Tumor Necrosis Factor Sustains the Generalized Lymphoproliferative Disorder (*gld*) Phenotype

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Abstract

Tumor necrosis factor (TNF) and Fas ligand (FasL) play major roles in the homeostasis of the peripheral immune system. This becomes dramatically obvious in the absence of a functional FasL. Mice with such a deficiency develop a profound lymphadenopathy, splenomegaly, hypergammaglobulinemia, and strain-dependent systemic autoimmune disease, and succumb to premature death. It is consequently termed generalized lymphoproliferative disorder (*gld*). By contrast, TNF deficiency alone does not result in a striking phenotype. Thus, we sought to determine what role TNF might play in contributing to the *gld* phenotype by creating C57BL/6.*gld*.TNF$^{-/-}$ mice. Contrary to the expected outcome, mice deficient for both FasL and TNF had a substantially milder *gld* phenotype with regard to mortality, lymphoaccumulation, germinal center formation, and hypergammaglobulinemia. To confirm these data in a strain highly permissive for the phenotype, C3H/HeJ.*gld* and C3H.HeJ.*lpr* mice were treated with a TNF-specific monoclonal antibody. This transient neutralization of TNF also resulted in a significantly attenuated lymphoproliferative phenotype. We conclude that TNF is necessary for the full manifestation of the lymphoproliferative disorder, in particular playing a critical role in lymphoaccumulation. Most importantly, absence of TNF protects *gld* mice against premature death.

Key words: lymphoproliferation • apoptosis • lymphadenopathy • Fas ligand • gene targeting

Introduction

Fas ligand (FasL), a member of the TNF family, and its receptor Fas, a member of the corresponding TNF receptor (TNFR) family, are essential in the homeostasis of the peripheral immune system (1–4). FasL is expressed predominantly on activated lymphocytes and is able to induce programmed cell death on virtually all Fas-expressing cells, if they are receptive for its signal (2, 3). The importance of this interaction for maintenance of lymphocyte homeostasis has been demonstrated in vivo in the dramatic phenotype of the disorders of generalized lymphoproliferative disorder (*gld*)[1] and lymphoproliferation (*lpr*) mice, two natural loss-of-function mutations of FasL and Fas, respectively (5, 6). In *gld* mice, a point mutation in the COOH-terminal region of FasL results in the expression of a nonfunctional form of FasL on the cell surface (7, 8). Mice homozygous for *lpr* and *gld* develop remarkably similar progressive nonmalignant lymphoproliferative diseases characterized by splenomegaly, severe lymphadenopathy, hypergammaglobulinemia, circulating autoantibodies, and premature death (5, 9–11). Lymphadenopathy results largely from the progressive accumulation of a population of thymus-derived CD45 (B220$^+$), TCR-α/β^+, CD4$^-$CD8$^-$ double negative (DN) T lymphocytes in spleen and LNs (5).

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[1]*Abbreviations used in this paper:* ABTS, 2.2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); B6, C57BL/6; C3H, C3H/HeJ; DN, double negative; ds, double-stranded; GC, germinal center; *gld*, generalized lymphoproliferative disorder; HRP, horseradish peroxidase; *lpr*, lymphoproliferation; mLN, mesenteric LN; pLN, peripheral LN; TPBS, Tween/PBS; WT, wild-type.

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TNF, the prototype member of this family, is also potentially important in the induction of programmed cell death (1). This cytokine, a product of many cell types but particularly of leukocytes (4, 12), not only has emerged as a mediator in the early stages of the inflammatory response, but also has been demonstrated to be of importance during the effector phase mediating cell death (1). However, TNF and its receptors, TNFR-1 and -2, have been deleted by homologous recombination, and the mouse lines so generated are normal with respect to size and composition of their lymphocyte compartments (13–17). The major phenotypic impact TNF and TNFR-1 mutations have in the unchallenged mouse is an absence of B cell follicles and a defect in the formation of germinal centers (GCs [16, 17]) that can be attributed to defects in induction of lymphocyte-homing chemokines (18).

When Fas and TNFR-1 mutations were combined by crossing TNFR-1–deficient mice onto the *lpr* background, an earlier onset and significant acceleration of the *lpr* pathology were observed (19). These results seemed to indicate that the TNF–TNFR-1 signaling pathway played some role in peripheral homeostasis, and in particular was compensatory in the absence of FasL–Fas interactions. To dissect these interactions further, we crossed C57BL/6 (B6).TNF$^{-/-}$ mice (17) with B6.*gld* mice (10) to generate a strain deficient for both ligands.

In contrast to the Fas/TNFR-1–deficient mice, the absence of TNF in FasL-inactive mice protected against lymphoaccumulation and, coincidently, premature death, which are characteristic of the *gld* mutation. The critical need for TNF in the development of the lymphoproliferative disorder was further confirmed by antibody-mediated blockade of TNF that significantly ameliorated lymphoaccumulation and hypergammaglobulinemia in C3H/HeJ (C3H).*gld* and C3H.*lpr* mice.

Materials and Methods

Mice. Inbred B6 wild-type (B6.WT), C3H.*gld*, and C3H.*lpr* mice were purchased from The Walter and Eliza Hall Institute of Medical Research. B6.*gld* (FasL mutant; breeding colonies obtained from The Jackson Laboratory), B6.TNF$^{-/-}$, and B6.*gld* × TNF$^{-/-}$ (B6.*gld*.TNF$^{-/-}$) mice were obtained from the Centenary Institute of Cancer Medicine and Cell Biology, and were bred at the Austin Research Institute Biological Research Laboratories and the Institut für Klinische Mikrobiologie. B6.*gld*.TNF$^{-/-}$ mice were established by crossing B6.*gld* and B6.TNF$^{-/-}$ mice and subsequently interbreeding the F_1 generation. The correct double mutant genotype was checked by PCR specific for the TNF deficiency (17) and the FasL point mutation (20). All B6 genotypes were bred on a genetically pure background. Mice of 4–48 wk of age were used in all experiments, which were performed according to Animal Experimental Ethics Committee guidelines of the Austin Research Institute and the University of Erlangen-Nürnberg.

TNF Blockade. To neutralize TNF in vivo, 3-wk-old C3H.*gld* or C3H.*lpr* mice were treated intraperitoneally every second day with 100 μg of anti-TNF mAb per mouse (XT3, rat IgG_1; provided by Dr. Geeta Chaudri, Department of Pathology, University of Sydney, Sydney, Australia) or control mAb (R3.34, rat IgG_1; PharMingen) until the mice were 14 wk of age.

Organ Weights and Serum ELISAs. Mice were weighed and then killed at the ages indicated. Sera were tested by ELISA for IgG, anti–double-stranded (ds)DNA, and anti-IgG autoantibody levels as follows. (a) IgG: ELISA plates (Costar) were precoated with 10 μg/ml goat anti–mouse IgG (Sigma Chemical Co.) in PBS for 1 h at room temperature. After seven washes in 0.05% Tween/PBS (TPBS), plates were blocked with 2% BSA/PBS for 1 h at room temperature. Subsequent steps with intervening washes included adding sera for 1 h at room temperature, horseradish peroxidase (HRP)-conjugated goat anti–mouse Ig (PharMingen) for 1 h at room temperature, and development with 2.2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) for 10–20 min (read at OD 405 nm). (b) Anti-dsDNA: ELISA plates (MaxiSorp; Nalge Nunc International) were precoated with 10 μg/ml methylated BSA in carbonate buffer overnight at 4°C. After four washes in 0.05% TPBS, 10 μg/ml herring sperm DNA was added in carbonate buffer overnight at 4°C. Plates were washed and blocked with 2% polyethylene glycol (PEG) 8,000/1% gelatine/1% BSA in TPBS for 30 min at 37°C. Subsequent steps with intervening washes included adding sera for 2 h at 37°C, HRP-conjugated goat anti–mouse Ig for 1 h at 37°C, and development with ABTS for 10–20 min (read at OD 405 nm). The level of anti-dsDNA antibodies of B6.*gld* at 28 wk of age was set at 100%, and the results of the other genotypes were recorded in relation to this level. (c) Anti-IgG: ELISA plates (MaxiSorp; Nalge Nunc International) were precoated with 10 μg/ml purified rabbit IgG (Sigma Chemical Co.) in carbonate buffer overnight at 4°C. After 4 washes in TPBS, plates were blocked with 1% BSA/PBS for 1 h at room temperature. Subsequent steps with intervening washes included adding sera for 1 h at room temperature, adding biotin-conjugated rat anti–mouse IgM (R6-60.2; PharMingen) for 1 h at room temperature, adding streptavidin-HRP for 1 h at room temperature, and development with ABTS for 30 min (read at OD 405 nm). For all ELISA assays, results were recorded as the mean ± SE of four to six B6.WT, B6.*gld*, B6.TNF$^{-/-}$, and B6.*gld*.TNF$^{-/-}$, or six or seven C3H.*gld* or C3H.*lpr* mice.

Organs were excised and weighed wet as follows: spleen, kidneys (two), mesenteric LNs (mLNs; two randomly chosen), and peripheral LNs (pLNs; two axillary, two cervical, and two inguinal). Organ weights of six mice of each genotype were used per time point. Additionally, to evaluate the anti-TNF mAb treatment, the two largest C3H.*gld* and C3H.*lpr* pLNs were selected, and lymphocytes were counted and the sum was totalled. These results were recorded as the mean ± SE of four to six mice. Statistical significance was determined using the Student's *t* test.

Immunofluorescence. Fluorescence immunohistology was performed on frozen sections. Spleen specimens were embedded in Tissue-Tek™ OCT compound (Sakura) and snap-frozen in the gas phase of liquid nitrogen. 6-μm sections were cut from tissue blocks and thaw mounted onto glass slides. Sections were air dried for 1 h, fixed in acetone for 30 min, and stored at −20°C. Before staining, sections were rehydrated with Tris-buffered saline (TBS), pH 7.6, and blocked with 20% horse serum. Slides were incubated with each antibody layer for 30–45 min at 37°C in a humidified chamber, then washed with three changes of TBS (5 min) before addition of the next layer. Primary antibodies used were anti-B220 (purified or biotinylated; RA3-6B2), anti-CD4 (clone H129.19), and anti-CD8α (clone 53-6.7), all from PharMingen, and biotinylated peanut agglutinin (PNA; Vector Laboratories). The staining was developed with anti–rat FITC, anti–rat Alexa™ 594, and streptavidin-Alexa™ 594 (all from Molecular Probes). After the final wash, slides were mounted and examined under a Leica confocal fluorescence microscope.

Flow Cytometry. For multiparameter analysis of splenic and blood lymphocytes, cells were stained with anti–TCR-β–PE (H57-597), anti-B220–FITC (RA3-6B2), anti-CD4–FITC (clone H129.19), and anti-CD8α–PE (clone 53-6.7; all from PharMingen), or anti-CD8α–Quantum red (clone 53-6.7; Sigma Chemical Co.). In Heidelberg and Erlangen, analysis was performed on a FACSCalibur™ (Becton Dickinson). Statistical significance was determined using the Student's *t* test.

Results and Discussion

Attenuation of the gld Phenotype in B6.gld.TNF$^{-/-}$ Mice. All B6.WT and B6 gene-deficient mice were observed for at least 420 d to assess the relative level of mortality of each strain. B6.TNF$^{-/-}$ mice (2.8% dead, $n = 36$) did not exhibit mortality over and above that observed in B6.WT mice (2.2% dead, $n = 45$). B6.*gld* mice developed symptoms of lymphoaccumulation in secondary lymphoid organs within 16–20 wk of age, and mortality was 31.9% ($n = 47$) within the first 60 wk of age.This is in agreement with the 33% mortality within the first 12 mo described for B6.*lpr* mice (11). By contrast, B6.*gld*.TNF$^{-/-}$ mice did not show obvious signs of generalized lymphoproliferation, and mortality levels were similar to B6.WT mice (4.1%, $n = 49$). To further analyze this observation, body weights and organ weights of mutant mice were monitored over the first 28 wk of life. The weight of the secondary lymphoid organs (mLNs, pLNs, and spleen) of B6.*gld* mice increased dramatically after 16 wk of age (Fig. 1, A–C). The lymphoid organs of B6.*gld*.TNF$^{-/-}$ mice did increase to a minor extent after 16 wk of age. However, they were never dramatically enlarged like the lymphoid organs in B6.*gld* mice (Fig. 1, A–C). Even in much older B6.*gld*.TNF$^{-/-}$ mice (>40 wk), the increase in secondary lymphoid organ weights never reached that observed in B6.*gld* mice (data not shown). Total body weights and the weights of the kidneys increased slightly over the 28 wk of observation, irrespective of the mouse genotype (data not shown).

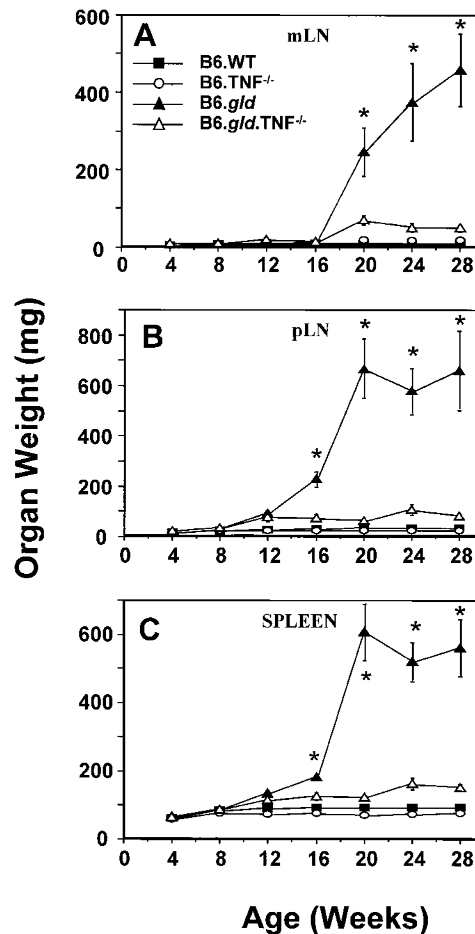

Figure 1. Mice doubly mutant for FasL and TNF have significantly reduced lymphoaccumulation. Organs were excised from individual B6.WT, B6.TNF$^{-/-}$, B6.*gld*, and B6.*gld*.TNF$^{-/-}$ mice, and wet weight (in milligrams) was determined for (A) mLNs, (B) pLNs, and (C) spleen. At each time point, organ weights of six B6.WT, B6.TNF$^{-/-}$, B6.*gld*, and B6.*gld*.TNF$^{-/-}$ mice were determined. Total body weights ranged from 16 to 36 g over the 4–28-wk period, regardless of the genotype. Combined kidney weights ranged from 250 to 550 mg over the 4–28-wk period, regardless of the genotype. Results were recorded as the mean ± SE of six mice. Asterisks indicate the groups of B6.*gld* mice that are significantly different from B6.*gld*.TNF$^{-/-}$ mice (*$P < 0.04$).

Hypergammaglobulinemia and circulating autoantibodies are features of the *gld* phenotype (5). Sera from all strains of mice at various ages were tested for IgG and autoantibodies specific for dsDNA and IgG. B6.*gld* mice had higher levels of IgG than B6.*gld*.TNF$^{-/-}$ mice after 16 wk of age (Fig. 2 A). Comparisons of serum IgG levels over weeks 16–28 revealed concentrations of serum IgG (Fig. 2 A) in B6.*gld*.TNF$^{-/-}$ mice that were ~20–65% less than that of B6.*gld* mice. Nevertheless, sera IgG levels were still elevated 10–20-fold in B6.*gld*.TNF$^{-/-}$ mice compared with B6.WT or B6.TNF$^{-/-}$ mice. B6.TNF$^{-/-}$ mice did not show Ig production higher than B6.WT mice, as described previously (16, 17).

Extensive analysis of the *lpr* mutation on different genetic backgrounds has demonstrated that this mutation results in the formation of various autoimmune specificities (5, 11). To study the effect of TNF deficiency on autoantibody production, we analyzed the level of anti-IgG (rheumatoid factor) and anti-dsDNA antibodies as representatives of autoimmune antibodies (10). There was considerable variation in the level of anti-IgG autoantibody (IgM) detected in various B6.*gld*.TNF$^{-/-}$ and B6.*gld* mice; therefore, individual mice were compared with the mean level of anti-IgG in 28-wk-old B6.*gld* mice (normalized to 1 U). Both groups of 24- and 28-wk-old B6.*gld*.TNF$^{-/-}$ and B6.*gld* mice contained some mice with at least 10–100-fold higher levels than observed in B6.TNF$^{-/-}$ or B6.WT mice (Fig. 2 B). We additionally examined the relative levels of circulating autoantibodies specific for dsDNA. No significant reduction in the relative levels of circulating autoantibodies specific for dsDNA was noted in B6.*gld*.TNF$^{-/-}$ mice compared with B6.*gld* mice, with both 24- and 28-wk-old B6.*gld*.TNF$^{-/-}$ and B6.*gld* mice displaying at least 10-fold higher levels than B6.TNF$^{-/-}$ or B6.WT mice (data not shown). Taken together, it would appear that TNF plays a minor role in hypergammaglobulinemia associated with *gld*, but that neither IgG nor autoantibody levels correlated well with

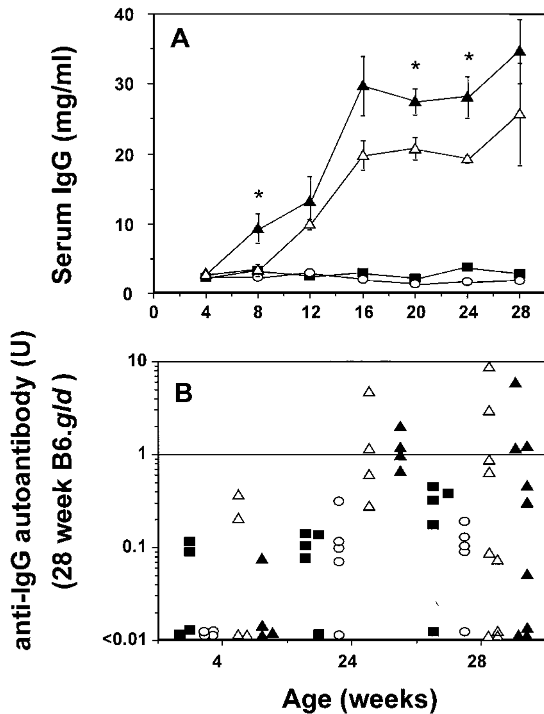

Figure 2. Mice doubly mutant for FasL and TNF have reduced serum IgG levels. Serum IgG (A) and anti-IgG (IgM, B) levels were determined by ELISA from B6.WT (■), B6.TNF$^{-/-}$ (○), B6.*gld* (▲), and B6.*gld*.TNF$^{-/-}$ (△) mice. Results were recorded as the mean ± SE of (A) four to six mice or (B) four to nine mice. In A, asterisks indicate the groups of B6.*gld* mice that are significantly different from B6.*gld*.TNF$^{-/-}$ mice (*$P < 0.05$). For B, 1 U was defined as the mean ± SE of B6.*gld* mice ($n = 9$) at 28 wk of age. Each mouse in B is represented by an individual symbol.

mortality rates in these strains of mice. It remains to be determined whether a qualitative, rather than quantitative, difference exists between autoantibodies in B6.*gld*.TNF$^{-/-}$ and B6.*gld* mice. Moreover, for the manifestation of drastic clinical symptoms that include lethal glomerulonephritis, additional genetic requirements seem to be necessary (11).

Analysis of Composition and Localization of Lymphocyte Populations. The *gld* phenotype is characterized by peripheral accumulation of B220$^+$ DN T cells. A flow cytometric analysis of B6.*gld* peripheral lymphocytes at different ages demonstrated an increase in the proportion of this population in the spleens of mice >20 wk of age (21). At the same time, the spleens of B6.*gld*.TNF$^{-/-}$ mice contained a comparable proportion of B220$^+$ DN T cells (data not shown). Not surprisingly, the absolute number of B220$^+$ DN T cells was lower in the spleens of B6.*gld*.TNF$^{-/-}$ mice, given the smaller size of these organs. In the blood, the results were different. At 12 wk of age, B6.*gld*.TNF$^{-/-}$ and B6.*gld* mice exhibited a substantially lower number of T cells, but no significant increase in B220$^+$ DN T cells (Table I). At 18–22 wk of age, B6.*gld* mice exhibited a significantly higher percentage of B220$^+$ DN T cells (11.3 ± 7%, $n = 8$; Table I) than the B6.*gld*.TNF$^{-/-}$ mice (4.1 ± 2.4%, $n = 9$, $P < 0.01$; Table I). B6.TNF$^{-/-}$ and B6.WT mice did not accumulate these abnormal lymphocytes (Table I). This indicates a significant retardation of the peripheral increase of B220$^+$ DN T cells in the B6.*gld*.TNF$^{-/-}$ genotype. The ratio of CD4$^+$ T cells to CD8$^+$ T cells in the peripheral pool was tested in parallel in the four genotypes, and was found to be comparable throughout and independent of the age of the mice (data not shown).

Histological analysis of the spleen demonstrated a clear difference in the microarchitectural structure between B6.WT and B6.TNF$^{-/-}$ mice (Fig. 3, A and B), as described previously (16, 17). TNF deficiency abolished the capacity of naive B cells to form organized follicles (compare Fig. 3, A and B). The compartmentalization of B and T cell areas was maintained, albeit with a relaxed demarcation between the populations (Fig. 3 B). In 12-wk-old B6.*gld* mice, follicle formation was clearly identifiable (Fig. 3 D). The B6.*gld*.TNF$^{-/-}$ strain displayed a combination of these struc-

Table I. *Proportional Changes in Lymphocyte Populations in the Blood of 12- and 18–22-wk-old B6.WT, B6.gld, B6.TNF$^{-/-}$, and B6.gld.TNF$^{-/-}$ Mice*

	Age of mice					
	12 wk			18–22 wk		
	T cells	B220$^+$ DN T cells	B cells	T cells	B220$^+$ DN T cells	B cells
B6.WT*	16 ± 2.6	0.7 ± 0.2	75 ± 1.3	ND	ND	ND
B6.TNF$^{-/-}$‡	ND	ND	ND	12.7 ± 4	0.8 ± 0.2	78.5 ± 6
B6.*gld*§	5 ± 2.2	2 ± 0.7	87 ± 1.7	16 ± 15	11.3 ± 7	63 ± 23
B6.*gld*.TNF$^{-/-}$‖	6 ± 2.2	2.1 ± 2	88 ± 3.8	5.1 ± 2	4.1 ± 2.4¶	1 ± 5

Peripheral blood lymphocytes were stained with anti–TCR-α/β and anti-B220. Data are recorded as mean percent ± SD.
*$n = 4$.
‡$n = 4$.
§$n = 5$ (<12 wk of age) and 8 (18–22 wk of age).
‖$n = 5$ (<12 wk of age) and 9 (18–22 wk of age).
¶The level of B220$^+$ DN T cells in B6.*gld*.TNF$^{-/-}$ mice is significantly different from that in B6.*gld* ($P < 0.01$) mice.

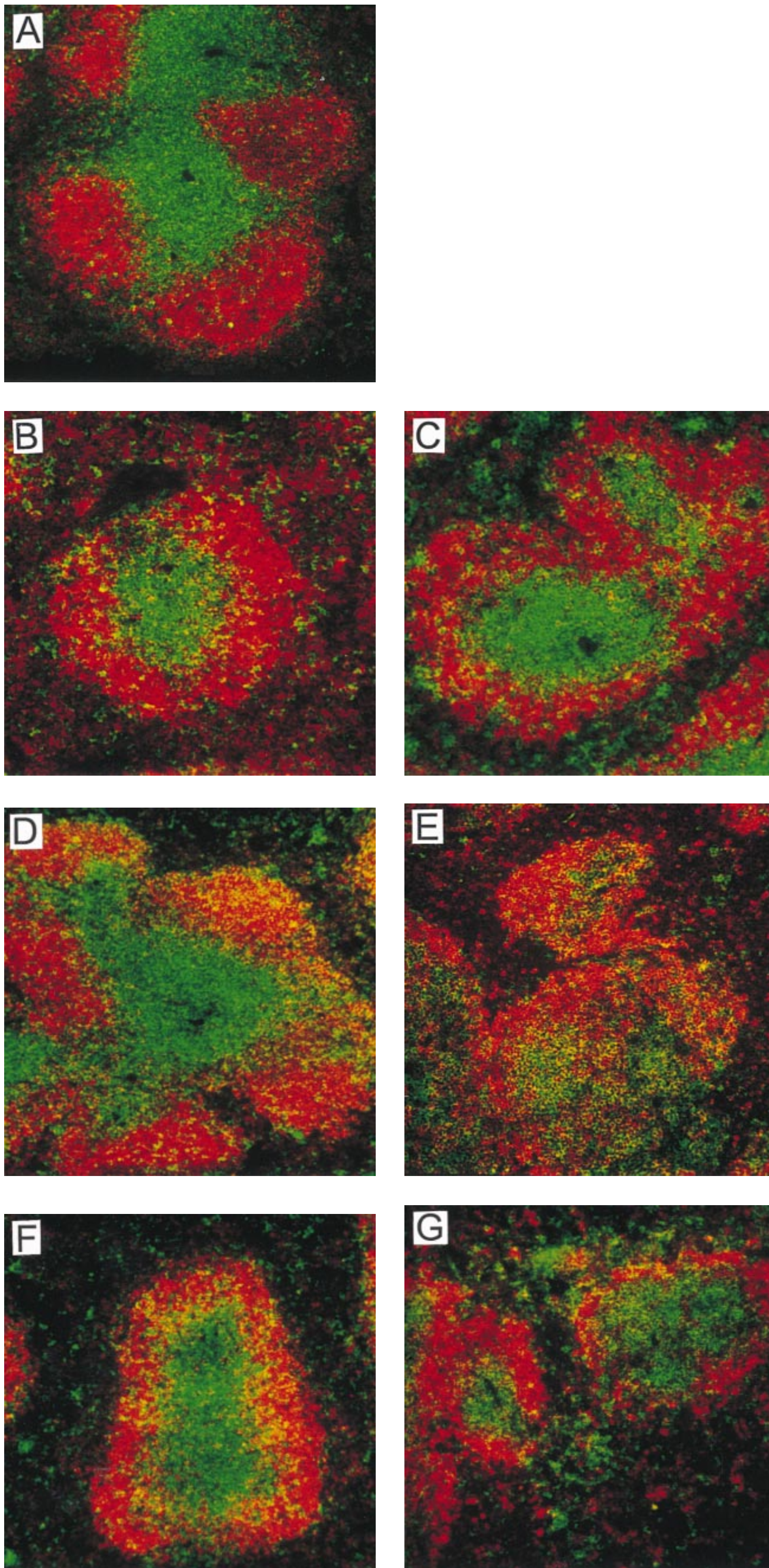

Figure 3. The loss of B cell follicles because of the TNF deficiency is dominant in the doubly mutant mice. This outcome does not change with age. T and B cell localization was investigated in the spleens of young (12 wk of age; A, B, D, and F) and old mice (44–48 wk of age; C, E, and G). The four genotypes were compared: (A) B6.WT; (B and C) B6.TNF$^{-/-}$; (D and E) B6.*gld*; and (F and G) B6.*gld*.TNF$^{-/-}$. T cell areas were stained in green (FITC), and B cell areas in red (Alexa™ 594). Original magnifications: ×200.

tural phenotypes, with separated T cell compartment and small rim-like structures of naive B cells (Fig. 3 F). The structures that were histologically visible were composed of naive, normal lymphocytes. At an older age (>40 wk), defined splenic structures in B6.*gld* mice diminished (Fig. 3 E), whereas demarcated but small and rim-like B cell areas were still observed in B6.*gld*.TNF$^{-/-}$ mice (Fig. 3 G).

The spleens of B6.*gld* mice at 12 wk of age displayed a strong GC reaction, as determined by staining with peanut agglutinin (Fig. 4 B). Naive B6.*gld* mice exhibited between 10 and 12 GCs per spleen section at the appropriate location in the B cell follicle (Fig. 4 B). B6.WT mice of the same age, and kept under the same conditions, displayed a maximum of two GCs per section (Fig. 4 A). A feature of TNF deficiency is the absence of the GC reaction (16, 17). TNFR-1 signaling is responsible for this phenotype (22, 23). Interestingly, this aspect of the TNF deletion was dominant in B6.*gld*.TNF$^{-/-}$ mice (Fig. 4 C) compared with B6.*gld* mice. Nevertheless, the lack of GCs in B6.*gld*.TNF$^{-/-}$ mice did not compromise their ability to produce high levels of sera Ig and autoantibodies, and this is consistent with our analysis of Ig production in challenged B6.TNF$^{-/-}$ mice, which was also not significantly diminished (17).

Short-Term Anti-TNF mAb Treatment Reduces the gld Phenotype. The severity of the *gld* phenotype is less in the B6.WT strain than in the highly susceptible C3H strain (11). To examine the effect of TNF deletion in another experimental setting, we treated C3H.*gld* and C3H.*lpr* mice with a neutralizing anti-TNF mAb or control mAb from age 3 to 14 wk. In concert with data from B6.*gld*.TNF$^{-/-}$ versus B6.*gld* mice, the serum IgG levels of C3H.*gld* and C3H.*lpr* mice treated with anti-TNF mAb were reduced at 14 wk of age, compared with untreated or control mAb–treated C3H.*gld* ($P < 0.05$) and C3H.*lpr* mice, respectively (Fig. 5 A). More evidently, continuous anti-TNF mAb treatment severely restricted lymphoaccumulation in the pLNs of C3H.*gld* and C3H.*lpr* mice, with pLN weights <30% of that observed in untreated or control mAb–treated mice (Fig. 5 B). Moreover, pLN cell numbers were significantly reduced (Fig. 5 C). Again, body and kidney weights of controls were comparable in all groups of mice (data not shown). Although anti-TNF mAb did not reduce lymphoaccumulation in *gld* mice to the same degree as gene targeting of TNF, it must be appreciated that treatment did not commence until mice were 3 wk of age, and that the *gld* phenotype is more permissive on the C3H than on the B6.WT background. These data support those obtained in B6.*gld*.TNF$^{-/-}$ mice, indicating that TNF is not necessary to sustain all features of the *gld* phenotype.

Concluding Remarks. These experiments demonstrate that the complete *gld* phenotype is dependent on the presence of TNF. When TNF was inhibited by antibody or deleted by gene targeting, the TNF signaling pathway was abrogated, and the *gld* phenotype was retarded in its onset and attenuated in its severity. In particular, the lymphoaccumulation characteristic of the *gld* and *lpr* phenotype was shown to be substantially dependent on TNF. In contrast, combination of the *lpr* phenotype and TNFR-1 deficiency led to an acceleration of lymphadenopathy and increased serum Ig production (19). This contrasting outcome was interpreted in the context of the loss of two pathways driving T cell apoptosis, thus concluding that TNF–TNFR-1 interactions normally compensate in part for the loss of the Fas–FasL signaling pathway. An alternative view offered by this study is that TNF actively drives *lpr* by signaling through TNFR-2, and in the absence of TNFR-1 and Fas–FasL signaling there is no mechanism to regulate this effect. This conclusion is supported by earlier studies suggesting that TNFR-2 transmits signals important for the proliferation of thymocytes and cytotoxic T cells (24). The dramatic increase in the severity of the *lpr*.TNFR-1 mutant phenotype compared with *lpr* alone could be explained by the absence of the biologically important buffer capacity of shedded, soluble TNFR-1. Indeed, recent work has demonstrated the importance of this soluble receptor in dominantly inherited autoinflammatory syndromes (25). Thus, one might also predict very high serum TNF levels in the Fas–TNFR-1 double-deficient mice, particularly as the lack of FasL alone leads to high levels of resting serum TNF (Wilhelm, P., and H. Körner, unpublished results).

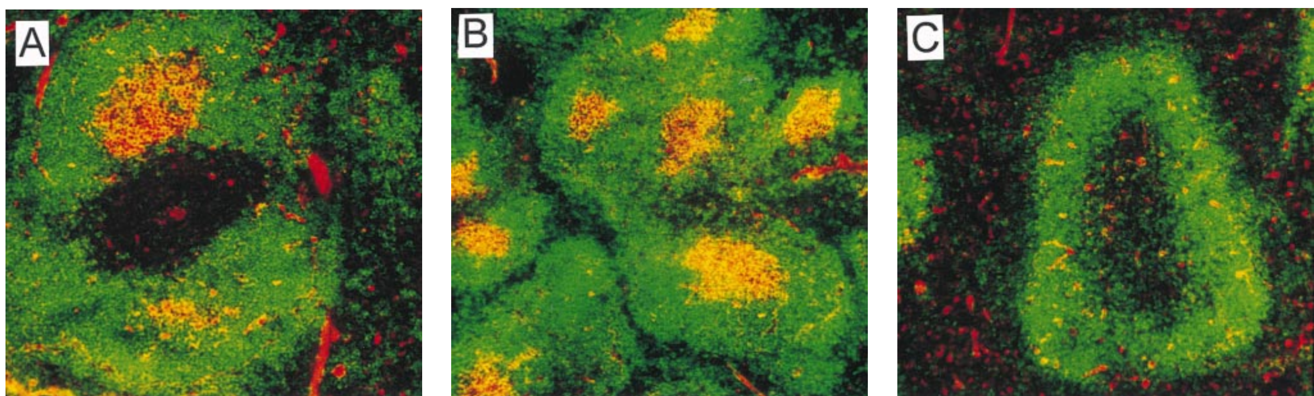

Figure 4. The *gld* phenotype exhibits a strong GC reaction that is abolished by TNF disruption. Spleens of young mice were analyzed (12 wk of age). B6.WT (A), B6.*gld* (B), and B6.*gld*.TNF$^{-/-}$ (C) mice were compared. The B cell area was stained in green (FITC) and the GC in red (Alexa™ 594). Because of the strong green fluorescence, the overlaying red GCs appear to be yellow. Original magnifications: ×200.

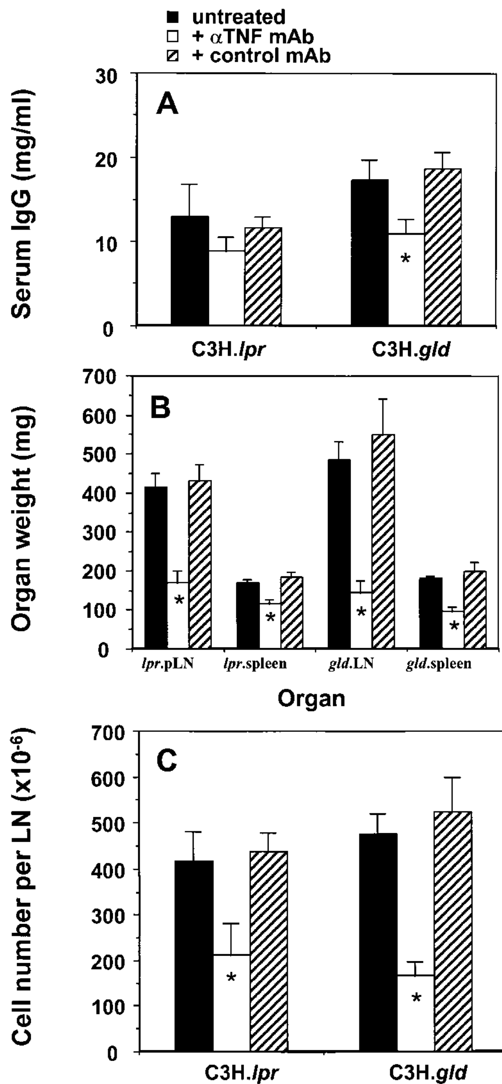

Figure 5. TNF inhibition reduces lymphoaccumulation and serum IgG levels in C3H.*gld* and C3H.*lpr* mice. 3-wk-old C3H.*gld* and C3H.*lpr* mice were treated intraperiteneally every second day with 100 μg of anti-TNF mAb (white bars) or control rat IgG_1 mAb (hatched bars) until the mice were 14 wk of age. Mice were killed, and (A) serum IgG levels, (B) pLN and spleen organ weights, or (C) pLN cell number ($\times 10^{-6}$) were determined. Total body and kidney weights ranged from 25 to 27 g and 361 to 407 mg, respectively, regardless of treatment. Results were recorded as the mean ± SE of six or seven (A and B) or four to six (C) mice. Asterisks indicate the groups that are significantly different from untreated C3H mice ($^*P < 0.05$).

It remains unclear how TNF signaling amplifies some aspects of the *gld* phenotype, particularly premature death. As the rapid accumulation of all lymphocyte subpopulations and, predominantly, $B220^+$ DN T cells is due to defective Fas-mediated apoptosis, the presence of TNF could act antagonistically and inhibit the apoptotic process. Indeed, antiapoptotic action of TNF on T cells has been described in different models (26, 27). Thereafter, TNF-driven lymphoaccumulation and, subsequently, either plasmacytoid tumors or interstitial pneumonitis would further exacerbate the phenotype (28, 29). Finally, Fas-mediated apoptosis could be dependent on the homing of cells to appropriate sites, and TNF is known to promote trafficking of lymphocytes to or within lymphoid tissues via induction of chemokines (18, 30). In this case, the accumulation of lymphocytes in lymphoid organs is a secondary event supported indirectly by TNF. Without TNF, this notable feature of the *gld* phenotype fails. The possibility that TNF acts as an antiapoptotic cytokine in some circumstances is in contrast to a study by Zheng et al. (31) that showed apoptotic actions of TNF via TNFR-2 in vivo. This study postulated that TNF–TNFR-2 interactions function in the maintenance of T cell homeostasis. However, it is possible that $B220^+$ DN T cells respond differently to long-lasting TNF stimulation, and their susceptibility to TNF-mediated apoptosis is not yet known. The issues will be addressed in future experiments.

Several treatments have been reported to decrease lymphoproliferative disease and to reduce the numbers of $B220^+$ DN T cells in LNs and spleen. These include neonatal thymectomy, chronic treatment with mAb specific for CD4, CD8, B220, or TCR Vβ, or chronic administration of cyclosporin A or staphylococcus enterotoxin B [SEB (32–36)]. (32–36). Nevertheless, our study is the first to describe a specific molecule that contributes to lymphoaccumulation and premature death associated with the *gld* phenotype. This work demonstrates a new role of TNF, and advances our understanding of the components leading to *gld* and the mechanisms that drive manifestation of the *gld* phenotype.

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