Gld mutation of Fas ligand increases the frequency and up-regulates cell survival genes in CD25+CD4+ TR cells

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Abstract

The Fas pathway and regulatory T (TR) cells play intertwining roles in controlling T cell tolerance through deletion and suppression of autoreactive T cells. Impairment of either mechanism causes severe T cell lymphoproliferation albeit with opposing outcomes. T cell lymphoproliferation induced by defective Fas pathway does not cause overt lymphocytic infiltration but rather prevents an important set of T cell-mediated autoimmune diseases. In contrast, deficiency in TR cells causes fulminant autoimmunity in very early life and fatal lymphocytic infiltration. These observations suggest existence of unidirectional fail/safe mechanism that compensate for defects in the Fas pathway but not in regulatory cells. To gain insights into how animals compensate for defects in the Fas system, we analyzed the impact of generalized lymphoproliferative disease (gld) mutation on survival, function and transcription profile of CD25+CD4+ TR cells. Our results show that all CD4 T cells expanded in gld mice. However, CD25+CD4+ TR cells are disproportionately increased in the pool of CD4 T cells perhaps due to their unique apoptosis phenotype. Freshly isolated CD25+CD4+ TR cells, unlike CD25−CD4+ T cells, are highly sensitive to FasL-induced apoptosis in the steady state. CD25+CD4+ TR cells that accumulate in gld mice express similar level of Foxp3, and have suppression potency and TR gene expression profile as wild-type CD25+CD4+ TR cells. Furthermore, the transcription profile of gld CD25+CD4+ TR cells is characterized by differential expression of genes involved in cell survival, metabolism and innate immune responses. These results provide a strong cellular and molecular basis for understanding why impaired Fas pathway prevents an important subset of T cell-mediated autoimmune diseases.

Introduction

Under normal circumstances, T cell autoreactivity is kept under tight control by a combination of deletion mechanisms and a system of regulatory cells. These mechanisms play intertwining roles in maintaining T cell homeostasis and tolerance. The Fas death pathway plays a major role in deleting autoreactive T cells that escape negative selection and enter secondary lymphoid organs (1, 2). CD25+CD4+ TR cells and other naturally occurring regulatory cells control pathogenicity of autoreactive and effector T cells that escape peripheral deletion (3-9). Massive lymphoproliferation occurs when either the Fas pathway or CD25+CD4+ TR cell homeostasis is impaired. However, there are stark differences in the consequences. Impaired Fas pathway does not cause overt lymphocytic infiltration and is associated with prevention of organ-specific autoimmune diseases such as type 1 diabetes and multiple sclerosis in animal models (10-14). By contrast, deficiency of CD25+CD4+ TR cells leads to an aggressive and fatal lymphocytic infiltration of multiple organs early after birth (15-18). However, very little is known about how direct T cell pathogenicity is held in check in mice with impaired Fas pathway. Understanding the mechanisms that underlie this paradox could lead to the design of novel approaches to
ameleriorate the lymphoproliferation and harness the Fas pathway for developing new therapeutics.

Toward this goal, we show in this study that generalized lymphoproliferative disease (gld) mutation leads to their disproportionate accumulation in lymphatic tissues of mutant mice due to reduced apoptosis. Freshly isolated CD25+CD4+ T\textsubscript{R} cells, unlike CD25−CD4+ T cells, are highly sensitive to FasL-induced apoptosis in the absence of concomitant TCR stimulation. Gld CD25+CD4+ T\textsubscript{R} cells maintain their suppressive function and signature genes. In addition, accumulation of CD25+CD4+ T\textsubscript{R} cells in gld mice is associated with the induction of a transcription program characterized by up-regulation of vascular cell adhesion molecule-1 (VCAM-1) and genes involved in survival, metabolism and innate immune responses. These results have important implications for understanding why impaired Fas pathway prevents rather than exacerbate an important subset of T cell-mediated autoimmune diseases.

**Methods**

**Mice**

C3H-gld/gld mice carrying homozygous loss-of-function gld mutations of FasL (hereafter referred to as gld mice), as well as C3H-HeJ wild-type (wt) control mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were bred and maintained at the Animal Care Facility of the Johns Hopkins School of Medicine. All mice used were between 16 and 20 weeks of age, unless otherwise noted.

**Flow cytometry**

Unless otherwise indicated, mAbs used in our analysis were purchased from PharMingen (San Diego, CA, USA). Anti-mGITR (glucocorticoid-induced tumor necrosis factor receptor family-related gene) goat IgG was obtained from R&D System (Minneapolis, MN, USA). In our four-color analysis, we used, unless otherwise stated, FITC anti-CD8, PerCP anti-CD4, APC anti-TCR and PE-conjugated mAb specific for the fourth molecule analyzed. Data were collected using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed by the CellQuest software (Becton Dickinson). Intracellular staining for cytotoxic T lymphocyte associated-4 (CTLA-4) was performed according to BD PharMingen’s protocol. Gating of positively stained populations in each instance was determined using isotype-matched antibodies.

**Cell preparations**

CD4 T cells were isolated by negative selection with dynabeads (Dynal®, Lake Success, NY, USA). Briefly, splenocytes were incubated with a cocktail of biotin-conjugated rat mAbs specific for murine CD8, I-E\textsuperscript{K}, CD16/CD32 and B220 surface molecules. Streptavidin-conjugated beads from Dynal® were used to capture and remove mAb-coated cells according to the manufacturer’s instructions. When desired, the CD25 subset of CD4 T cell population was depleted by adding biotin-conjugated anti-CD25 mAb to the cocktail. On the other hand, to isolate CD25+CD4+ T\textsubscript{R} cells, we used an isolation kit from Miltenyi Biotec (Auburn, CA, USA). B220+

**Cell proliferation and apoptosis assays**

For long-term proliferation assay, mice were given bromodeoxyuridine (BrdU) (Sigma Chemical Co., St Louis, MO, USA) in drinking water at a concentration of 0.8 mg ml\textsuperscript{-1}. BrdU was dissolved in sterile water and changed daily. For short-term proliferation assay, mice were intra-peritoneally injected with two doses of BrdU (2 mg each) within 24-h period. At the time of analysis, cells were harvested from indicated organs and analyzed for BrdU incorporation using the BrdU flow kit protocol (BD PharMingen). In brief, cells were surface stained with TCR, CD4 and CD25 antibodies, fixed, permeabilized, re-fixed and treated with DNase. Cells were then stained with anti-BrdU mAb and analyzed by four-color FACSCalibur. To determine the percentage of T\textsubscript{R} cells that are apoptotic, spleen and lymph node cells from gld and wt mice were surface stained for TCR, CD4 and CD25 and then stained for Annexin V binding using apoptosis detection kit from BD PharMingen according to the manufacturer’s protocol. To test sensitivity of gld or wt CD25+CD4+ T cell to FasL-mediated apoptosis, CD25+CD4+ and CD25−CD4+ T cells were isolated as described above. Cells were cultured overnight with 1–10 ng soluble recombinant human Fas ligand in the presence of 1 μg of cross-linking antibody (Alexis). As control, cells were incubated with cross-linking antibody without FasL. Percent of cells that are apoptotic was then determined by FACS analysis.
**Real-time PCR**

We performed real-time PCR to measure Foxp3 mRNA transcripts in CD25+CD4+ T₅ and CD25−CD4+ T cells isolated from wt and gld mice by using the manufacturer’s protocol (Milenyi Biotec). Total RNA was isolated from different cell types with the RNeasy kit (Qiagen, Valencia, CA, USA). Briefly, cells from each sample were disrupted, homogenized and re-suspended in an equal volume of 70% ethanol. Samples were loaded into mini columns; on-column DNase digestion was performed and columns were washed three times. Total RNAs were eluted with water and concentrations were determined. First-strand cDNAs were synthesized from total RNAs by using Superscript II (Invitrogen, Carlsbad, CA, USA). Briefly, 1 µg of total RNAs was reverse transcribed with 50 units of Superscript II reverse transcriptase and 150 ng of random hexamers as primers. Quantitative real-time PCR was performed by adding cDNA to the reaction mixture containing the Foxp3 primers. After 40 cycles, the relative copy numbers of Foxp3 mRNA were determined by normalizing the amount of total RNA added to each reaction with 18S rRNA.

The sequences of primer sets (5′−3′) were as follows (forward and reverse, respectively): Foxp3: GGCCCTTCTC-CAGGACAGA, GCTGATCATGCTGAGTTGTG; VCAM-1: TGA-CAAGTCCCATGTTGA, ACCTGCCAGCGCATATT; Axl: GGTCCGCTTGAAAGACATGA, CTCAAGGTACTCCATACACT; Casb: GGATGCATGACTCTGGAGGT, GAGAGGAGCCAGT-GCTGTG; Cxcl-10: GACGGGTCCGCTGCAACTG, GCTTC-

**DNA microarray analysis**

Total RNA was isolated from cells, as described above, by using the RNeasy Mini Kit (Qiagen). RNAs from control and experimental T cells were processed by using the two-round RNA amplification protocol described by Affymetrix (Affymetrix GeneChip Expression Manual Small Sample_2). Briefly, 100 ng of starting total RNA were used to synthesize first-strand cDNA with oligonucleotide probes with 24 oligo-dT plus T7 promoter as primer (Proligo LLC), and the SuperScript Choice System (Invitrogen). After synthesis of the double-stranded cDNA, the product was purified by phenol–chloroform extraction, and unlabelled ribonucleotides were used in a first round of *in vitro* transcription (MegaScript, Ambion). The following cycle of cDNA synthesis was started with random primers, and the oligo-dT with T7 promoter was again used as a primer at the second-strand cDNA synthesis step. The double-strand cDNA product was then purified again by phenol–chloroform extraction. Next, biotinylated anti-sense cRNA was generated through *in vitro* transcription by using the BioArray RNA High Yield Transcript Labeling kit (ENZO Life Sciences Inc.). Fifteen micrograms of the biotinylated labeled cRNA were fragmented at 94°C for 35 min (100 mM Tris acetate, pH 8.2, 500 mM K/OAc, 150 mM Mg/OAc), and 10 µg of total fragmented cRNA were hybridized to the Affymetrix murine genome GeneChip array MOE430 set for 16 h at 45°C with constant rotation (60 r.p.m.). Affymetrix Fluidics Station 400 was then used to wash and stain the chips, removing the non-hybridized target and incubating with a streptavidin–PE conjugate to stain the biotinylated cRNA. The staining was then amplified with goat IgG as blocking reagent and biotinylated anti-streptavidin antibody (goat), followed by a second staining step with a streptavidin–PE conjugate. Fluorescence was detected using the Affymetrix-GS300 GeneArray Scanner and image analysis of each GeneChip was done through the GeneChip Operating System software from Affymetrix (GCOs1.1.1), using the standard default settings. For comparison between different chips, global scaling was used, scaling all probe sets to a user-defined target intensity of 150. To ascertain the quality control of the total RNA from the samples, we used the Agilent Bioanalyzer, Laboratory on a Chip technology, and confirmed the rRNA ratios and clean run patterns of the samples. Likewise, this technology is used to confirm the quality of the RNA in the form of cRNA and fragmented cRNA. To assess the quality of the hybridization, GeneChip image and comparison among chips, we confirmed the following parameters: scaling factor values within comparable range (between 0.7 and 1.4), low background values (between 51 and 70), high percentage of present calls (between 36 and 50), consistent 3′/5′ ratios of glyceraldehyde-3-phosphate dehydrogenase as representation of housekeeping genes and presence or absence of Bio B and C as internal spike controls.

**Statistical analysis of microarray data**

The mRNA samples were interrogated with Affymetrix GeneChip Mouse genome 430A microarrays. Quality of the microarray experiment was assessed with the following statistical approaches: affyPLM, a bioconductor package in *R* according to the publication of Ihaka and Gentleman (20), and ‘AffyRNAdeg’ function in the bioconductor package ‘Affy’ in *R*. To estimate the gene expression signals, data analysis was conducted on the chips’ CEL file probe signal values at the Affymetrix probe pair (perfect match probe and mismatch probe) level, using the statistical algorithm ‘Robust Multiarray Analysis’ (21) with the bioconductor package Affy. This probe level data processing includes a normalization procedure utilizing quantile normalization (22) to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization and/or scanning. With the signal intensities estimated above, an empirical Bayes method with the Gamma–Gamma modeling, as implemented in the *R* package ‘EBarrays,’ was used to estimate the posterior probabilities of the differential expression of genes between the genotypes (23–25). The criterion of the posterior probability >0.5, which means that the posterior probability is larger than by chance, was used to produce the differentially expressed gene lists. All computation was performed under *R* environment and all bioconductor packages are available at http://www.bioconductor.org.

**Results**

**Disproportionate accumulation of CD25+CD4+ T cells in gld mutant mice**

T cells slowly cycle in the steady state in the absence of overt T cell activation. Such basal proliferation is necessary to...
induction of CD25+CD4+ TR cells in response to gld mutation

The disproportionate increase in BrdU+ CD25+CD4+ T cell in gld mice after long-term BrdU labeling could be due to increased proliferation or decreased apoptosis. To distinguish between these possibilities, we analyzed the effect of gld on cell cycling and apoptosis of CD25+CD4+ T cells in the steady state. Mice were injected twice with BrdU (2 mg each) within 24-h period. Lymph node and spleen cells were isolated and analyzed for BrdU incorporation. There were no significant differences in the proliferation rates between gld and wt CD25+CD4+ T cells (Fig. 2B). However, CD25+CD4+ T cells proliferated at higher rate than CD25−CD4+ T cells both in gld and wt mice [(28) and data not shown]. Therefore, proliferation rate of CD25+CD4+ T cells does not appear to be accelerated by gld mutation.

Next, we determined whether steady-state apoptosis of CD25+CD4+ T cells was affected by gld mutation. We examined and compared the percentage of CD25+CD4+ T cells undergoing apoptosis in 16- to 20-week old gld and wt mice using Annexin V. We observed a significant decrease in the percentage of apoptotic CD25+CD4+ T cells in gld mice compared with wt mice (Fig. 2B). There was 10-fold decrease in apoptosis of CD25+CD4+ T cells (3 ± 1%) in the lymph nodes of gld mice compared with CD25+CD4+ T cells (33 ± 2%) in the lymph nodes of wt mice. The decrease in the apoptosis of gld CD25+CD4+ T cells was limited to 2-fold in the spleen. The reduced apoptosis in the lymph nodes was consistent with the significantly higher increase in the absolute numbers of CD25+CD4+ T cells in the lymph node (100-fold) than in the spleen (5- to 15-fold) of gld mice (Fig. 2A). The basis for the disparity in apoptosis of T cells in the lymph nodes and spleen of gld mice is currently unclear. However, it could be related to the influence of gld mutation on antigen-presenting cells located in the lymph node and spleen, which subsequently modulate survival of neighboring T cells through cytokine release or cell–cell contact. Apoptosis of CD25−CD4+ T cells was also reduced in gld mice thereby contributing to the global increase in CD4 T cells in gld mice (data not shown). Therefore, reduced apoptosis appears to be an important factor that contributes to accumulation of CD25+CD4+ T cells in gld mice.

Next, we determined whether CD25+CD4+ T cells were more prone to Fas-mediated apoptosis than CD25−CD4+ T cells in the steady state and thus more beneficial from defective Fas pathway. Consistent with this idea, a recent human study shows that freshly isolated human CD25+CD4+ Tp cells, unlike CD25−CD4+ T cells, are highly sensitive to Fas-mediated apoptosis (30). Therefore, it is possible that homeostasis of CD25+CD4+ T cells is constantly regulated by Fas-mediated apoptosis due to their high cycling rate in the steady state. We directly compared susceptibility of CD25+CD4+ T cells and
CD25+CD4+ T cells for FasL-mediated apoptosis in vitro in the absence of TCR stimulation. We initially compared the expression level of Fas by freshly isolated CD25+CD4+ T cells and CD25−CD4+ T cells. We found that gld CD25+CD4+ T cells expressed higher levels of Fas receptor than autologous CD25−CD4+ T cells (Fig. 2C). Similar difference in surface Fas was observed between wt CD25+CD4+ T cells and wt CD25−CD4+ T cells (data not shown). Next, highly purified CD25+CD4+ T cell and CD25−CD4+ T cells were incubated for 16 h with soluble recombinant FasL in the presence of cross-linker followed by apoptosis analysis. Gld CD25+CD4+ T cells died at significantly higher rates (93%) than CD25−CD4+ T cells (35%) as shown in Fig. 2(C). There was no difference between gld and wt CD25+CD4+ T cells in their susceptibility to
FasL-mediated apoptosis (data not shown). These results confirm and extend the above-mentioned findings in human CD25+CD4+ T cells (30) and show that murine CD25+CD4+ T cells are susceptible to FasL-mediated apoptosis in the steady state. In addition, they provide novel insight into why CD25+CD4+ T cells could particularly benefit from defective Fas-mediated apoptosis in the steady state. The pool size of CD25+CD4+ T cells, which usually cycle at high rate, could be
Gld mutation does not affect the regulatory function of CD25+CD4+ T cells

The possibility that CD25+CD4+ T cells in gld mice were effector T cells that divided in response to foreign pathogens is unlikely as lymphadenopathy occurs in mutant mice that were kept under germ-free environment (31). In addition, gld CD25+CD4+ T cells were also unlikely to be autoreactive T cells reacting to self-antigens as uncontrolled response to self usually leads to T cell-mediated autoimmune diseases (3, 4, 32). On the other hand, sensitivity of gld CD25+CD4+ T cells to FasL-mediated apoptosis and absence of overt cell-mediated autoimmune disease in gld mice suggest that they were regulatory cells. Therefore, we analyzed the phenotype, lineage and function of CD25+CD4+ T cells in gld mice and compared their attributes with those in wt CD25+CD4+ T cells.

We first examined and compared CD25+CD4+ T cells from 16- to 20-week old gld and wt mice for expression of molecules (GITR, CD103 and CTLA-4) characteristic of TR cells (Fig. 3A). Our analysis shows a slight increase and more homogeneous expression of GITR in gld than wt CD25+CD4+ T cells. CTLA-4 was more expressed in gld CD25+CD4+ T cells than wt CD25+CD4+ T cells. Analysis of CD103 (itgae) identified two subsets of gld CD25+CD4+ T cells: a major subset that expressed high level of CD103, and a minor subset that expressed no or low level of CD103. A comparable number of wt CD25+CD4+ T cells expressed CD103 but its expression was heterogeneous and extended from low to high with no apparent distinction between the two subsets. Thus, phenotypic analysis of molecules characteristic of Treg cells shows that the majority of gld CD25+CD4+ T cells expressed markers associated with regulatory cells. However, for more definitive characterization, we examined gld CD25+CD4+ T cells for expression of the lineage-specific marker Foxp3 (3, 4, 33) using wt CD25+CD4+ T cells, and gld and wt CD25−CD4+ T cells as standard controls. Intracellular staining for Foxp3 and real-time PCR analysis show that CD25+CD4+ T cells from gld and wt mice expressed Foxp3 at comparable levels (Fig. 3B). Expression of Foxp3 by CD25+CD4+ T cells was confirmed at the protein level using flow cytometry. In both cases, expression of Foxp3 in CD25+CD4+ T cells was substantially higher than in corresponding CD25−CD4+ T cells. Thus, as judged by Foxp3 expression, it appears that the lineage of the highly proliferating gld CD25+CD4+ T cells is that of CD25+CD4+ TR cells.

The above findings were confirmed by the functional properties of gld CD25+CD4+ T cells. Similar to CD25+CD4+ T cells isolated from wt mice, CD25+CD4+ T cells from gld mice were anergic to stimulation with anti-CD3 alone, but proliferated vigorously on stimulation with anti-CD3 and -CD28 antibodies (data not shown). The capacity of gld CD25+CD4+ T cells to suppress proliferation of autologous gld CD25−CD4+ T cells was at least similar to the capacity of wt CD25+CD4+ T cells to suppress proliferation of autologous wt CD25−CD4+ T cells over a wide range of anti-CD3 doses (Fig. 3C). We used autologous systems (gld versus gld and wt versus wt) in the suppression assay to avoid plausible Fas/FasL effects that could result from interactions of FasL-deficient suppressor and responders cells. In addition, use of autologous CD25−CD4+ T cells as a suppression target is more appropriate as such cells represent the endogenous and physiologic targets of in vivo suppression for regulatory cells in each case. Nevertheless, normalized data show that, on per cell basis, CD25+CD4+ T cells from gld mice possessed somewhat more potent suppressive function than CD25+CD4+ T cells from wt mice. Taken together, these results show that gld CD25+CD4+ T cells, based on their surface phenotype, Foxp3 expression and suppressive function, were regulatory cells. Thus, gld mutation significantly increases steady-state numbers of CD25+CD4+ TR cells without negatively affecting their suppressive activity.

Analysis of the transcription profile of gld Treg cells

Next, we determined whether the transcription programs of CD25+CD4+ TR cells were affected by gld mutation. We analyzed the gene expression profiles of gld CD25+CD4+ TR cells, wt CD25+CD4+ TR cells and gld total CD25+/− T cells using Affymetrix GeneChip arrays. All subsets were isolated and purified by sorting or MACS beads as described in Methods. RNA was prepared from each subset, amplified and labeled for hybridization to Affymetrix arrays. Raw hybridization data generated from the different subsets were processed and normalized as described in Methods. The expression profiles of gld and wt CD25+CD4+ TR cells were identified and compared with each other using gld CD25− T cells as a common control (Fig. 4 and Table S1 and S2, Supplementary Table available at International Immunology Online). We identified 812 genes that were up-regulated in gld CD25+CD4+ T cells relative to gld CD25− T cells (Fig. 4A and Table S1A, Supplementary Table available at International Immunology Online).
Fig. 3. CD25+CD4+ T cells in gld mice display surface, transcription and functional characteristics of CD25+CD4+ T<sub>R</sub> cells. (A) Surface phenotype of gld and wt CD25+CD4+ T<sub>R</sub> cells. Spleen cells from 16- to 20-week old gld and wt mice were four-color stained for TCR, CD4, CD25 and GITR, CD103 or CTLA-4 (intracellular). TCR+ cells were gated for CD4 and CD25 (left dot plot) and proportion of CD25+CD4+ T cells expressing each of the indicated markers was determined. One of seven experiments is shown. (B) Expression of Foxp3 by gld CD25+CD4+ T cells (top). CD25+CD4+ T cells were isolated from spleens of gld and wt mice by sorting using FACS Vantage (Becton Dickinson, San Jose, CA, USA), whereas CD25<sup>−</sup>/CD25<sup>+</sup>CD4+ T cells were isolated by negative selection. All subsets were >90% pure. Foxp3 was measured by quantitative real-time PCR as described in Methods. Level of Foxp3 mRNA was normalized relative to internal 18S for each subset. Each point is the mean of three independent experiments ± SEM. Splenocytes from 16-week-old C3H gld/gld mice and age-matched wt mice were stained for TCR, CD4, CD25 and intracellular Foxp3 using mAb FJK.16S and manufacturer’s instruction (eBioscience, San Diego, CA, USA) (Bottom). CD4+ TCR+ cells were gated and the percentage of Foxp3+CD25+ T cells was determined. (C) Functional properties of gld CD25+CD4+ T cells. Increasing numbers of purified gld and wt CD25+CD4+ T<sub>R</sub> cells were cultured at 37°C in triplicate with 5 × 10<sup>4</sup> autologous gld or wt CD25<sup>−</sup>/CD25<sup>+</sup> T cells, respectively. Cultures were stimulated with 1 μg ml<sup>−1</sup> anti-CD3 in the presence of 5 × 10<sup>5</sup> irradiated autologous T cell-depleted splenocytes for 72 h. Proliferation in each culture was determined by measuring [3H]thymidine incorporation added in the last 18 h of stimulation. Left and middle panels show suppression by gld and wt CD25<sup>−</sup>/CD4+ T cells, respectively. The right panel shows normalized suppression by gld and wt Treg cells displayed as percentage inhibition of proliferation relative to proliferation in positive control cultures of gld or wt CD25<sup>−</sup>/CD4+ T cells, respectively, that were stimulated in the absence of CD25+CD4+ T cells. Results are shown as mean ± SEM. One of two representative experiments is shown.
Supplementary Table available at International Immunology Online), 783 genes were up-regulated in wt CD25+CD4+ T<sub>R</sub> cells relative to gld CD25<sup>-</sup> T cells (Fig. 4A and Table S1B, Supplementary Table available at International Immunology Online) and 1031 genes were up-regulated in gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells relative to wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells (Fig. 4A and Table S1C, Supplementary Table available at International Immunology Online). Posterior probability of >0.5 was used to identify differentially expressed genes as described in Methods.

Comparison of the gene expression profiles of gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells and gld CD25<sup>-</sup> T cells confirmed that gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells were regulatory cells that express a Treg-specific profile. The top 15 genes over-expressed by gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells relative to CD25<sup>-</sup> T cells are shown in Fig. 4(B). Foxp3 was the most differentially expressed gene by gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells. Genes encoding itgae (Cd103), Il2ra and ctla-4 proteins were also among the top most expressed 15 genes. Genes encoding Kruppel-like factor-4, (Klf4), pre-proenkaphalin, (penk1), Tnfrsf9 (4-1BB), Tnfrsf4 (OX40) and neuropilin (Nrp1) that were previously described to be associated with regulatory cells were also highly expressed in gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells (Fig. 4B). Treg cell signature genes were, as expected, highly expressed by wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells relative to gld CD25<sup>-</sup> T cells. However, the intensity of expression of signature genes (except ctla-4) in wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells was less than that of gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells—as compared in each case to gld CD25<sup>-</sup> T cells (Fig. 4B). This difference does not appear to be due to increased basal expression levels of these genes in gld CD25<sup>-</sup> T cells but rather due to higher expression levels of these genes by gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells. This is because differential expression of these genes by wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells relative to gld CD25<sup>-</sup> T cells (with the exception of GITR) was comparable to that reported previously when wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells were compared with wt CD25<sup>-</sup>CD4+ T cells (33–35). Expression of Treg-specific profile genes (Foxp3, Il2ra, ctla-4, itgae) was independently identified either by real-time PCR or by flow cytometry as described above (Fig. 3). Thus, gld mutation did not negatively alter expression of signature genes. However, there are notable specific changes in transcription programs of CD25<sup>+</sup>CD4+ T<sub>R</sub> cells. Six of the 15 most expressed genes in gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells relative to gld CD25<sup>-</sup> T cells (Man2b2, Cxcl10, VCAM-1, Sic40a1, CD81 and Hspa1a) were weakly or not expressed by wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells.

**Gld mutation induces expression of genes involved in cell survival, metabolism and innate immunity**

To better understand how gld mutation affects CD25<sup>+</sup>CD4+ T<sub>R</sub> cells, we directly compared the expression profiles of gld with wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells. Gld and wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cells had basically similar Treg-specific profile (Fig. 4B) and were equally suppressive (Fig. 3C). We therefore predicted that the genes that are essential for the suppression function are not affected by the gld mutation and should be equally expressed in gld and wt Treg cells. Consistent with this notion, none of regulatory cell signature genes was among the 56 genes that were up-regulated in gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells (<3 fold) relative to wt CD25<sup>+</sup>CD4+ T<sub>R</sub> cell (Fig. 4C and Table S1C, Supplementary Table available at International Immunology Online). We also identified 22 genes that were down-regulated in gld Treg cells by >3-fold (Table S2C, available at International Immunology Online). We thus assumed that the genes that are differentially regulated between the two Treg populations encode molecules that affect other attributes of T cell homeostasis such as proliferation and survival.

Indeed, up-regulated genes in gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells encode molecules involved in cell growth/survival (proto-oncogenes), glycolysis/metabolism and innate immune responses (Fig. 4C–E). VCAM-1 was highly expressed in gld T<sub>R</sub> cells relative to wt T<sub>R</sub> cells. Oncogene Runx2, tumor-associated calcium signal transducer 1 (Tacstd1), Myb and Axl were also highly expressed in gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells.

Expression of selected genes including VCAM-1 was confirmed using real-time PCR (Fig. 4D). Expression of VCAM-1 was also confirmed at the protein level using flow cytometry (Fig. 4E). VCAM-1 was expressed at high level on the surface of gld CD25<sup>+</sup>CD4+ T<sub>R</sub> cells both in the lymph nodes and spleens but interestingly CD25<sup>+</sup>CD4+ T<sub>R</sub> cells in the spleen but not lymph nodes of wt mice expressed high level of surface VCAM-1. Expression of genes that promote survival could be an important factor that supports steady-state T cell accumulation in gld mice. For example, Runx2 is a member of the RUNX (Runt-related transcription factor) family of genes that are also known as the acute myeloid leukemia family (36). Over-expression of Runx2 in the thymus leads to spontaneous lymphoma and is strongly synergistic with Myc (37). Axl is a receptor tyrosine kinase that is activated by Gas6, a growth factor that belongs to the vitamin K-dependent protein family. Gas6 binding to Axl has been shown to transmit mitogenic and/or antiapoptotic signals to a variety of cell types (38, 39).

Lymphoproliferation in gld mice, as discussed above, is associated with neither T cell-mediated disease nor immunosuppression despite the expansion of conventional and T<sub>R</sub> cells. Expansion of Treg cells, while important for self-tolerance, increases permissiveness for infections and tumor growth. Interestingly, CD25<sup>+</sup>CD4+ T<sub>R</sub> cells in gld mice up-regulated a set of genes coding molecules that are important for innate immune responses and host defense and thus could play dual roles in protecting hosts against T cell-mediated autoimmune diseases and infections. Complement component 1q (C1qa), was up-regulated in gld Treg cells (Fig. 4C). Genes preferentially induced by IFN-alpha (Stat1, Ifit1, G1p2, cxc10, vcam-1) (40) were also up-regulated in gld Treg cells, suggesting that gld mutation activates the IFN signaling pathway. Two proteins that is not cytotoxic by itself, but their complex induces apoptotic death in several tumor-derived cell lines even at sub-nanomolar concentrations (41–43), were expressed in gld Treg cells. These are peptidoglycan recognition protein (pdrn1/Tag7) and Hspa1a (component of Hsp70). Together, these data show that gld mutation of Fasl creates an environment that promotes growth and survival of CD25<sup>+</sup>CD4+ T<sub>R</sub> cells.

**Discussion**

T cell lymphoproliferation induced by defective Fas pathway does not cause overt lymphocytic infiltration but rather prevents an important set of T cell-mediated autoimmune
Induction of CD25+CD4+ T<sub>R</sub> cells in response to gld mutation

**A**

- gld/CD25n, gld/CD15n, wt/CD25n, wt/CD15n
- 168, 384, 308, 711

**B**

- gld, wt
- Log<sub>2</sub> (fold change)
- Foxy3, Man2c2, Penk1, Il7, Cxcl10, Kif4, Vcam1, Tnfrsf4, Slc4a1, CD81, Tnfrsf9, Hspa1a, Il2ra, Cila4, Nrp1

**C**

- Cell growth and survival
  - Axl, Myb, Tacsd1, Runx2
- Metabolism
  - Gskm1, Mkk7, Hk1, Top2a, Usp2, Ddc, Slc4a1
- Innate immune response
  - G1p2, Pdml1, Slat1, C1qa, Hspa1a, Cxcl10, Vcam1, If it 1

**D**

- Normalized mRNA levels (gld/wt)
- Gas6, Axl, Vcam1, cxcl10, cxcl11, ccr3

**E**

- Lymph node, Spleen
- CD25
- WT: 79.4, 15, 48, VCAM-1
- GLD: 45, 63, 45
diseases. To gain insights into how animals compensate for defects in the Fas system, we analyzed the impact of gld mutation on survival, function and transcription profile of CD25+CD4+ T R cells. Our results show that CD25+CD4+ T R cells are disproportionately increased in the pool of CD4 T cells that accumulate in gld mice. In addition, we show that freshly isolated CD25+CD4+ T R cells, unlike CD25−CD4+ T cells, are susceptible to FasL-induced apoptosis in the absence of TCR stimulation. CD25+CD4+ T R cells that accumulate in gld mice express similar level of Foxp3, and have suppression potency and T R gene expression profile as wt CD25+CD4+ T R cells. In addition, gld mutation induces expression of genes encoding cell survival, metabolism and homing and innate immune response functions. These findings provide novel insights into why gld-induced lymphoproliferation does not lead to T cell-mediated autoimmune diseases.

FasL has dual functions as a ligand for the Fas receptor and as an active signaling molecule in T cells (44–46) and innate immune cells (47, 48). Cross-linking of FasL co-stimulates CD8 T cell proliferation (44). Engagement of FasL on CD4+ T cells induces cell cycle arrest (46), proliferation or apoptosis (45) depending on the experimental circumstances. Our data extend these observations and show that gld mutation, which abrogates Fas/FasL interactions, causes profound effects on T cell transcription and homeostasis in the steady state. Genes-encoding molecules that facilitate nutrient uptake and glycolysis were up-regulated in gld CD25+CD4+ T R cells. Furthermore, induction of genes that regulate cell growth and survival and associated with oncogenesis (Axl, Runx2, Myb, and Tacstd1) could contribute to survival of gld CD25+CD4+ T R cells. Interestingly, expression of anti-apoptotic genes BCL-2 and BCL-X I was not significantly affected in gld T cells (Table S1 and S2, available at International Immunology Online). Perhaps this is because up-regulation of BCL2 and BCL-X I delays entry into the cell cycle and slows turnover of memory T cells.

VCAM-1 is normally expressed on endothelial cells to promote transmigration of activated T cells across the endothelium. Specific mAb against VCAM-1, which essentially traps T cells in the periphery, has a remarkable success in treatment of multiple sclerosis (49). Thus, expression of VCAM-1 on CD4+ T cells could affect migration of effector cells into inflamed tissues by promoting VCAM-1: VLA-4 interactions between activated T cells. Enhanced expression of VCAM-1 on gld T R cells (Fig. 4D) could also contribute to prevention of T cell-mediated autoimmune diseases by facilitating cell contact and inhibition of differentiation of activated T cells by regulatory cells (50). Consistent with this notion, genes for effector cytokines were not up-regulated in CD25− or CD25+ subsets of gld CD4+ T cells relative to each other or wt CD25+CD4+ T cells despite their continuous activation and proliferation. However, gld mutation induced genes of innate immune response molecules, as discussed above, which could help protect hosts against infections when effector T cells responding to invading pathogens are incidentally recruited and neutralized by T R cells. Future studies should examine the significance of gld-induced VCAM-1 expression.

Apoptosis of CD4 T cells and especially CD25+CD4+ T R cells in the steady state was reduced in gld mice as measured by Annexin V and DNA content (data not shown). However, superantigen (Sag)-reactive T cells were deleted and not converted into T R cells in gld mice. C3H/gld/gld mice express a mammary tumor virus 6 that encodes a Sag that recognizes and activates T cells bearing Vβ3 (51). We did not detect the expansion of CD25+CD4+ T R cells expressing Vβ3 in the thymi or periphery of mutant or wt mice (data not shown), indicating that endogenous Sag-reactive thymocytes were deleted in mutant mice and did not contribute to T cell expansion. Several decoy molecules have been identified and include decoy receptor 1 (Dcr1/RTRID, Dcr2/TRUNDD, Dcr3 and osteoprotegrin (52). The decoy molecules do not transduce apoptotic signals but rather compete with the death receptors for ligand binding and thereby inhibit ligand-induced apoptosis. It will be interesting in the future to determine whether Dcr3 that can bind to FasL could have similar effects as those induced by gld mutation albeit in specific or controlled manner.

The data sets used in this study will be freely available for download from (http://pathology2.jhu.edu/hamadlab/index.htm) and will be deposited in public databases. Because of the large number of genes identified in various analyses described in this study, it will be impossible to validate all these by ourselves. However, several parameters established the accordance of the variances identified in this study. These include independent verification of T reg signature genes (Foxp3, CD25, CD103 and CTLA-4) and another set of selected genes (Fig. 4D). Furthermore, our analysis correlated well with the published general transcriptional profile of T R cells. While gld mutation prevents T cell-mediated diseases such as autoimmune diabetes and multiple sclerosis in animal models, it is associated in certain backgrounds with lupus-like disease. In addition, polymorphism in FasL has been linked to susceptibility to lupus in humans (53). Therefore, it was interesting that a number of highly up-regulated genes in gld CD25+CD4+ T R cells has been associated with lupus (53–55).

Fig. 4. Comparison of gene expression levels in gld and wt CD25+CD4+ T R cells. (A) Venn diagram indicating the numbers of genes up-regulated in gld CD25+CD4+ T R cells relative to gld CD25− T cells (gld/CD25n), wt CD25+CD4+ T R cells relative to gld CD25− T cells (wt/CD25n) and gld CD25+CD4+ T R cells relative to wt CD25+CD4+ T R cells (gld/wt). Specific and shared up-regulated genes in each subset are shown. Posterior probability of >0.5 was used to identify differentially expressed genes. (B) Heat map showing expression levels of the top 15 most expressed genes in gld CD25+CD4+ T R cells and the level of expression of each gene in wt CD25+CD4+ T R cells using gld CD25−CD4− T cells as a common denominator. Well-characterized T reg cell-specific genes are shown in boldface. Colors indicate gene expression levels based on log2 signal intensity. (C) Selected (19/56) functionally clustered gene groups that were differentially expressed by at least 3-fold in gld CD25+CD4+ T R cells relative to wt CD25+CD4+ T R cells (left) or relative to gld CD25−−CD4+ T R cells (right) are shown. (D) Over-expression of VCAM-1 on gld CD25+CD4+ T R cells was validated by reverse transcription-PCR. Real-time PCR was performed as described in Methods. (E) Up-regulation of VCAM-1 by gld mutation. CD25−CD4+ T R cells were gated and analyzed for VCAM-1 expression in lymph nodes and spleens. Quadrants were drawn using PE-labeled isotype control. The percentage of VCAM-1+ cells was shown in each plot.
Analysis of genes modulated (up or down) by gld mutation could therefore be helpful for understanding the pathogenesis of lupus.

In summary, our data show that gld mutation is associated with significant increase in the absolute numbers and frequency of CD25+CD4+ T<sub>R</sub> cells. Gld mutation also induces expression of significant number of genes that control cell survival, metabolism and innate immune response. Together, these data provide a base for cellular and molecular analysis of mechanisms that control the paradoxical roles of gld and by extension lpr mutations in regulating T cell homeostasis and tolerance.

**Supplementary data**

Supplementary data are available at *International Immunology* Online.

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**Abbreviations**

BrdU bromodeoxyuridine  
CTLA-4 cytotoxic T lymphocyte associated-4  
Dcr1 decoy receptor 1  
DN double negative  
GITR glucocorticoid-induced tumor necrosis factor receptor family-related gene  
Gld generalized lymphoproliferative disease  
Sag supernigen  
Tacstd1 tumor-associated calcium signal transducer 1  
T<sub>R</sub> regulatory  
VCAM-1 vascular cell adhesion molecule-1  
w<sub>f</sub> wild type

**References**