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# Up-Regulation of Fas and the Costimulatory Molecules B7-1 and B7-2 on Peripheral Lymphocytes in Autoimmune B6/*gld* Mice[1]

**Jory P. Weintraub,* Robert A. Eisenberg,† and Philip L. Cohen[2]***

**C57BL/6-*gld/gld* (B6/*gld*) mice have a point mutation in the gene for Fas ligand (FasL) resulting in nonfunctional FasL protein. We hypothesized that the lack of normal Fas/FasL interactions in these mice might result in abnormalities of Fas expression. Thus, we compared spleen cells from B6/*gld* mice and normal B6 control mice. While B6 spleen cells consisted of two main populations, Fas$^{high}$ (high Fas expression) and Fas$^{low}$ (low Fas expression), nearly all B6/*gld* spleen cells were Fas$^{high}$. Two-color immunofluorescence revealed that the Fas$^{high}$ and Fas$^{low}$ populations in the B6 spleen were Thy-1.2$^{+}$ (T cells) and IgM$^{+}$ (B cells), respectively, whereas both T cells and B cells in the B6/*gld* spleen were Fas$^{high}$, indicating that Fas expression is increased on B cells in the B6/*gld* spleen. This phenomenon was age related and restricted to peripheral lymphocytes. In addition to Fas, B6/*gld* splenic B cells showed increased expression of the costimulatory molecule B7-2, while the related costimulatory molecule B7-1 was up-regulated on both B cells and T cells in the B6/*gld* spleen. In vitro, both B cells and T cells from the B6/*gld* spleen showed an increase in susceptibility to apoptosis mediated by soluble anti-Fas Ab. These results suggest that some lymphocytes in B6/*gld* mice are primed to undergo Fas-mediated apoptosis, but are unable to do so due to the absence of functional FasL. Further study of such abnormal lymphocytes in the B6/*gld* spleen may elucidate the nature of autoimmunity in these mice.** ***The Journal of Immunology,* 1997, 159: 4117–4126.**

Fas (CD95) is a 35-kDa type I transmembrane protein belonging to the TNF receptor family of proteins (1). Its expression is widespread and includes the thymus, lymph nodes (LN),[3] spleen, liver, lung, heart, kidney, and ovaries (2). The ligand for Fas (FasL) is a 40-kDa type II transmembrane protein that is a member of the TNF family (3). Expression of FasL is much more restricted; although it has recently been demonstrated in the testes (4) and anterior chamber of the eye (5), FasL expression in normal animals is generally limited to activated, mature T cells (6). Interaction of Fas with its ligand or cross-linking of Fas with Ab results in rapid apoptosis of the Fas-bearing cell (2).

The Fas/FasL system plays a crucial role in the maintenance of peripheral tolerance, as evidenced in mice lacking either of these two proteins. Mice homozygous for mutations in the genes for Fas or FasL (*lpr* (7) and *gld* (8) mutations, respectively) develop marked lymphadenopathy, primarily due to the accumulation of T cells that are CD3$^{+}$, Thy-1$^{+}$, B220$^{+}$, CD4$^{-}$, CD8$^{-}$ and are known as double-negative (DN) T cells (9). Although DN T cells appear to be chronically activated based on their cell surface markers, these cells are anergic and nonmalignant, show reduced survival in culture, and respond poorly to mitogens such as Con A (10, 11). The *lpr* and *gld* mice also produce autoantibodies similar to those seen in human systemic lupus erythematosus, presumably due to a failure to delete autoreactive lymphocytes via Fas/FasL (9).

The *fas ligand* gene has been shown to be constitutively activated in the accumulating DN T cells of *lpr* and *gld* mice, resulting in massive up-regulation of FasL on these cells (12, 13). Because FasL expression is normally restricted to activated T cells, constitutive expression of FasL by *lpr* and *gld* DN T cells is believed to reflect their status as a chronically activated subset of cells that are unable to undergo Fas/FasL-mediated apoptosis. The purpose of this study was to determine whether there is a similar dysregulation of Fas in *gld* mice. Spleen cells from B6 and B6/*gld* mice were isolated, and levels of Fas expression were compared. Our results show that Fas is up-regulated on the splenic B cells of B6/*gld* mice, and that this phenomenon is age related and coincident with increased expression of other lymphocyte activation markers. In addition, both splenic B cells and T cells from B6/*gld* mice showed an increased susceptibility to anti-Fas-mediated apoptosis in vitro.

## Materials and Methods

### Mice

C57BL/6 (B6), C57BL/6-*gld/gld* (B6/*gld*), and C57BL/6-*lpr/lpr* (B6/*lpr*) strains were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our breeding facility at the University of North Carolina School of Medicine. Except where noted, all animals were 6 to 8 mo old when the studies were performed.

### Antibodies

Anti-Fas-PE (Jo2, hamster IgG), anti-TNP-PE (UC8–4B3, hamster IgG), anti-B7.1-PE (16–10A1, hamster IgG), anti-B7.2-PE (GL1, rat IgG2a), anti-CD28-PE (37.51, hamster IgG), anti-CD40-PE (HM40–3, hamster IgM), anti-CD40L-PE (MR1, hamster IgG), anti-IgM-FITC (II/41, rat IgG2a), anti-Thy-1.2-FITC (53–2.1, rat IgG2a κ), and purified anti-Fas (Jo2, hamster IgG) were obtained from PharMingen, Inc. (San Diego, CA). Biotinylated anti-mouse IgD (Hbδ6, rat IgG2a) was obtained from

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[3] Abbreviations used in this paper: LN, lymph node; FasL, Fas ligand; DN, double negative; B6, C57BL/6; B6/*gld*, C57BL/6-*gld/gld*; B6/*lpr*, C57BL/6-*lpr/lpr*; PE, phycoerythrin; CD40L, CD40 ligand; PL, peritoneal lavage; BM, bone marrow; Fas$^{low}$, low Fas expression; Fas$^{high}$, high Fas expression; GC, germinal center.

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F. Finkelman. Anti-Fcγ III/II receptor Ab (2.4G2, rat IgG2b) was obtained from American Type Culture Collection (Rockville, MD).

### *Preparation of cells*

Mice were killed by cervical dislocation, and spleens were removed to cold complete medium (RPMI 1640 supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids (University of North Carolina Cancer Center Tissue Culture Facility), and $5 \times 10^{-5}$ M 2-ME (Sigma Chemical Co., St. Louis, MO)). Single cell suspensions were obtained by pressing spleens through sterile nylon mesh with a 3-cc syringe plunger. RBCs were lysed in $NH_4Cl$ for 5 min on ice, and cells were washed twice in cold medium and counted. Peritoneal lavage (PL) cells were obtained by injecting 10 ml of cold complete medium using a 25-gauge needle into the peritoneal cavity of mice after cervical dislocation, gently massaging the abdomen, and withdrawing the medium. This was performed twice for each animal, and peritoneal washings were pooled. The RBCs were lysed as described above, and cells were washed twice in cold medium and counted. To obtain bone marrow (BM) cells, the marrow cavities of femurs and tibias were flushed multiple times with cold complete medium using a syringe with a 19-gauge needle. The RBC were lysed, and cells were washed twice in cold medium and counted. For stimulation of spleen cells, LPS (Sigma Chemical Co.) was added at 100 μg/ml, and cells were cultured at 37°C and 5% $CO_2$ for 72 h and then washed twice in cold medium and counted.

### *Immunofluorescence and flow cytometry*

Cells were stained in 96-well microtiter plates at $1 \times 10^6$ cells/well. All samples were pretreated with 25 μl of overgrown TC supernatant of anti-Fcγ III/II receptor Ab for 15 min at 4°C to block nonspecific binding. Seventy-five microliters of fluorochrome-conjugated or biotinylated Ab (diluted in PBS/0.1% $NaN_3$) was then added to each well (except for unstained control samples), and cells were incubated for an additional 45 min at 4°C, then washed twice in cold PBS/0.1% $NaN_3$ and, if biotinylated Abs were used, incubated for 30 min at 4°C with streptavidin-PE (Southern Biotechnology Associates, Inc., Birmingham, AL). Cells were then washed twice in cold PBS/0.1% $NaN_3$ and fixed in an equal volume of PBS/0.1% $NaN_3$ and 2% paraformaldehyde. Multicolor flow cytometric analyses were performed on a FACScan (Becton Dickinson Co., Mountain View, CA) flow cytometer with Cytomation data acquisition and software (Fort Collins, CO). In all cases, at least $2 \times 10^4$ events were recorded, with size gating on the lymphocyte population.

### *Induction of apoptosis*

After isolation and washing, spleen cells were placed into culture in a 24-well microtiter plate ($4 \times 10^6$ cells/well in 1 ml of complete RPMI 1640 medium). Cells were treated with dexamethasone (Sigma Chemical Co.) ($10^{-6}$ M, final concentration), anti-Fas Ab (1 μg/ml, final concentration), or medium alone (control). Cells were cultured for 18 to 22 h at 37°C in 5% $CO_2$ and then washed twice with PBS/0.1% $NaN_3$. For quantitation of apoptosis, cells were surface stained as described above; however, instead of fixing in 2% paraformaldehyde, cells were fixed for 60 min in ice-cold 70% ethanol, washed twice with PBS/0.1% $NaN_3$, and resuspended in 100 μl of 1 mg/ml RNase A (Sigma Chemical Co.) followed by 200 μl of 0.1 mg/ml propidium iodide (Sigma Chemical Co.). Cells were then analyzed on a FACScan, with apoptotic cells appearing as a hypodiploid peak. Data were analyzed by a two-sample *t* test.

## Results

### *The* gld *spleen cells display an abnormal pattern of Fas expression*

Our initial observation was that, when compared with spleen cells from a normal, 6- to 8-mo-old B6 mouse, age-matched B6/*gld* spleen cells displayed a distinctly different pattern of Fas expression. In one-color analysis, B6 spleen cells consistently showed a bimodal distribution of Fas (Fig. 1*a*). When compared with the pattern of staining obtained with an irrelevant isotype control Ab (dashed line), the two populations appeared to be $Fas^{low}$ (56.0 ± 1.5%) and $Fas^{high}$ (42.5 ± 1.5%), rather than $Fas^-$ and $Fas^+$. In contrast, when spleen cells from comparable B6/*gld* mice were analyzed (Fig. 1*b*), a single $Fas^{high}$ peak was seen, with the majority of the cells appearing $Fas^{high}$ (69.5 ± 2.8%), and a much lower percentage (28.6 ± 2.7%) appearing $Fas^{low}$. In addition, the fluorescence intensity of the $Fas^{high}$ cells in the B6/*gld* spleen revealed that some of these cells (designated $Fas^{high+}$) stained more brightly for Fas than any cells seen in the B6 spleen (compare the areas to the *right* of the dashed vertical lines in Fig. 1, *a* and *b*). These results suggested that the $Fas^{low}$ cells seen in the B6 spleen are either absent from B6/*gld* mice or have up-regulated Fas, some to levels higher than those in normal mice.

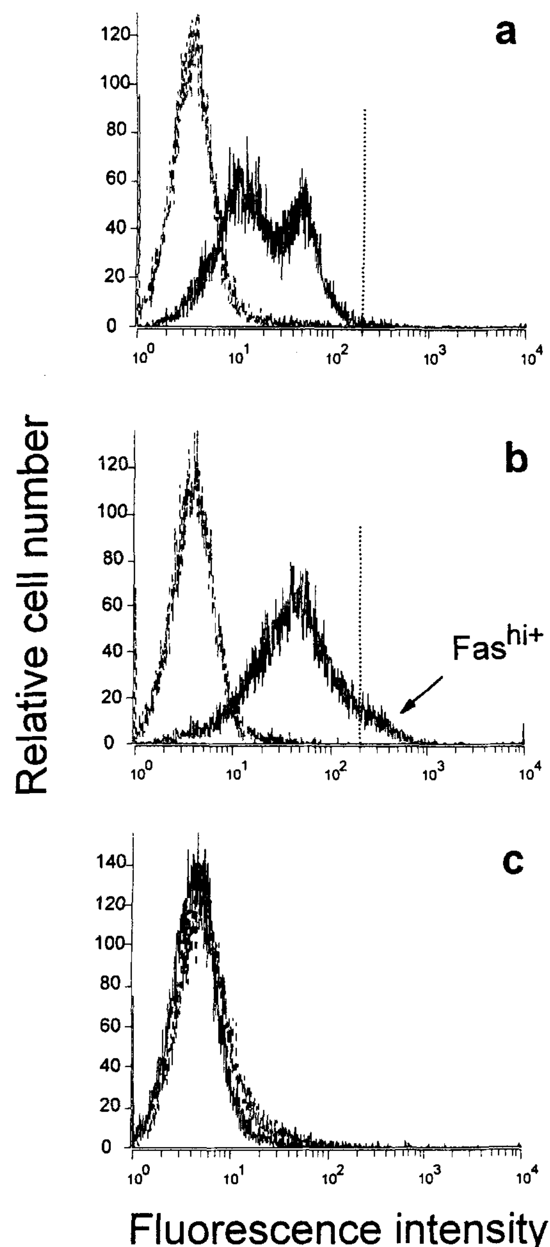


**FIGURE 1.** Anti-Fas staining of spleen cells from B6 (*a*), B6/*gld* (*b*), and B6/*lpr* (*c*) mice revealed an abnormal pattern of Fas expression caused by the *gld* mutation. Each histogram shows an overlay of anti-Fas (solid line) and isotype control (anti-TNP, dashed line) profiles. All mice were 6.5 to 8 mo old at the time of study. Representative data from one mouse are shown in each case (*n* = 10).

As a negative control, spleen cells from B6/*lpr* mice (which have a mutation in the *fas* gene resulting in early termination of transcription and greatly reduced levels of Fas protein) (14) were analyzed for Fas expression. As shown in Figure 1*c*, age-matched B6/*lpr* spleen cells stained with the anti-Fas Ab appeared almost identical to those stained with an isotype control Ab (dashed

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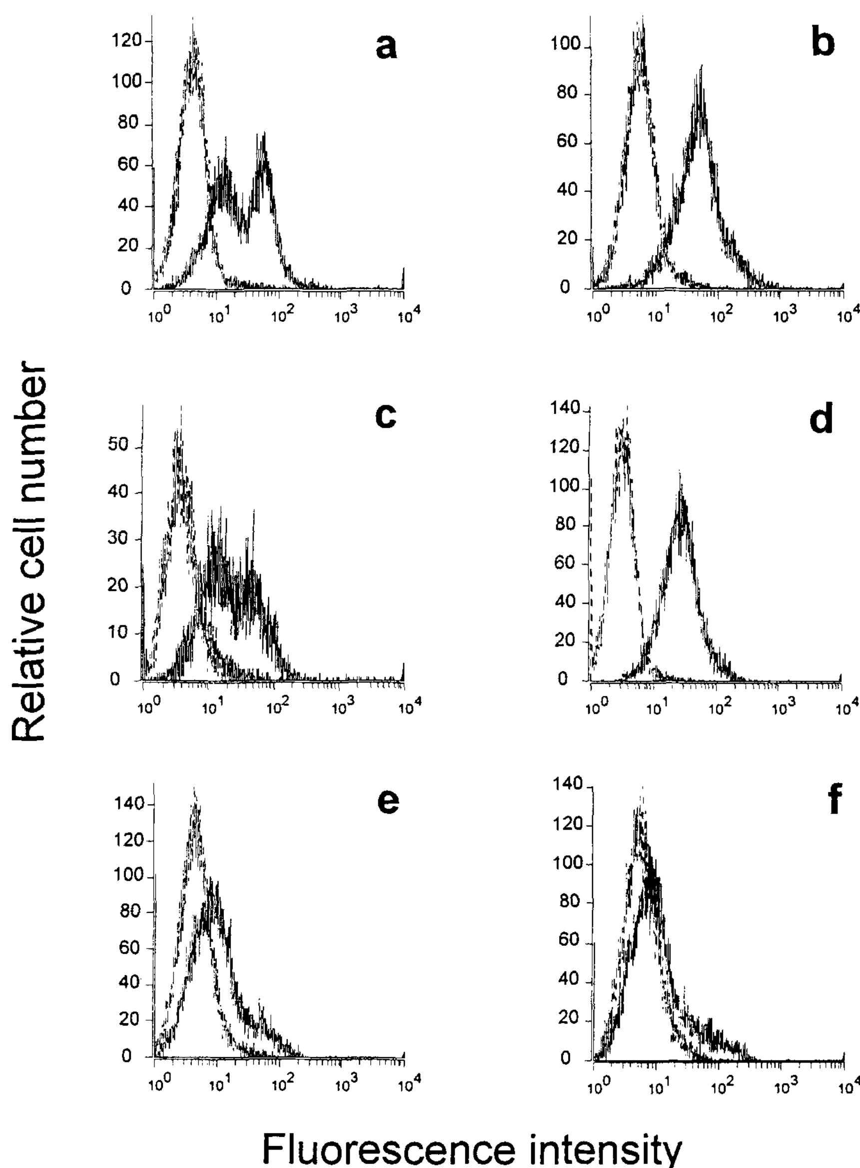


**FIGURE 2.** Abnormal Fas expression in B6/*gld* mice was restricted to peripheral lymphocytes, while Fas expression in the BM appeared similar in B6 and B6/*gld* mice. Anti-Fas (solid line)/isotype-control (dashed line) profiles are shown for B6 (*a*, *c*, and *e*) and B6/*gld* (*b*, *d*, and *f*) mice. Lymphocytes shown are from spleen (*a* and *b*), peritoneal cavity (*c* and *d*), or BM (*e* and *f*). All mice were 6.5 to 8 mo old at the time of study. Representative data from one mouse are shown in each case ($n = 5$).

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line), indicating that the staining of B6 and B6/*gld* spleen cells in Figure 1, *a* and *b*, was indeed Fas specific.

### *Fas is overexpressed in* gld *peritoneal lymphocytes and in LN, but not in BM*

We next wanted to determine whether the altered *gld* Fas staining profile shown in Figure 1 was restricted to the spleen cells, or if lymphocytes from other locations would show similar patterns of Fas expression. We therefore compared Fas expression on LN cells, PL lymphocytes, and BM lymphocytes to that on spleen cells. The peritoneal cavity contains a resident population of cells, including T cells, B cells, and macrophages, that are similar in many ways to other peripheral lymphocytes (i.e., those found in the spleen and LN), but with some distinguishing characteristics (15).

PL lymphocytes from B6 and B6/*gld* mice exhibited the same distinctive pattern of Fas expression as that observed for B6 and B6/*gld* spleen cells (compare Fig. 2, *a* and *b*, with Fig. 2, *c* and *d*). Specifically, PL lymphocytes from B6 mice showed a bimodal distribution with $Fas^{low}$ (51.4 ± 1.3%) and $Fas^{high}$ (47.9 ± 1.0%) populations, whereas PL lymphocytes from B6/*gld* mice appeared as a single $Fas^{high}$ peak (68.2 ± 2.6%). However, the $Fas^{high+}$ cells seen in the B6/*gld* spleen appeared to be absent from PL lymphocytes in B6/*gld* mice (compare Fig. 2, *b* and *d*). Similar results were seen when LN cells were stained for Fas (data not shown).

In contrast, BM lymphocytes from both B6 and B6/*gld* mice (Fig. 2, *e* and *f*) expressed very low levels of Fas (slightly above background levels in most cases), and the overall pattern of Fas expression appeared to be quite similar in both strains. Interestingly, Fas expression on BM lymphocytes showed more mouse-to-mouse variation than that on splenic lymphocytes, which consistently displayed the Fas expression profiles shown in Figure 1, perhaps reflecting a mixture of naive BM lymphocytes and recirculating mature lymphocytes that have migrated to the BM from the periphery. However, despite slight variations in Fas expression on BM lymphocytes from some animals, the general patterns were similar in B6 and B6/*gld* mice, suggesting that increased expression of Fas in B6/*gld* mice is restricted to peripheral lymphocytes.

### *Splenic B cells in* gld *mice overexpress Fas*

Two-color immunofluorescence was then used to determine whether the differences in Fas expression between B6 and B6/*gld*

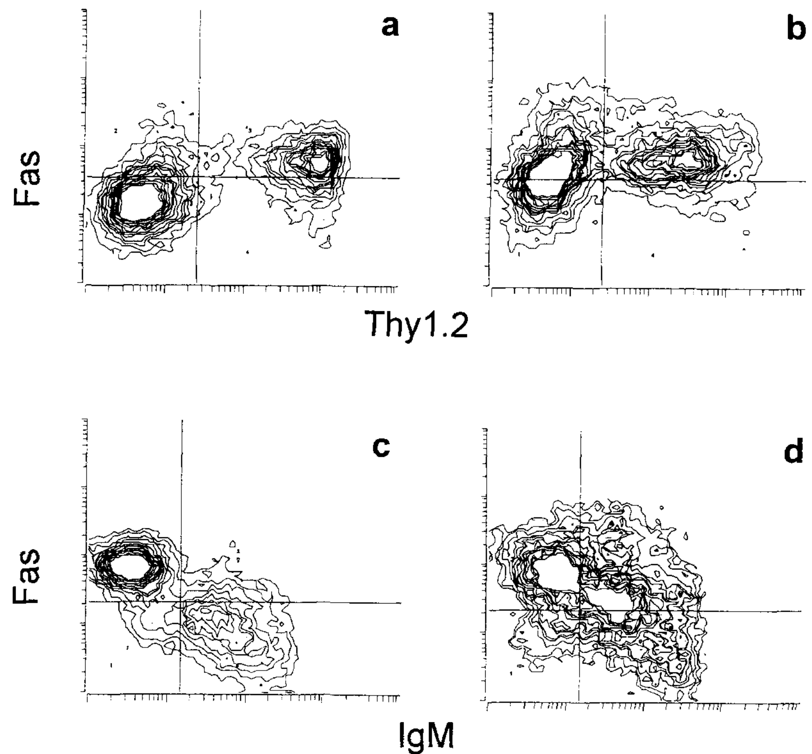


**FIGURE 3.** Abnormal Fas expression in the B6/*gld* spleen was restricted to IgM$^+$, Thy-1.2$^-$ cells (B cells). Two-color flow cytometric analysis of B6 (*a* and *c*) and B6/*gld* (*b* and *d*) spleen cells, measuring expression of Fas and Thy-1.2 (*a* and *b*) or Fas and IgM (*c* and *d*), revealed up-regulation of Fas in the IgM$^+$, Thy-1.2$^-$ population of the B6/*gld* spleen. All mice were 6.5 to 8 mo old at the time of study. Representative data from one mouse are shown in each case ($n$ = 8).

could be attributed to a particular lymphocyte subset. Cells from both B6 and B6/*gld* spleens were stained with anti-Fas and with either anti-Thy-1.2 (to identify T cells) or anti-IgM (to identify B cells). Figure 3*a* shows that when B6 spleen cells were stained for Fas and Thy-1.2, the Thy-1.2$^+$ cells were Fas$^{high}$ (42.9 ± 2.6%), and the Thy-1.2$^-$ cells were Fas$^{low}$ (57.1 ± 2.6%). In contrast, when B6/*gld* spleen cells were stained for Fas and Thy-1.2, nearly all Thy-1.2$^+$ cells and the majority of Thy-1.2$^-$ cells were Fas$^{high}$ (79.1 ± 2.2%), while a much lower percentage of Thy-1.2$^-$ cells (20.9 ± 2.2%) was Fas$^{low}$ (Fig. 3*b*).

These results suggested that it was the B cells in the B6/*gld* spleen that expressed abnormally elevated levels of Fas. This was confirmed by double staining for Fas and IgM (Fig. 3, *c* and *d*). The IgM$^-$ population appeared to be Fas$^{high}$ in both strains, while the IgM$^+$ population in B6/*gld* mice showed a marked increase in Fas expression compared with the IgM$^+$ population in B6 mice (46.2 ± 2.0% were Fas$^{high}$ and 53.8 ± 2.0% were Fas$^{low}$ in the B6 spleen, while 73.0 ± 2.8% were Fas$^{high}$ and 27.0 ± 2.8% were Fas$^{low}$ in the B6/*gld* spleen). Similar results were observed in double-staining experiments with PL cells and LN cells (data not shown).

## *Overexpression of Fas on splenic B cells in* gld *mice is age related*

All the data shown to this point were generated from studies on animals that were 6 to 8 mo of age. However, the severity of the disease caused by the *gld* mutation in mice is age related (9). B6/*gld* mice appear normal at birth. At 4 to 6 wk, DN T cells begin to appear in the LN, and by 4 to 5 mo they make up >75% of the LN cells. Autoantibody formation occurs in roughly the same time frame. We, therefore, wanted to determine whether the overexpression of Fas on splenic B cells in B6/*gld* mice was similarly age related.

Spleen cells from B6 and B6/*gld* mice at 1, 3, and 5 mo of age were double stained with anti-Fas Ab and either anti-Thy-1.2 or anti-IgM Abs, and examined by flow cytometry. The results presented in Figure 4 show that in B6 mice, the IgM$^-$ spleen cells were Fas$^{high}$, and the IgM$^+$ cells were Fas$^{low}$ at all three time points (Fig. 4, *a–c*). (The percentages of Fas$^{high}$ cells at 1, 3, and 5 mo were 30.1 ± 1.6, 36.4 ± 1.2, and 38.9 ± 1.4%, respectively, while the percentages of Fas$^{low}$ cells at these same time points were 69.9 ± 1.6, 63.6 ± 1.2, and 61.1 ± 1.4%, respectively.) The results were comparable to the staining profiles seen in 6- to 8-mo-old mice (Fig. 3*c*). In contrast, while the IgM$^-$ B6/*gld* spleen cells were Fas$^{high}$ at 1, 3, and 5 mo (Figs. 4, *d–f*), the IgM$^+$ cells showed a marked change in Fas expression as the mice aged. At 1 mo (Fig. 4*d*), IgM$^+$ B6/*gld* spleen cells showed a diffuse staining pattern, with Fas expression ranging from bright to nearly negative (34.0 ± 4.5% of all B6/*gld* spleen cells were Fas$^{high}$ and 66.0 ± 4.5% were Fas$^{low}$). By 3 mo, IgM$^+$ B6/*gld* spleen cells were showing less diffuse Fas staining, and the overall levels of Fas expression already appeared brighter than those in their age-matched B6 counterparts (compare Fig. 4, *b* to *e*; 58.5 ± 4.8% of all B6/*gld* spleen cells were Fas$^{high}$ and 41.5 ± 4.8% were Fas$^{low}$). At 5 mo, Fas expression on IgM$^+$ B6/*gld* spleen cells had increased slightly above the level seen at 3 mo (compare Fig. 4, *e* to *f*), although it was still not as bright as that in the 6- to 8-mo-old animals (Fig. 3*d*; 64.0 ± 4.2% of all B6/*gld* spleen cells were Fas$^{high}$ and 36.0 ± 4.2% were Fas$^{low}$). Similar results were obtained when spleen cells from B6 and B6/*gld* mice at 1, 3, and 5 mo of age were double stained with anti-Fas and anti-Thy-1.2, with the Thy-1.2$^-$ B6/*gld* spleen cells increasing their levels of Fas expression with age (data not shown).

Interestingly, at 1 mo both B6 and B6/*gld* mice contained a population of spleen cells that were IgM$^-$ and Fas$^-$ (see *lower left quadrants* of Fig. 4, *a* and *d*), but were absent in both strains at 3 and 5 mo. These cells did not appear to be T cells, as no corresponding Thy-1.2$^+$, Fas$^-$ cells were seen when 1-mo-old spleen cells from B6 and B6/*gld* mice were double stained with anti-Fas

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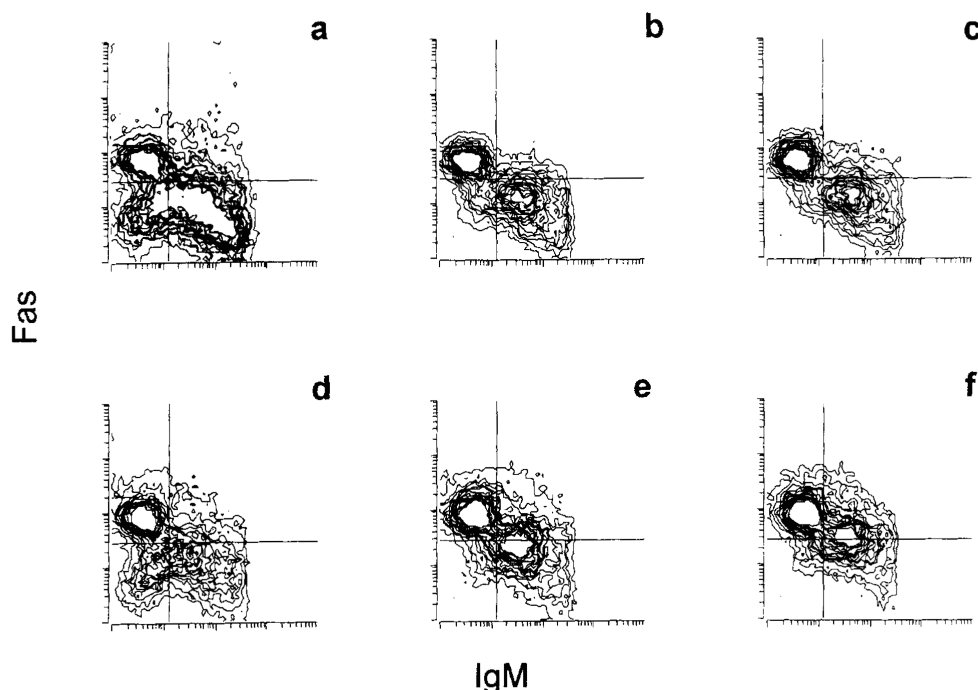


**FIGURE 4.** Up-regulation of Fas in splenic B cells of B6/*gld* mice was age related. Spleen cells from B6 (*a–c*) and B6/*gld* (*d–f*) mice at 1 mo (*a* and *d*), 3 mo (*b* and *e*), and 5 mo (*c* and *f*) were stained for Fas and IgM. No differences were seen in Fas expression on the $IgM^+$ cells from B6 mice (*a–c*), while a gradual increase was seen in age-matched B6/*gld* mice (*d–f*). Similar results were seen in the Thy-$1.2^-$ populations of B6 and B6/*gld* mice (data not shown). Representative data from one mouse are shown in each case ($n = 4$).

and anti-Thy-1.2 Abs (data not shown). The identity of this population of cells is unclear; they may be hemopoietic precursors, macrophages, or possibly a combination of both.

In summary, B cells in the B6/*gld* spleen showed an age-dependent up-regulation of Fas expression that was not seen in their B6 counterparts and that coincided with the known time course for the accumulation of DN T cells and autoantibodies.

### *B6/*gld *spleen cells express elevated levels of the costimulatory molecules B7-1 and B7-2*

Because Fas was expressed at elevated levels in B6/*gld* splenic B cells, we measured other activation-related cell surface markers on B and T cells in B6/*gld* mice. Using Abs specific for CD40, CD40L, CD28, B7-1, and B7-2, flow cytometric studies were performed on freshly isolated, unstimulated spleen cells from 6- to 8-mo-old B6 and B6/*gld* mice. Immunofluorescence studies revealed no significant differences in levels of expression of CD40 and CD40L (data not shown). In contrast, while B7-2 was not expressed at significant levels in unstimulated B6 spleen cells, this B cell marker appeared to be up-regulated significantly in the $IgM^+$ population of the B6/*gld* spleen. Figure 5 shows an overlay of the B7-2 expression profiles for $IgM^+$ spleen cells from B6 and B6/*gld* mice as well as LPS-stimulated spleen cells from B6 mice. While the unstimulated B6/*gld* B cells did not express as much B7-2 as LPS-stimulated cells, they up-regulated B7-2 relative to that in unstimulated B6 B cells, suggesting that the B cells in the B6/*gld* spleen may be chronically activated. No significant B7-2 expression was observed on Thy-$1.2^+$ spleen cells from either B6 or B6/*gld* mice.

Interestingly, one-color immunofluorescence using an anti-B7-1 Ab revealed a significant up-regulation of this molecule in the B6/*gld* spleen relative to that in the B6 spleen (data not shown), seemingly in contrast to previous reports from our laboratory (16) in which no differences were seen in B7-1 expression on B cells. Surprisingly, when this was followed by two-color immunofluorescence studies using anti-B7-1 and anti-IgM Abs or anti-Thy-1.2 Abs, it was revealed that the up-regulation of B7-1 in the B6/*gld* spleen was primarily occurring on Thy-$1.2^+$ spleen cells (compare Fig. 6, *b* and *d*). (In B6 mice, $17.3 \pm 0.7\%$ of Thy–$1.2^+$ spleen cells were B7-$1^+$, and $82.4 \pm 0.7\%$ were B7-$1^-$, while in B6/*gld* mice $60.0 \pm 4.3\%$ of Thy-$1.2^+$ spleen cells were B7-$1^+$, and $38.7 \pm 4.2\%$ were B7-$1^-$.) A slight increase in B7-1 expression was consistently seen in the $IgM^+$ population in the B6/*gld* spleen compared with that in the B6 spleen (compare Fig. 6, *a* and *c*); however, the shift was not as dramatic as that seen in the Thy-$1.2^+$ population of spleen cells, suggesting that the up-regulation of B7-1 on B6/*gld* spleen cells is primarily restricted to the T cell population. Three-color flow cytometric analysis of B6/*gld* spleen cells using Abs specific for IgM, B7-1, and B7-2 demonstrated that the small population of B7-$1^+$ B cells was a subset of the B7-$2^+$ B cells (data not shown).

CD28, the counter-receptor for B7-1 and B7-2, is constitutively expressed on essentially all T cells found in the spleen, LN, and peripheral blood of normal mice (17). Our results from anti-CD28 staining of B6 and B6/*gld* spleen cells were consistent with previous results reported by Giese et al. (18), who found that *gld* T cells not only express CD28, but do so at slightly higher levels than those in normal spleen cells (data not shown).

### *The* gld *spleen contains an increased percentage of $IgM^+$/$IgD^-$ (activated) B cells*

In addition to up-regulating certain costimulatory molecules, activated B cells down-regulate the expression of surface IgD, becoming $IgM^+$/$IgD^-$ (19). We, therefore, performed two-color immunofluorescence staining of freshly isolated spleen cells using anti-IgM and anti-IgD Abs to determine whether there was an increase in $IgM^+$/$IgD^-$ (activated) B cells in B6/*gld* mice. Figure 7 shows representative data ($n = 6$) in which the mean percentage of $IgM^+$/

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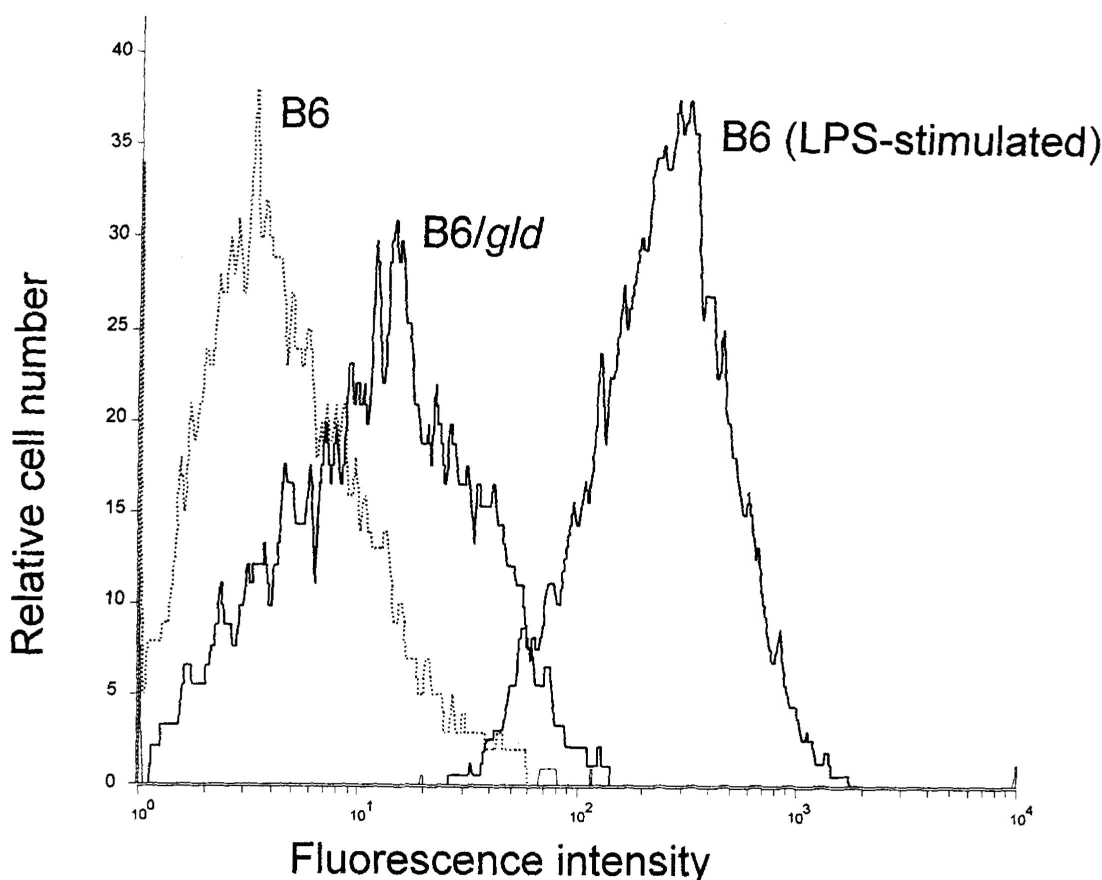


**FIGURE 5.** Expression of the costimulatory molecule B7-2 was elevated in splenic B cells of B6/*gld* mice. Two-color immunofluorescence using anti-IgM and anti-B7-2 Abs was performed on B6, B6/*gld*, and LPS-stimulated B6 spleen cells. Shown are overlays of B7-2 expression profiles after gating on $IgM^+$ cells. Representative data from one mouse are shown in each case ($n = 6$).

$IgD^-$ cells (*lower right quadrant*) was 2.9 ± 0.3% in the B6 spleen and 10.1 ± 1.9% in the B6/*gld* spleen ($p < 0.005$). These results indicated that there was a significantly higher percentage of $IgM^+/IgD^-$ (activated) B cells in B6/*gld* mice, consistent with other phenotypic data suggesting chronic activation of lymphocytes in these animals.

### *gld spleen cells show increased susceptibility to apoptosis mediated by a soluble anti-Fas Ab*

To explore the functional significance of the up-regulation of Fas on B6/*gld* spleen cells we examined whether these cells would show an increased susceptibility to anti-Fas-mediated apoptosis. Freshly isolated B6 or B6/*gld* spleen cells were cultured in the presence or the absence of soluble Jo2 Ab (an anti-Fas Ab that has been shown previously to induce lymphocyte apoptosis both in vivo (20) and in vitro (21)) or in the presence of the glucocorticoid dexamethasone as a positive control for apoptosis induction. Following overnight culture the cells were stained with Abs to IgM or Thy-1.2, fixed in ethanol, and treated with RNase A and propidium iodide. Flow cytometric quantitation of apoptosis of splenic T cells and B cells was then performed by gating on $Thy\text{-}1.2^+$ cells and $IgM^+$ cells, respectively, and measuring the hypodiploid (apoptotic) DNA peak. Figure 8 shows the percentage of cells that underwent specific apoptosis due to Jo2 treatment, after correcting for background (spontaneous) apoptosis.

When examining the total spleen cell population, B6/*gld* spleen cells were nearly 4 times more susceptible than B6 spleen cells to Jo2-induced apoptosis (specific apoptosis equaled 3.5 ± 0.4% for B6 spleen cells and 13.5 ± 0.7% for B6/*gld* spleen cells; $p < 0.0001$). Splenic B cells from B6/*gld* mice were 4.6 times more susceptible than B6 B cells to Jo2-induced apoptosis (specific apoptosis equaled 2.8 ± 1.1% for B6 B cells and 12.9 ± 2.7% for B6/*gld* B cells; $p < 0.05$). Splenic T cells from B6/*gld* mice were 2.6 times more susceptible than B6 T cells to Jo2-induced apoptosis (specific apoptosis equaled 5.6 ± 0.6% for B6 T cells and 14.6 ± 1.0% for B6/*gld* T cells; $p < 0.0005$).

Although there was not a statistically significant difference between B cells and T cells with respect to their susceptibility to anti-Fas-induced apoptosis, both B and T cells from B6/*gld* mice showed significantly greater susceptibility to anti-Fas-mediated apoptosis than their B6 counterparts. Levels of background (spontaneous) apoptosis did not differ significantly between the two strains. As a positive control, parallel cultures were treated with dexamethasone, resulting in high levels of apoptosis in spleen cells from both strains of mice (data not shown).

## Discussion

In this report we demonstrate that the apoptosis-signaling molecule Fas is up-regulated on splenic B cells of B6/*gld* mice, while the splenic T cells in these animals express Fas at levels similar to those in their normal B6 counterparts. This phenomenon is age related and appears to be restricted to peripheral lymphocytes. In addition to overexpressing Fas, splenic B cells in B6/*gld* mice express elevated levels of the costimulatory molecules B7-1 and B7-2, while splenic T cells in B6/*gld* mice only express elevated

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**FIGURE 6.** Expression of the costimulatory molecule B7-1 was elevated slightly in splenic B cells and markedly in splenic T cells of B6/*gld* mice. Two-color immunofluorescence using anti-IgM or anti-Thy-1.2 and anti-B7-1 Abs was performed on B6 (*a* and *b*) and B6/*gld* (*c* and *d*) spleen cells. IgM$^+$ cells (*a* and *c*) and Thy-1.2$^+$ cells (*b* and *d*) were gated separately, and B7-1 expression on these populations is shown. Representative data from one mouse are shown in each case ($n$ = 8).

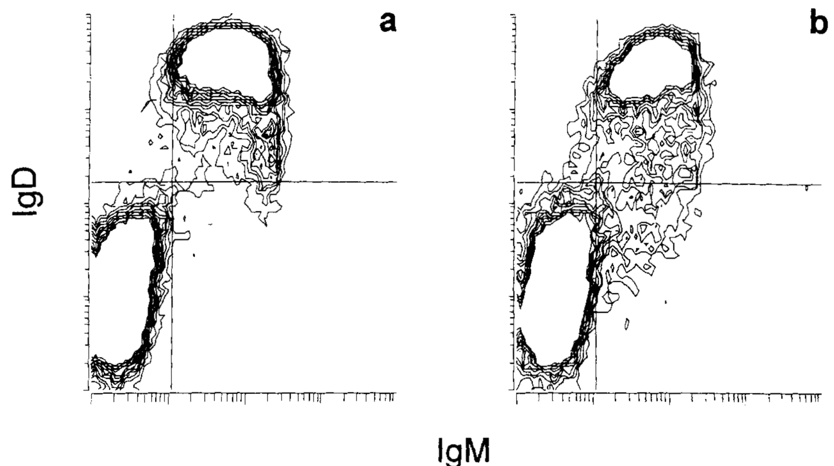


**FIGURE 7.** The B6/*gld* spleen contains an increased percentage of IgM$^+$/IgD$^-$ (activated) B cells. Two-color immunofluorescence using anti-IgM and anti-IgD Abs was performed on freshly isolated B6 (*a*) and B6/*gld* (*b*) spleen cells. Activated B cells are found in the *lower right quadrant* of each histogram. Quadrant regions were established based on the staining profile of normal (B6) splenic lymphocytes. All mice were 6.5 to 8 mo old at the time of study. Representative data from one mouse are shown in each case ($n$ = 6).

levels of B7-1. We also demonstrate an increased percentage of IgM$^+$/IgD$^-$ (activated) splenic B cells in B6/*gld* mice. Finally, we demonstrate that B6/*gld* spleen cells (both T and B cells) show a significant increase in susceptibility to soluble anti-Fas-mediated apoptosis in vitro compared with B6 spleen cells. Several findings from the present study are worthy of further consideration.

It is unclear why the lack of functional FasL results in dysregulation of Fas only on splenic B cells, while splenic T cells in these

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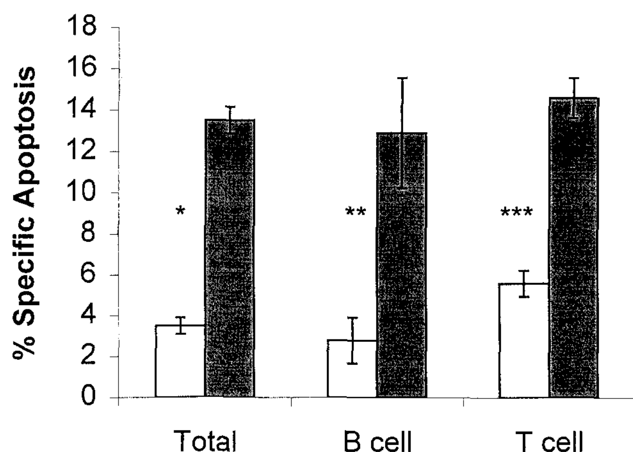


**FIGURE 8.** B6/*gld* spleen cells showed a significant increase in susceptibility to soluble anti-Fas-mediated apoptosis in vitro compared with B6 spleen cells. Spleen cells were cultured for 18 to 22 h in medium alone, medium plus Jo2 (anti-Fas; 1 μg/ml), or medium plus dexamethasone ($10^{-6}$ M). Shown is specific anti-Fas-induced apoptosis of total spleen cells, splenic B cells, and splenic T cells from B6 mice (white bar) and B6/*gld* mice (gray bar). Apoptosis was quantitated by staining cells with propidium iodide/RNase A and measurement of hypodiploid peaks. The *p* values were determined by two-sample *t* test (* indicates $p < 0.0001$; ** indicates $p < 0.05$; *** indicates $p < 0.0005$). Levels shown represent the mean percent apoptosis after subtraction of background (spontaneous) apoptosis ($n = 6$). Error bars represent 1 SEM.

animals appear relatively normal with respect to Fas expression. Based on studies from our laboratory as well as others (9, 22, 23), the *lpr* and *gld* mutations are known to result in a severely altered T cell phenotype (e.g., the DN T cell), while the B cell phenotype has appeared to be more stable (16). It might be the case that splenic T cells in B6 mice normally express maximal levels of Fas, and the *gld* mutation cannot result in significantly increased Fas expression. It is also possible that the propensity of T cells in *lpr* and *gld* mice to become DN T cells is an alternative to elevation of Fas in these cells above the already high levels seen in T cells of normal B6 mice.

It is also interesting to note that our findings suggest that the effects of the FasL mutation on Fas expression are peripheral, rather than central. PL lymphocytes appeared similar to spleen cells with respect to Fas expression (except for the unexplained absence of the $Fas^{high+}$ cells in PL lymphocytes in B6/*gld* mice), while similar patterns were not seen when Fas expression profiles on B6 and B6/*gld* BM lymphocytes were examined. This observation is consistent with previous reports from our laboratory and others that the Fas/FasL system is involved primarily in the establishment of peripheral tolerance, while other mechanisms are employed in the maintenance of central tolerance (24, 25).

The fact that Fas up-regulation in splenic B cells of B6/*gld* mice is age related is not surprising. Several other aspects of the *lpr* and *gld* diseases are age related, including accumulation of DN T cells and development of autoantibodies, and all seem to follow roughly the same time scale (9). We propose that $Fas^{high}$ B cells in the B6/*gld* spleen represent cells that are primed to undergo Fas/FasL-mediated apoptosis, but are unable to do so because of the lack of functional FasL. Since the Fas/FasL system is known to be involved in maintenance of peripheral tolerance, it might be the case that at least some of the persisting $Fas^{high}$ B cells are autoreactive cells that would have been deleted if functional FasL were present. Such a scenario is supported by the observation that the onset of $Fas^{high}$ B cells coincides with the development of autoantibodies in B6/*gld* mice. Our data, therefore, provide further support for the idea that the Fas/FasL system is crucial in the elimination of autoreactive B cells and the prevention of autoimmunity.

While it is possible that some of the $Fas^{high}$ B cells in *gld* mice represent nondeleted autoreactive cells, it is unlikely that all the B cells in these animals are autoreactive. Therefore, the up-regulation of Fas on the nonautoreactive *gld* B cells is an issue that requires further consideration. Mandik et al. (26) recently showed that in normal mice, Fas is expressed on B cells at all stages of development, from early progenitors in the BM through mature peripheral B cells, and expression is highest in germinal center (GC) B cells, which are undergoing affinity maturation. Martinez-Valdez et al. (27) further showed that in humans, both the $CD77^+$ centroblasts and $CD77^-$ centrocytes in the GC were $Fas^{high}$. These results suggest that a $Fas^{high}$ phenotype on normal B cells exists during affinity maturation and somatic hypermutation. It is, therefore, possible that the nonautoreactive $Fas^{high}$ B cells in *gld* mice represent an increase in GC B cells or in B cells undergoing somatic hypermutation.

In a previous study (16), >30 Abs to various B cell markers (including the costimulatory molecule B7-1) were used to examine potential differences in phenotype between splenic B cells from normal B6 mice and those from autoimmune B6/*lpr* and B6/*gld* mice. There was a general lack of phenotypic differences, with the one exception being an increase in $CD23^{low}$ B cells in the B6/*lpr* and B6/*gld* spleens.

In this study we demonstrate that the costimulatory molecule B7-2 is up-regulated on B6/*gld* splenic B cells. This finding is consistent with the idea that lymphocytes in B6/*gld* mice are chronically activated and is not an entirely unexpected result. Indeed, while it is clear that the Fas/FasL system is capable of deleting all types of activated B cells, one of the crucial roles of the Fas/FasL system in normal mice might be to eliminate $B7\text{-}1^+$/$B7\text{-}2^+$ (i.e., activated) B cells that are autoreactive. Alternatively, the Fas/FasL system might serve to eliminate autoreactive B cells before they become $B7\text{-}1^+$/$B7\text{-}2^+$ activated cells and establish themselves as a population of cells that are dangerous to their host.

However, in light of B7-2 up-regulation, it is surprising that B6/*gld* splenic B cells do not up-regulate B7-1 expression to the same extent. The slight up-regulation of B7-1 on B6/*gld* splenic B cells is apparently in contrast to a previous report (16) in which splenic B cells from B6, B6/*lpr*, and B6/*gld* mice were examined for B7-1 expression, and no differences were found. It is unclear why a difference between B6 and B6/*gld* mice was seen in these studies and not in the previous studies.

The more unexpected finding is that B7-1 expression is significantly increased on T cells in the B6/*gld* spleen. While there have been reports of B7-1 expression on T cells, most of these have described T cell lines that have received prolonged stimulation (28, 29) or were infected with HTLV-1 (30). However, the *lpr* and *gld* mutations are known to result in dramatically altered, chronically activated T cell phenotypes (e.g., DN T cells), and B7-1 expression on *lpr* and *gld* T cells may represent another example of this phenomenon.

Whether these $B7\text{-}1^+$ T cells in the B6/*gld* spleen are normal T cells, DN T cells, or perhaps both is currently under investigation. However, the observation that T cells in *gld* mice express B7-1 raises the possibility that these cells might be providing their own costimulatory signal through CD28, independent of T cell-B cell interaction. Consistent with this scenario is the observation by Giese et al. (18) as well as our own unpublished observations that CD28 is expressed at slightly elevated levels on *lpr* and *gld* T cells. This autocostimulation of *gld* T cells could contribute to their

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chronic activation and perhaps to T-dependent autoimmunity in these mice.

In a larger sense, the up-regulation of both B7-1 and B7-2 in the B6/*gld* spleen may represent a general increase in costimulation in these mice, resulting in activation of autoreactive lymphocytes and production of autoantibodies. There is, in fact, evidence for B7/CD28 involvement in other murine models of lupus. Finck et al. (31), showed that selective inhibition of the B7/CD28 and B7/CTLA4 costimulatory pathways by a CTLA4-Ig fusion protein resulted in blocked autoantibody production and prolonged life in lupus-prone New Zealand Black/New Zealand White mice. Nakajima et al. (32) demonstrated similar results in New Zealand Black/New Zealand White F1 mice, showing a near-complete blockage of autoantibody production with anti-B7-2 treatment. When anti-B7-1 and anti-B7-2 Abs were injected simultaneously, autoantibody production was blocked completely.

B7/CD28 interactions may be contributing to disease in human lupus as well, although the exact role(s) of these molecules is unclear. Some studies have demonstrated a decrease in $CD28^+$ T cells (33, 34) and B7 expression (35) in human systemic lupus erythematosus patients, suggesting that diminished B7/CD28 signaling is involved in this disease. Contrasting reports, however, have described increased B7-1 expression in human lupus (36) as well as preferential expression of B7 and CD28 in skin lesions of lupus patients (37). While it is unclear whether B7/CD28 signaling is diminished or augmented in human lupus, it does appear that a perturbation in this signaling pathway is somehow involved in autoantibody production and disease.

Our findings that B7-1, B7-2, and CD28 are up-regulated on *gld* lymphocytes have been reproduced in recent experiments with *lpr* lymphocytes (data not shown). Interestingly, while B7-1, B7-2, and CD28 are all increased on *lpr* and *gld* lymphocytes, expression of the costimulatory molecules CD40 and CD40L does not appear to be altered in these animals. The reason for this is unclear, although it may reflect a preferential involvement of the B7/CD28 molecules in Fas-mediated apoptosis and/or the regulation of autoimmunity. Supporting these ideas are several recent reviews describing the involvement of B7/CD28 in both apoptosis (38) and autoimmunity (39–41).

In addition to demonstrating an increase in B7-2 and, to a lesser extent, B7-1 on splenic B cells of B6/*gld* mice, we demonstrated an increase in $IgM^+/IgD^-$ (activated) B cells in the B6/*gld* spleen. This appears to be yet another example of certain *gld* lymphocytes displaying a chronically activated phenotype. Additionally, these cells may be another example of lymphocytes in *gld* mice that fail to be deleted due to a lack of functional FasL. Unlike the very large increase in the percentage of $Fas^{high}$ B cells (which, as discussed earlier, were probably not all autoreactive), the more subtle increase in $IgM^+/IgD^-$ B cells may reflect the accumulation of autoreactive B cells in these animals. Future experiments to determine whether the accumulation of $IgM^+/IgD^-$ B cells in B6/*gld* mice is age related and whether these cells are a subset of the $B7\text{-}1^+/B7\text{-}2^+$ B cells may elucidate the nature and function of these cells.

One final issue to consider is the increased susceptibility of B6/*gld* spleen cells to anti-Fas-mediated apoptosis. The increase might simply be due to elevated levels of Fas resulting in a threshold of apoptosis susceptibility being reached. Previous work with cultured human glioma cells has shown that simply increasing Fas expression can result in a greater sensitivity to anti-Fas treatment (42). If this is the case, it would suggest that the increased apoptosis in the B6/*gld* spleen would be restricted to the $Fas^{high}$ B cells; however, our results indicated that both T and B cells in B6/*gld* mice were significantly more susceptible to anti-Fas-mediated apoptosis in vitro than were their normal B6 counterparts. This leaves unanswered the question of why splenic T cells from normal B6 mice persist in vivo when they are $Fas^{high}$ and functional FasL is present in these animals. One possibility is that a death signal, much like an activation signal, requires a second signal, and in its absence, signaling through Fas cannot result in apoptosis. Alternately, the state of activation of peripheral T cells may determine their susceptibility to Fas-mediated cell death. Suda et al. (43) showed recently that naive peripheral T cells could be killed by soluble FasL, while activation of naive splenic T cells by an anti-CD3/TCR Ab induced resistance to killing mediated by the same soluble FasL molecule.

In conclusion, our findings further support the idea that the Fas/FasL system is crucial in maintaining homeostasis and peripheral tolerance, and perturbation of this system can significantly affect lymphocyte phenotype and function. Additional study of the unusual B cells and T cells in the B6/*gld* spleen should contribute to our understanding of the nature of autoimmunity in *gld* mice.

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