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+, MHC class II restricted helper T cells, or CD8+, MHC class I–restricted cytolytic T cells (for reviews, see references 1 and 2). This complex developmental pathway has been delineated in part by the expression of molecules such as CD4, CD8, CD24 (heat stable antigen), preTα, and the TCR α and β chains. Double negative (DN;* CD4−CD8−) thymocytes include the earliest T cell precursors, and can be further subdivided on the basis of CD44 and CD25 expression into DN1 (CD44+CD25−), DN2 (CD44+CD25+), DN3 (CD44−CD25+), and DN4 (CD44−CD25−) populations. These give rise to TCRlow then TCRhi double positive (DP) CD4+CD8+ CD24hi cells, that finally, after traversing transitional CD4+CD8low or CD4lowCD8+ 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 (TCR β paired with preTα) 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

TNF superfamily. This molecule is not only capable of inducing death through its receptor Fas (for reviews, see references 9 and 10), but of enhancing the in vivo and in vitro proliferative capacity of antigen-reactive peripheral T cells (11, 12). As such, FasL is capable of bipolar signaling, delivering a death signal in one direction and a positive costimulatory signal in the other. Both CD4^{+} and CD8^{+} T cells are susceptible to FasL-mediated costimulation (13), although little is known about the nature of this costimulatory signal.

The thymus is one of the few organs in the body that coexpresses Fas and FasL (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 efficient differentiation of class I– and class II–restricted T cells expressing TCRs with moderate affinities for self-MHC/peptide. Thus, in addition to its other functions, FasL acts as an accessory molecule in positive selection, presenting a novel means for modulating this complex process.

Materials and Methods

Mice. C57BL/6 (B6), B6Smn.C3H-*Fasl^gld* (B6.*gld*), B6.MRL-*Fas^lpr* (B6.*lpr*), and B6.SJL-*Ptprc^a* Pep3^b/BoyJ (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 TCR 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.*lpr*) were generated by cross/backcross breeding of OT-1 and B6.*lpr* mice, and screening for Vβ5 expression by flow cytometry and for the *lpr* mutation by PCR (17). OT-1 and OT-1.*gld* mice on the H-2^d or H-2K^bm1 backgrounds were obtained by cross/backcross breeding of OT-1 and OT-1.*gld* mice with Balb/cByJ mice (H-2^d) or B6C-H2^bm1/By (H-2K^bm1), both from The Jackson Laboratory, and screening as above for TCR and *gld*. Homozygosity for H-2^d was assessed by screening PBLs for expression of H-2K^d and absence of H-2K^b by flow cytometry. Homozygosity for H-2K^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′-GCAGTACGC-CTACGACGGCT and the reverse primer 5′-TTACTGAATT-CTTCAGGTATCTGCGGAG. Reaction products were digested with PstI and run on a 6% polyacrylamide gel to reveal a single band of 190 bp for H-2K^b or two bands of 115 and 75 bp for K^bm1. AND and AND.*gld* mice on an I-E^k background were obtained by cross/backcross breeding of AND with C3H/HeJ or with C3H/HeJ-*Fasl^gld* mice (both from The Jackson Laboratory) and screening for absence of Vβ3-deleting endogenous superantigens and for Vβ3, I-E^k, and I-A^b expression as above. Ly5.1 congenic OT-1 mice (OT-1.Ly5.1) were generated by cross/back-

cross 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 10^5 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 choloropyll-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-2K^d (clone SF1–1.1), all from BD Biosciences, anti-H-Y TCR (clone T3.70, provided by Dr. A. Farr, University of Washington), and Tri-Color (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 A19–3; 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 FACS-Vantage™ 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′-ATG-CAGCAGCCCATGAATTAC and 5′-CCATATCTGTC-CAGTAGTGC. 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′-GTGGCTCTGGTTGGAATGGGA to detect a fragment of 710 bp.

BM Chimeras. BM chimeras were constructed as described previously (25). Recipients were irradiated at 950 rads and reconstituted i.v. ≤6 h later with 15 × 10^6 T cell–depleted BM cells (single BM chimeras) or a 1:1 mixture of 8 × 10^6 cells of each donor type (mixed BM chimeras). Recipients of mixed BM were Ly5.1xLy5.2 F$_1$, while donors were either Ly5.1 (OT-1) or Ly5.2 (OT-1.*gld*). Thymocytes were analyzed at various time points, and host contribution to the thymocyte pool was negligible.

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–2K[b])-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
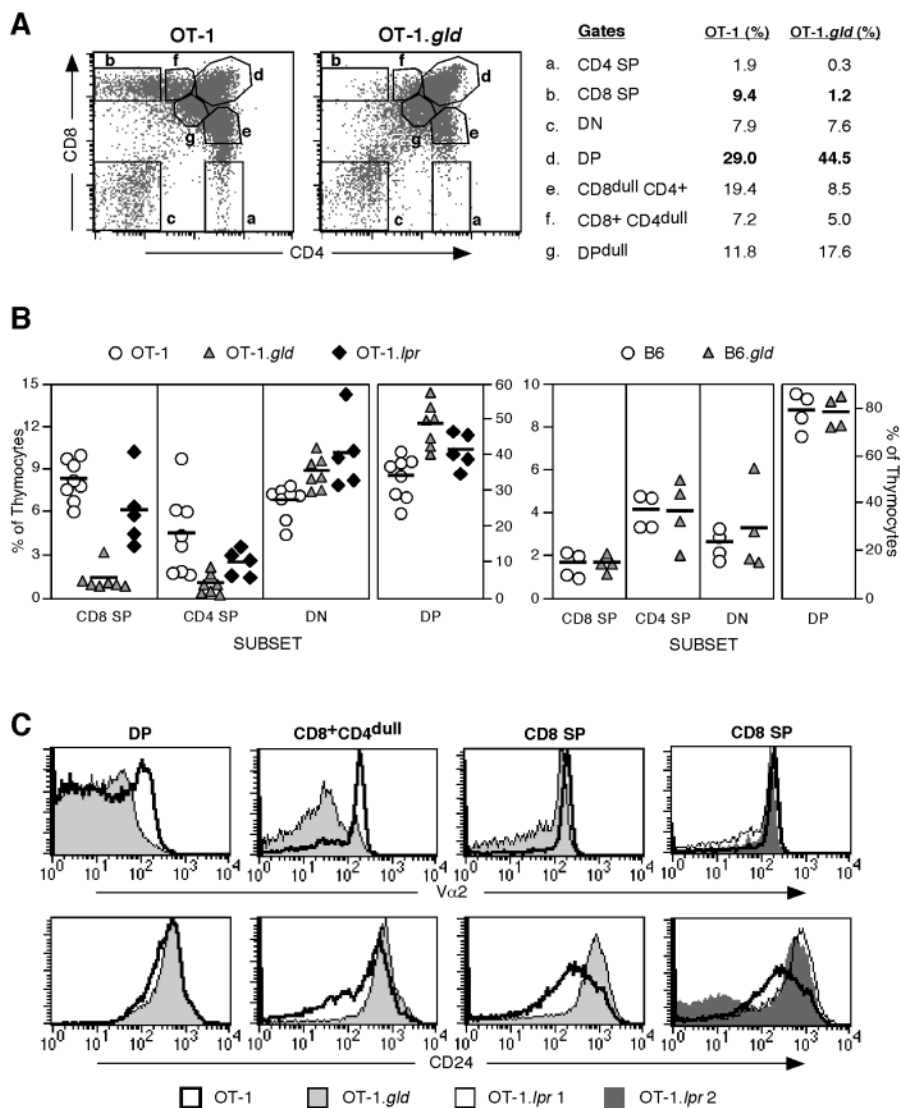

Figure 1. Defects in FasL alter thymocyte maturation in OT–1 TCR Tg mice. (A) CD4/CD8 profiles of thymocytes from OT–1 (left) and OT–1.*gld* (middle) mice are compared. Gates designated by letters in the dot plots represent thymocyte subpopulations described in the table (right), listing percentages of thymocytes in each compartment. Bold numbers highlight major differences observed. (B) Percentages of thymocytes in the indicated subcompartments 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) Vα2 (top) and CD24 (bottom) expression in DP, CD8^{+}CD4[dull] (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.

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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 expression accounts for the differential influence of 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
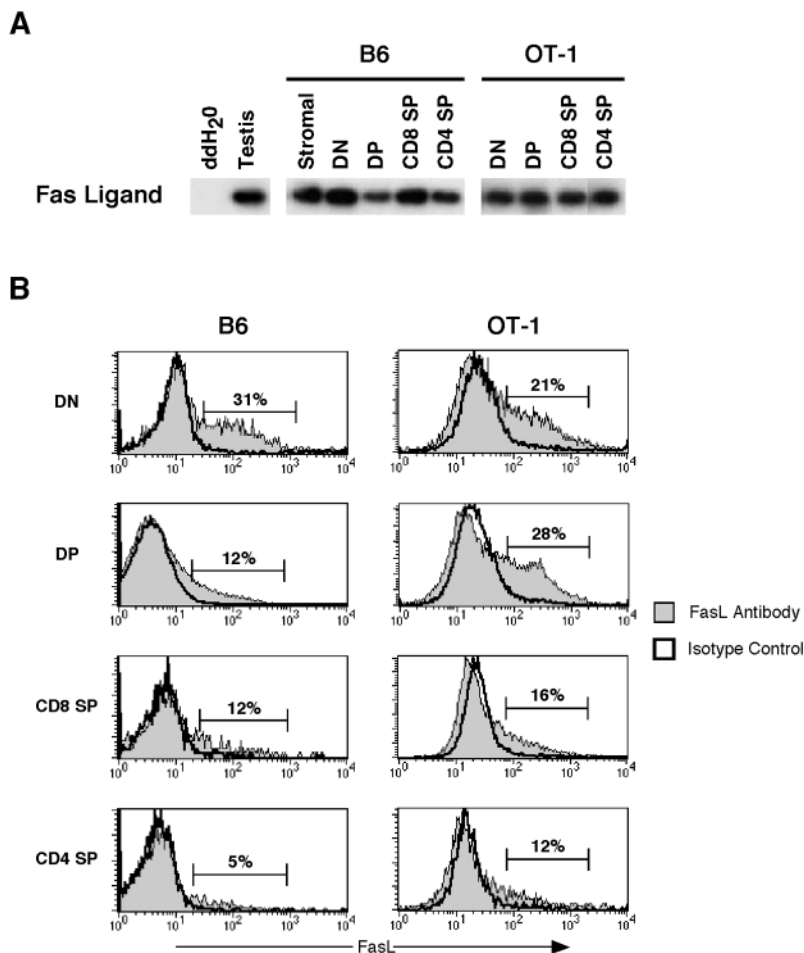

Figure 2. FasL RNA and surface protein are expressed by wild-type OT-1 and B6 thymocytes. (A) RT-PCR was performed to assess FasL expression in thymocytes from wild-type OT-1 and B6 mice sorted on the basis of CD4 and CD8 expression, using gates shown in Fig. 1 A. Testis was used as a FasL-positive control. Stroma represents thymic capsule debris treated with anti–Thy-1 plus complement to remove contaminating thymocytes. Serial dilutions of cDNA from each subpopulation were amplified in HPRT-specific PCR reactions to titrate relative amounts of cDNA. In FasL PCR reactions, equivalent amounts of cDNA were amplified for testis, stroma, CD4 SP (B6 and OT-1), and DN (B6). 3-fold more cDNA was required for amplification of DN (OT-1), 6-fold for DP (B6), 9-fold for DP (OT-1) and CD8 SP (OT-1), and 18-fold for CD8 SP (B6). FasL PCR products were transferred to hybridization membranes and probed with end-labeled oligonucleotide. (B) Thymocytes from wild-type B6 (left panels) and OT-1 (right panels) mice were stained for CD4 and CD8 by conventional methods and for FasL by EAS. FasL expression (gray histograms) is compared with isotype control (open histograms) for gated DN, DP, CD8 SP, and CD4 SP thymocytes. Numbers represent the percentage of FasL+ cells falling within the indicated markers.

FasL expression by radioresistant 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-2K^b), negatively selected (H-2^d), or not selected (H-2K^{bm1}). The CD4/CD8 profiles of thymocytes on the H-2^d and H-2K^{bm1} backgrounds are indistinguishable in OT-1 and OT-1.*gld* mice (Fig. 4 A). As expected (31), OT-1 thymocytes on the H-2^d and H-2K^{bm1} backgrounds are depleted for CD8 SP and enriched for DP cells, as are OT-1.*gld* thymocytes on the H-2K^b background (Fig. 4 B). The total thymic cellularity in wild-type mice is lower in the negatively selecting H-2^d background and higher in the neutral H-2K^{bm1} compared with H-2K^b 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-2^b 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

A SINGLE BONE MARROW CHIMERAS

OT-1 → B6   OT-1.*gld* → B6
OT-1 → B6.*gld*   OT-1.*gld* → B6.*gld*

B MIXED BONE MARROW CHIMERAS

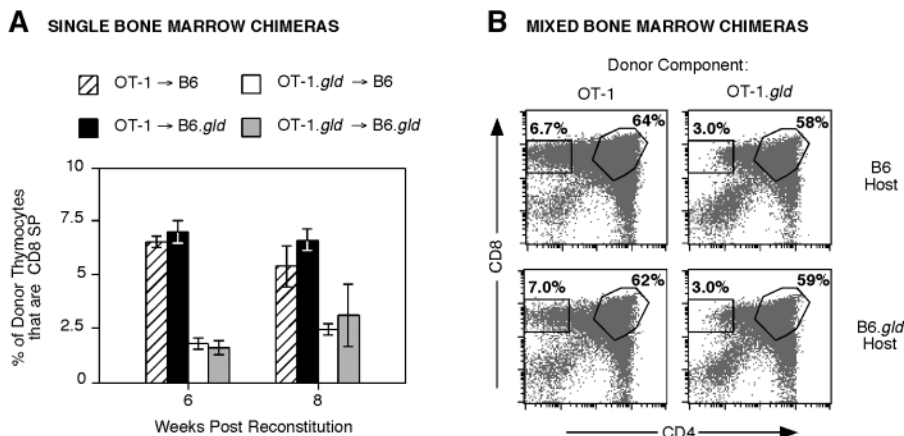Donor Component:

OT-1   OT-1.*gld*

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 host. Bottom 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.
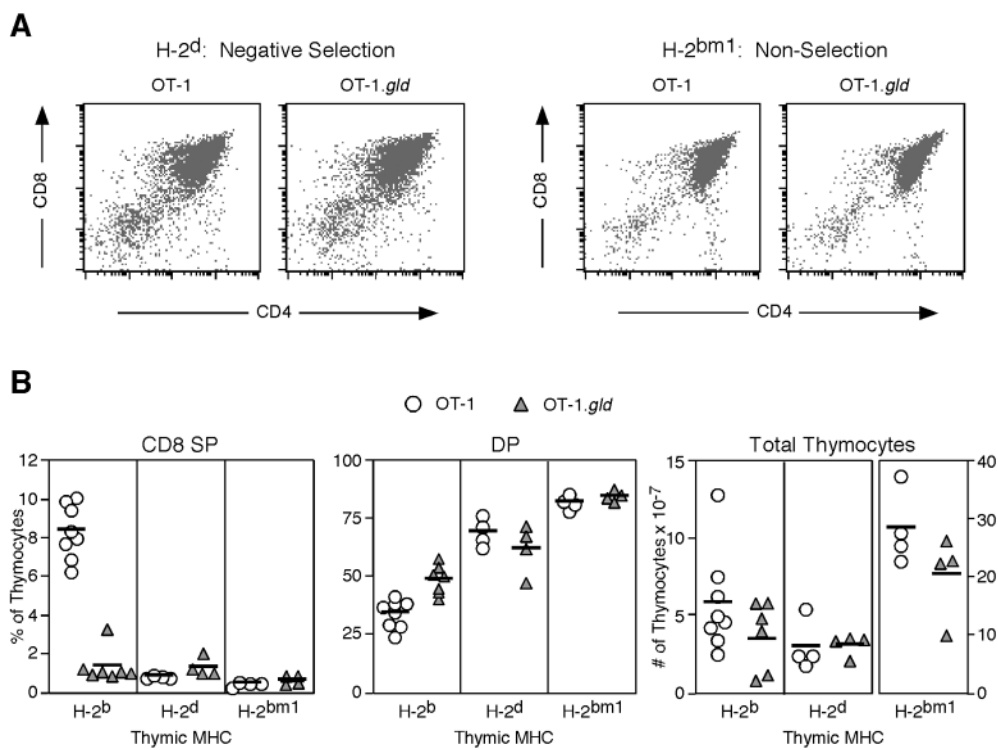
Figure 4. FasL deficiency does not affect negative selection or death by neglect. (A) Comparison between CD4/CD8 profiles of thymocytes from OT-1 and OT-1.*gld* mice on an H-2^d (left panels) or an H-2K^{bm1} background (right panels). (B) OT-1 mice on an H-2K^b background (left of each panel; OT-1 $n = 8$, OT-1.*gld* $n = 7$) were compared with those on an H-2^d (middle of each panel; OT-1 and OT-1.*gld* $n = 4$) and an H-2K^{bm1} (right of each panel; OT-1 and OT-1.*gld* $n = 4$) background. For each strain, percentages of CD8 SP (left panel), DP (middle panel), and total thymocytes (right panel) are shown for individual OT-1 (circles) and OT-1.*gld* mice (triangles). Means are designated by solid bars. Note change in y-axis scale for H-2K^{bm1} total thymocytes.

stronger positive selection for this TCR, respectively (32, 33). FasL expression on wild-type AND thymocytes on an I-E^k background is similar to that on an I-A^b background (Fig. 5 C). However, the level of CD5 expression is higher in both DP TCR^{hi} and CD4 SP TCR^{hi} subsets from I-E^k AND 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-E^k– than in I-A^b–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-E^k 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 B). 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, Vβ 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 Vβ3 (the β chain of AND), and a slight overrepresentation of Vβ6$^+$ CD8 SP in B6.*gld* relative to wild-type B6 mice. In addition, CD8 SP thymocytes expressing Vβ5 (the β 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
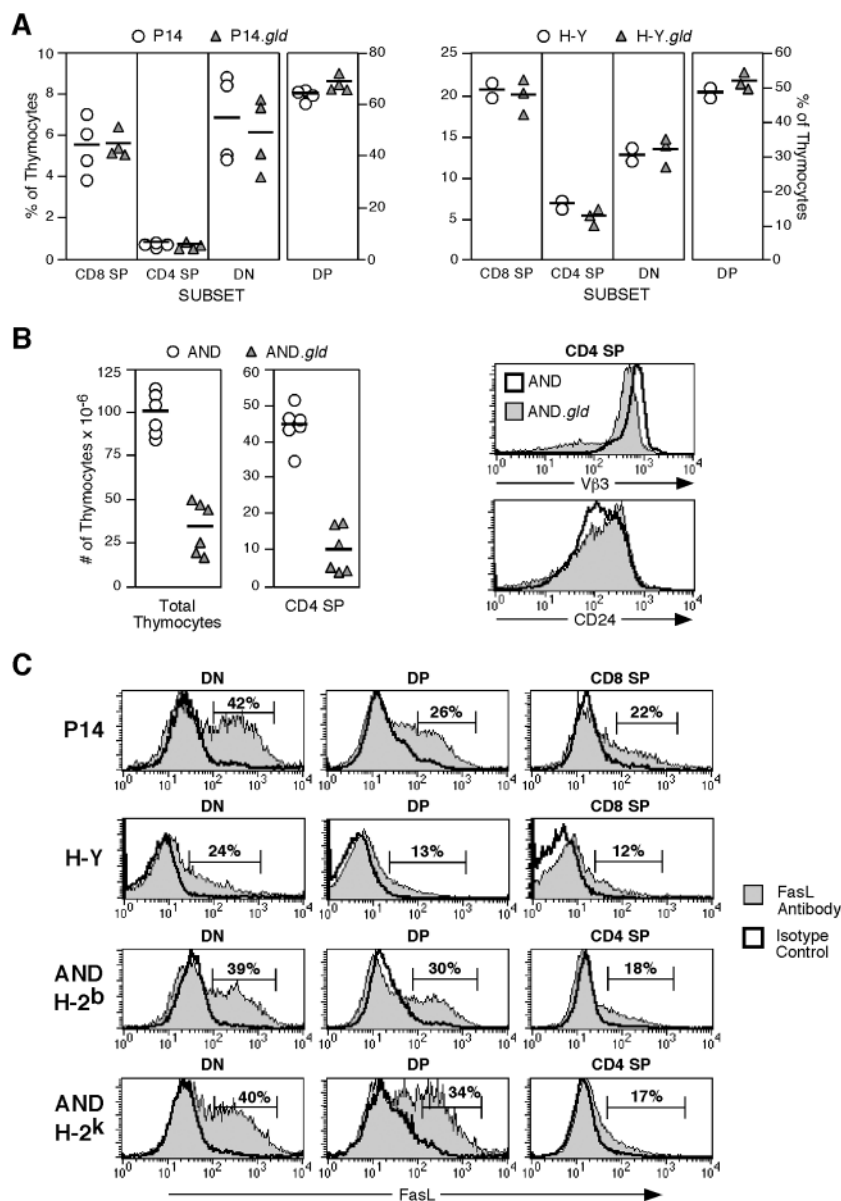
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Figure 5. Maturation of thymocytes expressing some, but not all, TCRs is influenced by FasL. (A) Percentages of thymocyte subpopulations based on CD4/CD8 expression were compared between wild-type (circles) and *gld* H-2^b mice (triangles) Tg for the 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-2^b 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 mice. (C) Thymocytes from wild-type P14 (top row), H–Y (second row), and AND on H-2^b (third row), and H-2^k backgrounds (bottom row) mice 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.

thymocyte maturation. 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-2^b background are unexpectedly similar to those in AND mice on an H-2^k background (Fig. 5 B, and Fig. 6, B and C). These data are perhaps more consistent with a role for FasL as a negative
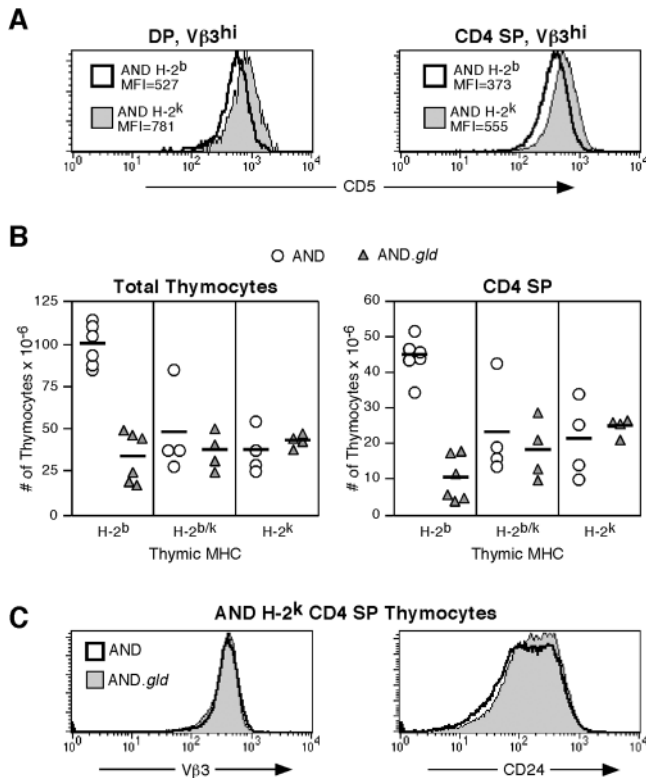
Figure 6. Increasing the strength of the positive selection signal bypasses the need for FasL during thymocyte development. (A) CD5 expression is compared between wild-type AND TCR Tg mice on H-2^b (open histograms) and H-2^k backgrounds (gray histograms). Histograms represent DP Vβ3^{hi} (left) or CD4 SP Vβ3^{hi} thymocytes (right); mean fluorescence intensity (MFI) for each is shown. (B) AND TCR Tg mice on H-2^b (wild-type and *gld* n = 6), H-2^b/H-2^k heterozygous (wild-type and *gld* n = 4) and H-2^k 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-2^k background.

regulator of TCR signaling. According to this alternate model, thymocytes from AND mice on an H–2^b background that express mutant FasL might receive a stronger signal than wild-type mice, and would display a mixed positively and negatively selected phenotype characteristic of cells from AND mice on an H–2^k background. Arguing against this interpretation is the consistently lower number of SP thymocytes in H–2^b AND.*gld* mice relative to AND and AND.*gld* mice on an H–2^k background (10 relative to 21–24 × 10^6; Fig. 6 B) and the dissimilarity between the TCR expression levels in total DP thymocytes from these backgrounds (Fig. 7 A). While it is clear that the TCR^{hi} DP cells in mice on the H–2^k background are depleted, such is not the case in mice on the H–2^b background.

Several additional predictions that follow from this alternate model are not borne out experimentally. If FasL serves as an attenuator of TCR signals, CD5 levels should be higher in *gld* than in wild-type thymoctyes in all of the TCR Tg strains. However, CD5 levels on DP thymocytes

from H-2^b 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-2^b AND.*gld* thymocytes should be similar to H-2^k 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-2^b AND.*gld* and on H-2^k AND thymocytes. However, CD24 levels are highest for H-2^b AND.*gld*, intermediate for H-2^k AND.*gld*, and lowest for AND on both H-2^b and H-2^k backgrounds (with mean fluorescence intensities [MFIs] of 296, 220, 198, and 192, respectively; Figs. 5 B and 6 C). 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 both 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-
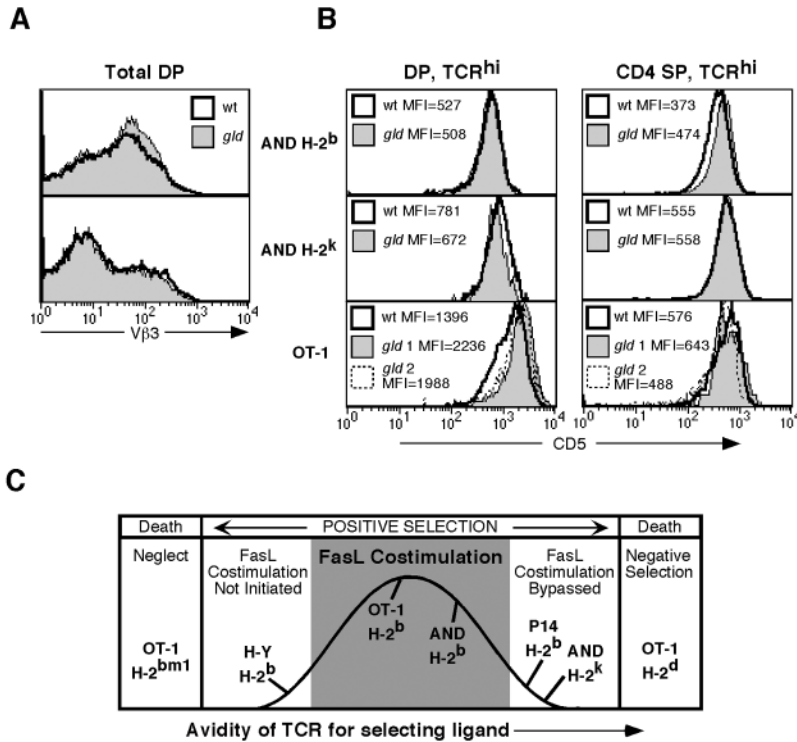
Figure 7. FasL engagement likely enhances rather than represses positive selection. (A) TCR expression levels on total DP thymocytes from AND and AND.*gld* mice on the H-2^b background are dissimilar to those on the H-2^k background. Vβ3 expression is compared between wild-type (open histograms) and *gld* (gray histograms) AND mice on the H-2^b (top panel) and H-2^k backgrounds (bottom panel). (B) CD5 levels on thymocyte subpopulations do not correlate with expression of wild-type FasL. CD5 expression is shown for DP TCR^{hi} and CD4 SP TCR^{hi} thymocytes from wild-type (open histograms, thick lines) and *gld* (gray histograms or open histograms, dotted lines) AND mice on the H-2^b (top panels) and H-2^k backgrounds (middle panels), and OT-1 mice (bottom panels). MFI for each is shown. (C) Model depicting the correlation between thymocyte sensitivity to FasL costimulation and avidity of the TCR for its selecting ligand. Thymocytes expressing TCRs of very low avidity for selecting ligands die by neglect (left) while those expressing TCRs with the highest avidity for selecting ligands die by negative selection (right). Thymocytes expressing TCRs with avidities between these two extremes will be positively selected. Within this range, thymocytes expressing TCRs with lower avidity, such as H-Y, or those expressing TCRs with sufficiently high avidity (P14 or AND selected on I-E^k) are not influenced by FasL costimulation during T cell development. Thymocytes expressing TCRs with intermediate avidity, such as OT-1 and AND selected on I-A^b, will benefit from the costimulatory boost delivered by FasL to develop into mature SP thymocytes (middle, gray area).

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-2K^b–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 DP^{dull} from DP^{bright} 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-2^d) and nonselecting (K^{bm1}) 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-Y Tg 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-A^b 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.

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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^k 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 on 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^k 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 CD3ζ 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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