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In Vivo Depletion of Thy-1-Positive Cells Originating from Normal Bone Marrow Abrogates the Suppression of *gld* Disease in Normal-*gld* Mixed Bone Marrow Chimeras[1]

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Mice homozygous for *gld* develop an autoimmune syndrome characterized by hypergammaglobulinemia, massive accumulation of abnormal T cells and the production of autoantibodies. Previous studies in our laboratory have shown that reconstitution of lethally irradiated B6/gld recipients with a mixture of normal and *gld* bone marrow (BM) suppresses the *gld*-induced syndrome. In this report we extend this observation by demonstrating that the depletion of normal Thy-1^{+} cells, but not normal B cells, restores *gld* disease in mixed BM chimeras congenic for Thy-1 and IgH alleles. These results strongly suggest that normal T cells suppress the development of *gld*-related abnormalities. It is probable that the mechanism by which normal Thy-1^{+} cells mediate the suppression is Fas ligand dependent. *The Journal of Immunology*, 1995, 154: 444–449.

as is a 35-kDa cell-surface type I transmembrane protein having similarity with members of the TNF/nerve growth factor receptor family (1). Fas mediates apoptosis after interacting with a Fas-specific ligand, shown recently to be a 40-kDa type II transmembrane protein having homology with TNF-α (2). Activation of a Fas-mediated apoptosis pathway is believed to be an important process by which autoreactive cells are deleted to maintain immune cell tolerance (3). Mice homozygous for the mutations *lpr* or *gld* develop similar autoimmune syndromes characterized by the formation of autoantibodies to nuclear Ags and by the marked accumulation of an abnormal double negative (DN)[3] (CD4^{−}CD8^{−}) T cell population in the lymph nodes (LN) and spleen (4). Because *lpr* and *gld* induce almost indistinguishable autoimmune syndromes, it had been proposed that *lpr* and *gld* represent defects in an interacting pair of molecules, i.e., *gld*

may encode a mutant Fas ligand (5). Recent studies confirmed this hypothesis and identified *lpr* and *gld* as mutant forms of the *Fas* and *Fas-ligand* genes, respectively (6–8). Homozygosity at the *lpr* locus results in aberrant Fas transcription and markedly diminished cell surface expression of Fas protein (6). In contrast, the *Fas ligand* transcript appears normal in *gld* homozygotes; however, the presence of a single point mutation in the extracellular domain of the *Fas-ligand* gene results in the production of a nonfunctional mutant molecule (7).

Consistent with the *lpr-gld* model is the observation that autoimmune and cellular defects associated with *gld* expression are suppressed in irradiated recipients reconstituted with a mixture of bone marrow (BM) from normal and *gld* donors (9, 10). On the other hand, normal BM added to *lpr* BM had no effect on the development of *lpr* disease in *lpr* graft recipients. The nonresponsiveness of *lpr* cells to the suppressive effects of normal BM is consistent with the recent report that CD4^{+} T cell lines derived from *lpr* strain mice are resistant to apoptosis induced by cross-linking of the TCR/CD3 complex (11). These data suggested that normal BM-derived cells corrected the *gld* defect by providing normal Fas ligand. Here we demonstrate that Thy-1^{+} cells originating from the normal BM donor are necessary to suppress the *gld* syndrome in such mixed chimeras. These data strongly implicate T cells as the cell type responsible for mediating the suppression. Several mechanisms through which normal Thy-1^{+} cells expressing Fas ligand would be able to suppress the development of *gld* disease are discussed.

[3] Abbreviations used in this paper: DN, double negative; BM, bone marrow; LN, lymph node; B6/gld, C57BL/6-gld/gld; B6/TC, C57BL/6-IgH[a],Thy-1[a],Gpi-1[a]; SEA, staphylococcal enterotoxin A.

Materials and Methods

Mice

The single congenic strain C57BL/6-*gld/gld* (B6/gld) and the triple congenic strain C57BL/6-*IgH^a,Thy-1^a,Gpi-1^a* (B6/*TC*) were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our breeding facility.

Abs

The hybridoma cell lines HB63 (mIgG2a anti-huIgA), HB138 (mIgG1 anti-human IgM) and HB162 (AF6–78.25.4: mouse IgG1 anti-mouse IgM^b) were obtained from American Type Culture Collection, Rockville, MD. 1A14 (mouse IgG2a anti-Thy-1.1) and MmT1 (mIgG2a anti-Thy-1.2) were obtained from Dr. C. Badger, Fred Hutchinson Cancer Research Center, Seattle, WA. 53.6.7 (rat IgG2a anti-CD8) was obtained from Dr. G. Haughton, University of North Carolina at Chapel Hill, NC; 172–4 (rat IgM anti-CD4) from Dr. David Harris, University of Arizona, Tucson, AZ and 145–2C11 (hamster IgG anti-CD3) from Dr. J. Bluestone, University of Chicago, Chicago, IL. DS-1 (mouse IgG1 anti-IgM^a) was obtained from Dr. D. Sieckmann, Naval Medical Research Institute, Bethesda, MD.

Experimental design

Mixed BM chimeras were generated by injecting lethally irradiated B6/gld mice with BM harvested from B6/*TC* and B6/gld donors. The cell-surface expression of Thy-1.1 and IgH^a by B6/*TC*-derived T cells and B cells, respectively, made these cells sensitive to depletion in vivo by allotype-specific mAbs. The effect of depleting normal T cells and B cells on the suppression of *gld*-associated autoimmunity was determined by measuring autoantibody production and the number of abnormal DN T cells accumulating in peripheral lymphoid organs, as compared with B6/gld mice injected with isologous BM.

Chimeras

Mixed BM chimeras were prepared as previously described (12). Briefly, BM was harvested from 8- to 10-wk old B6/*TC* and B6/gld donors and depleted of T cells with a mixture of cytotoxic mAbs (anti-CD4, anti-CD8, anti-Thy-1.1, and anti-Thy-1.2) in the presence of rabbit C. The BM preparations were mixed at a ratio of 1:1, 3:1, or 10:1 (B6/*TC*:B6/gld) and a total of 10^7 cells injected via the tail vein into lethally irradiated (900 rad) age- and sex-matched B6/gld recipients. In preliminary experiments, the co-transfer of equal numbers of BM cells resulted in similar numbers of T cells originating from either strain, yet B cell reconstitution was found to favor *gld* marrow-derived B cells (IgH^{b+}) by a ratio of 3:1 (data not shown). Control recipients that later developed disease were given 10^7 *gld* BM cells alone. Beginning the day after transplant, mixed-BM chimeras were injected (i.p.) either weekly with anti-Thy-1.1 (1.5 mg/wk) or twice weekly with anti-IgH^a (3.0 mg/wk). Ab control chimeras were injected with isotype-matched mAbs possessing irrelevant specificities.

ELISAs

The methods for the determination of allotype-specific serum concentrations of IgM, IgG, IgG2a antichromatin and rheumatoid factors (IgM anti-IgG1 and -IgG2b) have been described (12). Because similar results were obtained with all BM mixtures, only the data from chimeras reconstituted at 3:1 are reported.

Flow cytometric analysis

LN and spleen cell suspensions prepared from 5-month-old mixed chimeras were used in two-color analysis to determine T cell and B cell chimerism, as well as the number of DN T cells. Cells (10^6) were incubated first with either DS-1-FITC and biotinylated AF6–78.25 (B cell chimerism); MmT1-FITC and biotinylated 1A14 (T cell chimerism); or anti-CD3 culture supernatant and biotinylated Abs specific for CD4 and CD8 (DN T cells). After a 30-min incubation at 4°C, the appropriate second step reagents (goat anti-hamster IgG-FITC and avidin-phyco-erythrin) were added and incubated for an additional 30 min at 4°C. The cells were then fixed in phosphate-buffered 2% paraformaldehyde and analyzed on a Becton Dickinson FACScan using Cicero software (Cytomation, Fort Collins, CO). FITC-labeled and biotinylated Abs not available commercially were prepared using standard methods. As with the serologic analysis, similar results were obtained with all BM mixtures so only the data from chimeras reconstituted at 3:1 are given.

Statistical analysis

Significant differences between experimental groups were measured using Student's *t*-test.

Results

Chimerism and DN T cells in T and B cell-depleted chimeras

Spleen and LN cells, harvested from chimeric mice 5 mo after lethal irradiation and reconstitution, were examined by two-color flow cytometry for T cell subsets and for the BM source of T cells and B cells. In LN cell suspensions from mixed BM chimeras reconstituted at 3:1 (normal to *gld*) and treated with either control mAb HB63 or HB138, 22% of the cells expressed Thy-1.1 and 10% expressed Thy-1.2 (Table I). Roughly equal numbers of B cells expressed the a or b IgH allotypes. LN and spleen weights of these chimeras were not significantly increased compared with unmanipulated B6/*TC* mice (data not shown). Depletion with 1A14 effectively removed all Thy-1.1$^+$ cells and caused a smaller reduction in the number of IgM^a B cells. In contrast to control mAb-treated chimeras, these chimeras exhibited significant LN ($p < 0.0005$) and spleen ($p < 0.0005$) enlargement, with organ weights comparable with those of B6/gld recipients given isologous BM. The treatment of similarly prepared chimeras with DS-1 completely removed IgM^a B cells and left Thy-1.1$^+$ T cell numbers intact. No significant increase in LN and only a marginal increase in spleen weight ($p < 0.05$) were observed, as compared with mice treated with control mAbs.

Phenotypic analysis of T cell subsets also demonstrated the reversal of disease suppression in 1A14-treated chimeras (Fig. 1). For example, DN (CD3$^+$,CD4$^-$,CD8$^-$) T cells (quadrant 4) accounted for more than 50% of the CD3$^+$ LN cells (quadrant 2 + 4) in the chimeras treated with 1A14 (Fig. 1*B*); and this closely resembled the fraction of DN T cells observed in LN cell suspensions prepared from B6/gld recipients injected with *gld* marrow (Fig. 1*D*). In contrast, no change in the proportion of DN T cells in LN was observed in DS-1-treated mice (11%), compared with control mAb-treated chimeras (8.4%) (Fig. 1, *A* and *C*). Similar results were seen in spleen cell suspensions (data not shown). The increase of lymphadenopathy combined with the elevated percentage of DN T cells observed in Thy-1.1-depleted chimeras resulted in 20-fold and fourfold increases in the absolute numbers of DN T cells in LN and spleen, respectively, as compared with chimeras treated with control mAbs (Fig. 2).

Table I.   *Chimerism of LN T and B cells, and adenopathy in B6/TC-B6/gld BM chimeras (3:1)[a]*

| mAb Rx[b] | Chimerism (% ± SE) | | | | Organ Weight (mg ± SE) | |
| | T cells | | B cells | | | |
	Thy-1.1	Thy-1.2	IgM[a]	IgM[b]	LN[c]	Spleen
1A14	<1 (5)	51.3 ± 4.2 (5)	2.0 ± 0.4 (9)	28.7 ± 2.2 (9)	292.8 ± 40.8 (9)	260.6 ± 29.4 (9)
DS-1	23.4 ± 1.5 (8)	7.1 ± 1.6 (8)	<1 (12)	38.0 ± 2.0 (12)	75.9 ± 16.4 (14)	143.2 ± 8.0 (14)
HB63 and HB138	21.6 ± 2.3 (7)	10.0 ± 1.0 (7)	19.3 ± 1.3 (8)	18.7 ± 1.6 (8)	45.6 ± 14.3 (8)	118.1 ± 9.3 (8)
NT[d]	<1 (5)	63.2 ± 3.9 (5)	<1 (5)	27.3 ± 3.4 (5)	280.0 ± 86.0 (5)	270.0 ± 90.1 (5)

[a] Two color flow cytometry was used to determine T and B cell chimerism of LN cells isolated from B6/*TC*-B6/gld chimeras 5 mo after BM transfer. Values in parentheses indicates the sample size.
[b] Rx, treatment.
[c] Mean LN weights were calculated from the combined weight of axillary, brachial, inguinal, and mesenteric LN from each animal.
[d] NT, irradiated B6/gld mice reconstituted with B6/gld BM and not treated with mAb. All animals were killed 5 mo after BM reconstitution. Data were compiled from two separate experiments.
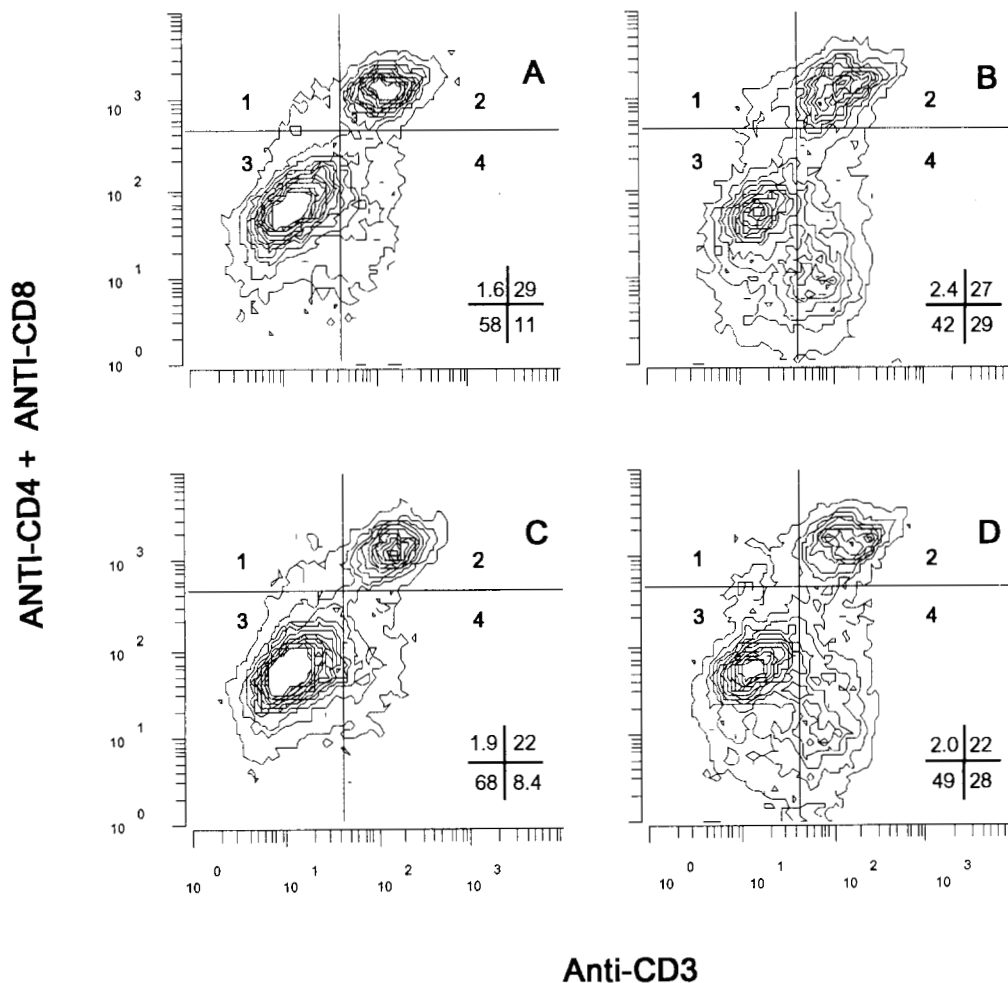

Anti-CD3

FIGURE 1.   Two-color flow cytometric analysis of LN cells isolated from B6/*TC*-B6/gld chimeras depleted of normal T or B cells. LN cells were harvested from mixed BM chimeras treated with DS-1 (anti-IgM[a], Fig. 1*A*), 1A14 (anti-Thy-1.1, Fig. 1*B*), with either of the isotype-matched control mAb HB63 or HB138 (Fig. 1*C*) and B6/gld animals reconstituted with B6/gld BM (Fig. 1*D*). Values indicate the percentage of cells in each quadrant. A total of 10⁴ events was counted for each plot. Each plot is representative of at least five mice.
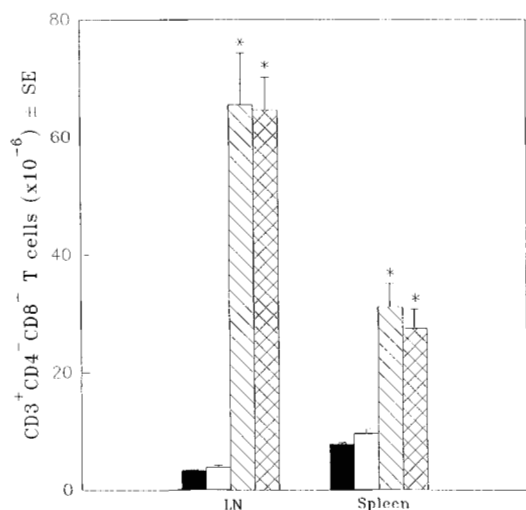
FIGURE 2. The effect of Ab treatment on the absolute number of DN T cells in the LN and spleens of B6/*TC*-B6/gld chimeras. Chimeras treated with anti-Thy-1.1 (1A14, hatched bar, LN n = 5 and spleen n = 6) and B6/gld recipients reconstituted with B6/gld BM (cross-hatched bar, n = 5 for both LN and spleen) exhibited a significant increase (*p < 0.0005, t-test) when compared with control-Ab-treated chimeras (HB63 or HB138, solid bar, n = 7 for both LN and spleen). In contrast, no significant change in DN T cells was observed in chimeras treated with anti-IgM^a (DS-1, open bar, LN n = 9 and spleen n = 10). The absolute number of DNT cells for each lymphoid organ represents the fraction of the total number of cells in each cell suspension that are CD3$^+$,CD4$^-$,CD8$^-$ as determined by two-color flow cytometry. Results are given as the arithmetic mean ± SE.

Autoantibody levels in T and B cell-depleted chimeras

Allotype-specific ELISAs were used to measure autoantibodies and total IgG2a and IgM in 5-month-old chimeras. High levels of IgG2a^b anti-chromatin, comparable with those observed in B6/gld controls (p > 0.4), were observed in 1A14-treated chimeras, whereas DS-1-treated mice exhibited levels less than those measured in control mAb-treated animals (Fig. 3A). Similar results were also observed for IgM^b rheumatoid factor against IgG1a (p > 0.1, Fig. 3B) and IgG2b^b (p > 0.1, Fig. 3C). In contrast, only low levels of IgG2a^a anti-chromatin and IgM^a anti-IgG2b^b rheumatoid factor were detected in the sera of 1A14-treated chimeras. 1A14-treated chimeras exhibited a marked increase in the total amount of both IgG2a^b (p < 0.0005) and IgM^b (p < 0.005), as compared with control mAb-treated chimeras (Fig. 3, *D* and *E*). Chimeras treated with DS-1 showed a significant (p < 0.0005) increase only in the serum levels of IgG2a^b (Fig. 3D).

Discussion

The co-transfer of normal BM with *gld* BM suppresses the development of lymphadenopathy and autoantibody pro-

duction in lethally irradiated *gld* recipients (9). In this report, we demonstrate that in vivo depletion of normal Thy-1$^+$ cells, but not normal B cells, from such chimeras abrogates the suppressive effects of the normal BM-derived cells, as indicated by the appearance of adenopathy, the accumulation of DN T cells, and the production of hypergammaglobulinemia and autoantibodies. Because Thy-1 is recognized as being predominantly a T cell marker, it is likely that the Thy-1$^+$ cells mediating the suppression are in fact T cells. The possibility that T cells are the normal Thy-1$^+$ cells is in agreement with recent studies showing that: SEA-activated CD4$^+$ splenic *gld* T cells, but not CD4$^+$ cells taken from normal mice, were refractory to SEA and immobilized anti-CD3 directed suicide (13); normal and *lpr*, but not *gld*, LN cells in the presence of PMA and ionomycin induced cytolysis of Fas$^+$ cell lines (14); and cloning of the *Fas ligand* was accomplished using a cDNA library generated from the CTL hybridoma, d10S (2). Additional experiments utilizing other means to remove normal T cells, including the depletion of T cell subsets with allotype-specific mAb (anti-CD8) and CD4 "knockout" mice, will be needed to confirm whether T lymphocytes are the only cells that suppress abnormal lymphoproliferation and autoantibody production. Thy-1 expression can also be demonstrated on certain B cells (see below) and a subpopulation of NK cells (15). Therefore, it remains possible that non-T cells can mediate the suppressive effects of normal marrow. Mixed BM chimeras, similar to those presented in this report, treated in vivo with anti-NK1.1, a mAb directed against an NK-specific marker, should resolve this issue.

The removal of normal B cells by Ab treatment enabled us to measure the effect of normal Thy-1$^+$ cells alone on the development of *gld* disease. Similarly, the depletion of normal Thy-1$^+$ cells was expected to leave the normal B cell population intact and thereby allow us to determine the effect of normal B cells on the suppression of *gld* disease. However, the effect of B cells alone was difficult to evaluate in these chimeras, because the normal B cells failed to reconstitute in a proportion equal to that of *gld* donor BM and treatment with anti-Thy-1.1 further reduced normal B cell numbers, presumably because BM stem cells and pro B cells express low levels of Thy-1 (16). In B6/gld mice reconstituted with 10 times more normal BM than *gld* BM and treated with 1A14, normal IgM$^+$ cells comprised 3% of the LN cells. The presence of normal B cells had no significant effect on the levels of autoantibodies produced, when compared with chimeras depleted of both normal T cells and normal B cells by treatment with a combination of 1A14 and DS-1 (data not shown). Although these results do not exclude the possibility that normal B cells might have an effect in greater numbers, they are in agreement with others who have shown that B220$^+$ LN cells, containing less than 5% CD3$^+$ cells, were relatively ineffective in mediating the in vitro killing of Fas-expressing target cells (14). It is interesting to note
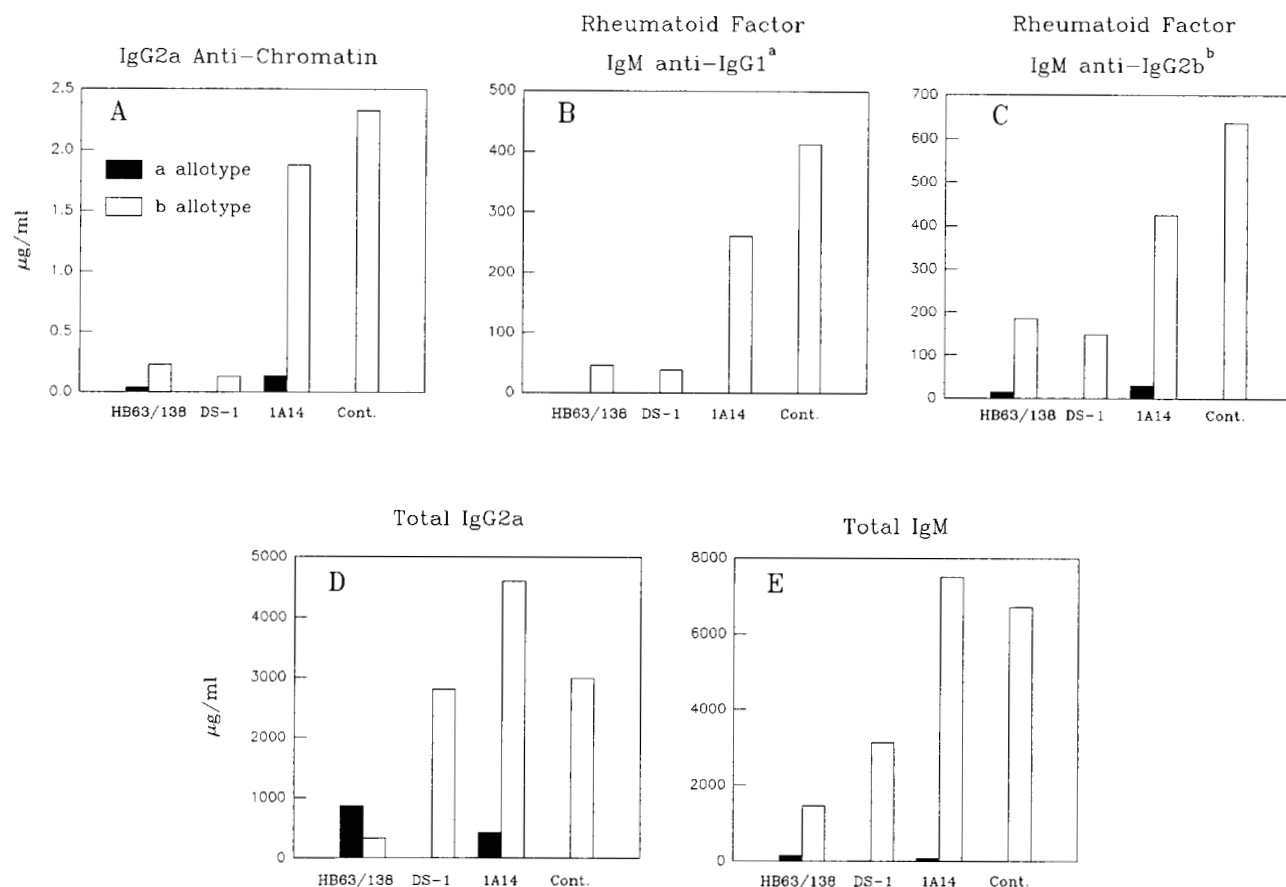
FIGURE 3. Serologic analysis of 5-mo-old B6/*TC*-B6/gld BM chimeras that had been treated with control Ab (HB63 or HB138, $n = 8$), DS-1 (anti-IgM^a, $n = 14$), or 1A14 (anti-Thy-1.1, $n = 10$). Control B6/gld recipients were reconstituted with B6/gld BM ($n = 5$). The results are presented as geometric means.

that although depletion of normal B cells did not restore *gld* autoimmunity, IgG2a^b serum levels were elevated in anti-IgM^a-treated mice, as compared with control-Ab-treated chimeras. The reason for this is not readily apparent; however, the over-production of b allotype Ig may be caused by compensation in response to the absence of a allotype Ab.

The ability of normal BM-derived Thy-1⁺ cells to correct the *gld* defect in this report is consistent with the idea that Fas ligand, which mediates Fas-induced apoptosis, originates from the normal Thy-1⁺ cell population and is therefore extrinsic to autoreactive *gld* cells. How normal Thy-1⁺ cells could correct *gld* autoimmune disease in mixed BM chimeras by expressing normal Fas ligand is not evident from these experiments; however, several possibilities do exist. One explanation is that Fas⁺ autoreactive cells require contact with Thy-1⁺ cells expressing normal Fas ligand in order for these cells to be deleted. Our previous work with *lpr*/normal mixed BM chimeras defines the extent of these proposed cellular interactions. Specifically we have shown that *lpr* gene expression in T cells is necessary for the accumulation of DN T cells (17) and for autoantibody production (18). Furthermore,

lpr B cells, but not normal B cells, are responsible for autoantibody production in mixed BM chimeras (12). Taken together, these data strongly suggest that both T and B cells from *gld* marrow express Fas Ag and therefore, should be subject to deletion induced by normal Thy-1⁺ cells expressing Fas ligand. Our data do not prove, however, that both populations involved in autoantibody formation are in fact suppressed by normal cells because deletion of either would be sufficient to prevent the appearance of autoantibodies. Nevertheless, the fact that removal of normal Thy-1⁺ cells restores Ab formation demonstrates that this is the only normal cell population that could interact through Fas/Fas ligand with such autoreactive B and T cells. Whether this cell-to-cell interaction would involve T cells from different subsets or from the same subset is at present under study (see above). Another possibility is that Fas ligand is secreted or shed, in vivo, from the surface of the Thy-1⁺ cell, so that cells expressing Fas may be deleted once they come into contact with soluble Fas ligand. This hypothesis is supported by the observations that Fas ligand has been identified in the culture supernatants of d10S cells and Fas ligand-transfected COS cells and that the supernatant alone was shown to be

cytotoxic for target cells expressing Fas (2). In our mixed BM model, soluble Fas ligand could function in either a paracrine and/or autocrine manner to delete autoreactive cells (19).

In summary, the in vivo depletion of the normal Thy-1$^+$ cells, but not normal B cells, from mixed BM chimeras reconstituted with normal and *gld* marrow abrogates the suppressive effects of normal BM-derived cells, as indicated by the appearance of *gld*-associated features (adenopathy, the accumulation of DN T cells, hypergammaglobulinemia, and the production of autoantibodies). Furthermore, these data suggest that the normal Thy-1$^+$ effector cells mediating the suppression of *gld* disease are T cells.

Acknowledgment

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