, gld mice with a diverse TCR repertoire (references 16, 28, and 29; Fig. 1 B), we have detected subtle but reproducible maturational defects. The expression levels of several, but not all, V J252 elements are depressed in SP thymocytes of B6, gld mice compared with their wild-type counterparts (unpublished data), mirroring the decrease in TCR expression levels seen in OT-1, gld and AND, gld mice. Minor repertoire differences were also detected, with a lower fraction of both CD4 and CD8 SP thymocytes from B6, gld mice expressing VB3 (the $\beta$ chain of AND), and a slight overrepresentation of VB6+ CD8 SP in B6, gld relative to wild-type B6 mice. In addition, CD8 SP thymocytes expressing VB5 (the $\beta$ chain of OT-1) are underrepresented in B6, gld relative to wild-type mice (unpublished data), thus extending our findings beyond TCR Tg mice.

It is possible that FasL on the surface of a BM-derived cell binds Fas on an epithelial cell, thereby initiating positive signaling through Fas, signaling that has been demonstrated in human T cells (35, 36). Such an interaction could induce the secretion of a positive mediator, influencing...
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However, the fact that thymocytes expressing wild-type FasL cannot rescue the maturation of neighboring gld thymocytes, even those sharing the same TCR (and thus recognizing the same ligands), renders this explanation unlikely (Fig. 4 B). We instead favor the simpler interpretation that Fas and/or some other molecule expressed by BM-derived cells or thymic epithelial cells binds to FasL on thymocytes and initiates delivery of a positive reverse signal through FasL. This interpretation is consistent with our data, offers a unifying role for FasL in thymocyte maturation and peripheral T cell activation (11–13), and may also explain how Fas can influence T cell maturation by a mechanism that does not involve cell death pathways (37). It is interesting that thymocyte maturation in OT-1.lpr mice is intermediate between that of OT-1 and OT-1.gld mice (Fig. 1, B and C). This attenuated phenotype may be due to the known leakiness in the lpr phenotype in the thymus (18) or the possible existence of an alternate FasL binding partner (our unpublished data). Although it is possible that the gld mutation is permissive for the FasL function on stromal cells but not on thymocytes, our data strongly suggest that the defect in thymocyte maturation in OT-1.gld relative to OT-1 mice is T cell autonomous (Fig. 3) and likely results from abortive FasL interactions with Fas (or an alternate partner), rather than dysregulated FasL expression (Fig. 1, B and C, and Figs. 2 and 5 C).

Two of our observations do not fit well with the notion that FasL delivers a positive signal that costimulates positive selection. The total number and TCR expression levels of thymocytes in AND.gld mice on an H-2b background are unexpectedly similar to those in AND mice on an H-2k background (Fig. 5 B, and Fig. 6, B and C). These data are perhaps more consistent with a role for FasL as a negative

Figure 5. Maturation of thymocytes expressing some, but not all, TCRs is influenced by FasL. (A) Percentages of thymocyte subpopulations based on CD4/CD8 expression were compared between wild-type (circles) and gld H-2b mice (triangles) Tg for the P14 TCR (left panel; wild-type and gld n = 4) and the H-Y TCR (right panel; wild-type n = 2, gld n = 3). Means are designated by solid bars. (B) Left: numbers of total and CD4 SP thymocytes were compared between AND (circles; n = 6) and AND.gld (triangles; n = 6) TCR Tg H-2b mice. Means are designated by solid bars. Right: Vβ3 (top) and CD24 (bottom) expression were compared between wild-type (open histograms) and gld (gray histograms) CD4 SP thymocytes from AND TCR Tg H-2b mice. Means are designated by solid bars. (C) Thymocytes from wild-type P14 (top row), H-Y (second row), and AND on H-2b (third row), and H-2k backgrounds (bottom row) were stained for CD4, CD8, and FasL as in Fig. 2 B. FasL expression (gray histograms) is compared with isotype control (open histograms) for gated DN (left histograms) and DP (middle histograms) thymocytes in all strains, CD8 SP in P14 and H-Y, and CD4 SP in AND (right histograms) mice. Numbers represent the percentage of FasL+ cells falling within the indicated markers.
regulator of TCR signaling. According to this alternate model, thymocytes from AND mice on an H-2b background express mutant FasL might receive a stronger signal than wild-type mice, and would display a mixed positively and negatively selected phenotype characteristic of CD5 levels on DP thymocytes from AND mice on an H-2b background.

Figure 6. Increasing the strength of the positive selection signal bypasses the need for FasL during thymocyte development. (A) CD5 expression is compared between wild-type AND TCR Tg mice on H-2b backgrounds (open histograms) and H-2b backgrounds (gray histograms). Histograms represent DP Vβ3hi (left) or CD4 SP Vβ3hi thymocytes (right); mean fluorescence intensity (MFI) for each is shown. (B) AND TCR Tg mice on H-2b (wild-type and gld n = 6), H-2b/H-2k heterozygous (wild-type and gld n = 4) and H-2k backgrounds (wild-type and gld n = 4) were compared. Numbers of total (left panel) and CD4 SP thymocytes (right panel) are shown for AND (circles) and AND.gld (triangles). Means are designated by solid bars. (C) Vβ3 (left) and CD24 (right) expression were compared between wild-type (open histograms) and gld (gray histograms) CD4 SP thymocytes from AND TCR Tg mice on an H-2b background.

from H-2b AND.gld are the same as wild-type, while CD5 levels on SP thymocytes from AND.gld are higher than on wild-type (Fig. 7 B). The reverse is true for OT-1: CD5 expression levels are higher in OT-1.gld DP, but overlapping with wild-type thymocytes at the SP stage (Fig. 7 B). This model also predicts that CD5 levels on H-2b AND.gld thymocytes should be similar to H-2b AND. However, CD5 levels on the former cells are lower than on the latter at all stages (Fig. 7 B). The absence of a consistent relationship between CD5 levels and expression of mutant FasL may suggest that while CD5 senses the strength of signal through the TCR, it may not sense the modulation of that signal by FasL or other accessory molecules. Finally, this model would predict that maturation of the remaining SP thymocytes should be efficient in all strains of mice. As such, CD24 levels should be similar on thymocytes from H-2b AND.gld and on H-2b AND thymocytes. However, CD24 levels are highest for H-2b AND.gld, intermediate for H-2b AND.gld, and lowest for AND on both H-2b and H-2k backgrounds (with mean fluorescence intensities [MFIs] of 296, 220, 198, and 192, respectively; Figs. 5 B and 6). OT-1.gld SP thymocytes also display aberrantly high CD24 expression compared with their wild-type counterparts. We therefore favor a model invoking a role for FasL as a positive modulator of thymocyte maturation as most compatible with the data described above and with previous studies demonstrating a costimulatory role for FasL in mature peripheral T cells (11–13).

Fas is expressed in the thymus by embryonic day 16.5 (14) and in adult DP and SP thymocytes (38–40). FasL RNA is expressed at low levels in embryonic and adult thymus (references 14, 15, 38, 39, and 41; Fig. 2 A) as well as in stromal cells. In addition to FasL message, we have also detected FasL surface expression by flow cytometry (Figs. 2 B and 5 C) using a technique to enhance weak staining obtained by conventional methods (30). Newly synthesized FasL travels directly to secretory lysosomes in mature T cells and undergoes targeted release upon vesicle degranulation (42). Such a directional release of FasL could explain why DP thymocytes do not commit suicide despite their expression of Fas and their known susceptibility to death mediated by anti-Fas antibodies and by FasL (39, 40). SP thymocytes coexpressing Fas and FasL are not susceptible to Fas-mediated death (39), clearly indicating that additional control is exerted over this pathway, perhaps by expression of Fas splice variants lacking the death domain (43) or decoy receptors for FasL that do not induce death (10).

What is the basis for the differential influence of FasL on T cell maturation in polyclonal B6 mice and the various lines of TCR Tg mice? Given that thymocyte maturation defects are evident in TCR Tg mice, TCR locus accessibility to the recombinase machinery is not an issue. Likewise, lineage commitment does not appear to be skewed by the Fas/FasL interaction, because entry into both the mature CD4 and CD8 compartments is impaired by the gld mutation (Figs. 1 and 5 B). Instead, this differential impact is likely due to the nature of the TCRs expressed by the developing thymocytes, as FasL expression levels in thy-
mocyte subpopulations are not correlated with defective maturation in the absence of functional FasL (Figs. 2 and 5 C). Several possible explanations may account for why thymocytes expressing only some TCRs are sensitive to FasL-mediated signals.

**FasL Encodes a Peptide that Interacts with Certain TCRs, and This Peptide Is Altered by the gld Mutation.** Defective T cell maturation in OT-1.lpr mice and the fact that maturation of both class I- and class II–restricted T cells is enhanced by functional FasL renders this explanation unlikely. Although an H-2Kb–binding peptide within wild-type FasL is destroyed by the gld mutation (unpublished data), this peptide does not appear to influence positive selection when assayed in vitro for the ability to generate DPPhifrom DPbright OT-1 thymocytes (S. Jameson and K. Hogquist, personal communication).

**Mutant FasL Is Incapable of Initiating Fas-mediated Death of Negatively or Nonselected Thymocytes.** Previous studies (16, 18) and the normal representation of thymocytes in B6.gld mice weigh against this explanation for the defect in TCR Tg gld mice. In addition, no reproducible differences in either the uptake or loss of bromodeoxyuridine in OT-1.gld relative to OT-1 or AND.gld relative to AND thymocytes were apparent (unpublished data), indicating that movement of AND.gld and OT-1.gld thymocytes into and out of cellular subcompartments is not grossly disrupted. Thus, cell cycle arrest of CD4 T cells (44) is an unlikely explanation for our findings. Furthermore, crossing both wild-type and gld OT-1 TCR Tg mice onto negative-selecting (H-2b) and nonselecting (Kbm1) backgrounds emphasizes that mutant FasL does not influence thymocyte death either by neglect or by negative selection (Fig. 4). Inhibition of death pathways is therefore not the means by which mutant FasL alters T cell maturation in a TCR–specific manner.

**The Precise Timing of TCR Expression During Development Dictates the Influence of FasL on Thymocyte Maturation.** TCRαβ transgenes are expressed early in the DN stage, before rearrangement of the endogenous TCRβ locus (45), while asynchronous expression of first TCRβ and then TCRα is required for normal thymocyte maturation and proliferation (46, 47). Although expression of the Tg TCR is particularly early in the H-2Tg line, OT-1, P14, and AND Tg mice all show a similar pattern of TCR expression in the DN3 and DN4 compartments (unpublished data). Thus, it is unlikely that FasL influences T cell maturation in OT-1 and AND I-Aβ TCR Tg mice because the timing of TCR expression meets some requirement not satisfied by the other transgenes.

**The Avidity of Interaction Between the TCR and Its Positively Selecting Ligand Determines Whether FasL Costimulates Maturation.** FasL costimulation occurs in peripheral CD8+ T cells in conjunction with signals delivered through the TCR, and this costimulation plays a greater role under conditions of suboptimal activation (11). We speculate that this notion of an upper and lower boundary also holds true for FasL costimulation during thymocyte development. According to this hypothesis (Fig. 7 C), the interaction between the P14 TCR and its positively selecting ligand is of high enough avidity (34) to maintain sustained signaling for successful positive selection, and although FasL is up-regulated in thymocytes of these mice (Fig. 5 C), Fas/FasL interactions become redundant at avidities of this strength.
OT-1 (in a K\(^b\) environment) and AND on an I-A\(^b\) background fall within the boundaries that delineate the range of avidities for which FasL costimulation is required for efficient positive selection, and T cell development in these strains would, therefore, suffer in the absence of functional FasL. The AND TCR on the I-E\(^b\) background increases the efficiency of positive selection (32), and nudges this receptor out of the window of affinities sensitive to FasL costimulation (Fig. 7 C). Thus, while FasL surface expression is up-regulated in OT-1, P14, and AND TCR Tg mice on both backgrounds, its relevance is not dictated solely by its presence, but is also dependent on the avidity of the particular TCR for its selecting ligand. The comparison of AND TCR Tg mice on the I-A\(^b\) versus the I-E\(^k\) background, where maturation is induced by weak versus strong positive selection signals delivered through the identical TCR, provides the strongest evidence in support of our model.

More speculative are the events that define the lower boundary of FasL costimulation, beyond which lies the maturation of thymocytes in H-Y TCR Tg mice. Several explanations may account for the lack of influence of FasL on the differentiation of thymocytes bearing such a TCR with low avidity for its selecting ligand. It is possible that H-Y thymocytes, while expressing TCRs capable of being positively selected, receive a signal too weak to initiate the entire program of events driving efficient differentiation, a program which may involve the up-regulation and subsequent utilization of FasL. If FasL is never properly up-regulated, its absence would not alter thymocyte maturation. This notion is compatible both with the lower expression of CD5 in postselection thymocytes and peripheral T cells of H-Y mice compared with those from P14 (34) and OT-1 mice (our unpublished data), and with the low level of FasL surface expression by H-Y Tg thymocytes relative to the other TCR Tg lines (Fig. 5 C). The connection between up-regulation of FasL expression and its relative contribution to positive selection is compatible with the fact that mice with a polyclonal TCR repertoire also have fewer FasL\(^+\) thymocytes, especially at the DP stage (Fig. 2 B). This lower expression level is likely due to a much lower efficiency of positive selection in polyclonal B6 compared with TCR Tg mice, in which all thymocytes bear TCRs capable of positive selection.

While we favor the unified model that avidity of the TCR for its positively selecting ligand defines both the upper and lower limits for the influence of FasL-mediated costimulation on thymocyte maturation (Fig. 7 C), there is an alternative explanation for the H-Y phenotype. Perhaps the aberrantly early expression of the H-Y TCR delivers a positive selection signal before the stage at which FasL-mediated costimulation can be engaged, and thus bypasses the influence of FasL observed in other strains.

Our data are compatible with the notion that Fas/FasL interaction enhances the efficiency of thymocyte selection for TCR:MHC/peptide complexes within a particular affinity window. The decrease in the number of mature thymocytes, the aberrant expression of CD24, and the paucity of DP TCR\(^{hi}\) cells all suggest that the multi-step process of positive selection is defective in the absence of functional FasL. An alteration in signaling may be involved and is suggested by the balance of TCR/CD5 levels observed in AND I-A\(^b\) versus AND I-E\(^b\) mice.

The nature of the costimulatory signal delivered through FasL is unknown, although the structure of FasL suggests signaling capacity. Its highly conserved, proline-rich, cytoplasmic tail is distinct from that of other costimulatory molecules such as CD28 and LFA-1, and contains several SH3 consensus binding domains (48), a casein kinase I motif (49), other putative interaction domains (50), and motifs that target FasL to secretory vesicles (42). The Src-family protein tyrosine kinase Fyn has been shown to bind to peptides containing the FasL SH3 domains (48). Using chemical inducers of dimerization (51), our preliminary experiments show that cocrosslinking the cytoplasmic tails of CD3\(\varepsilon\) and FasL in Jurkat T cells enhances IL-2 promoter activity (unpublished data). While the undoubtedly complex signaling pathways invoked during positive and negative selection have yet to be fully unraveled, it is clear that structural motifs in the TCR can differentially affect positive and negative selection (52), and that qualitatively or quantitatively distinct signaling pathways may also contribute (for a review, see reference 53). Add the bipolar molecule FasL to this list of modulators and thymocyte maturation deepens in complexity.

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