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Autoantibody production in *lpr/lpr gld/gld* mice reflects accumulation of CD4+ effector cells that are resistant to regulatory T cell activity[1]

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Abstract

In Fas/FasL deficient mice anti-chromatin Ab production is T cell dependent and is not apparent until after 10 weeks of age. Early control of anti-chromatin antibodies may be due to the counterbalancing influence of Treg cells. Here we show that Treg cells block *lpr/lpr gld/gld* Th cells from providing help to anti-chromatin B cells in an *in vivo* transfer system. Interestingly, the percentage and absolute numbers of Foxp3$^+$ Treg cells is elevated in BALB/c-*lpr/lpr gld/gld* mice and increases with age compared to BALB/c mice. The majority of Foxp3 expression is found in the B220$^-$ CD4$^+$ T cell population, and Foxp3-expressing cells are localized in the splenic PALS (periarteriolar lymphocyte sheath). Strikingly, although the lack of functional Fas/FasL does not affect the ability of Treg cells to block Th cell proliferation, Treg cells can block the IFN-γ differentiation of Th cells from BALB/c or young BALB-*lpr/lpr gld/gld* mice but not of pre-existing Th1 cells from older BALB/c-*lpr/lpr gld/gld* mice. Thus, we suggest autoantibody production is not caused by the lack of Treg cells but by a defect in activation-induced cell death that leads to the accumulation of T effector cells that are resistant to regulatory T cell activity.

Keywords

autoantibody; Fas/FasL; IFN-γ; T regulatory

Introduction

VH3H9/λ_1 anti-chromatin B cells are present in the periphery of non-autoimmune BALB/c mice and young Fas (*lpr/lpr*) or FasL (*gld/gld*) deficient mice but in both cases autoAbs are not secreted; in the Fas/FasL deficient mice autoAbs are not detected until after 10 weeks of age [1–3]. Although anti-chromatin B cells appear to be held in check in both scenarios, the localization and cell surface phenotype of anti-chromatin B cells in non-autoimmune and autoimmune mice are distinct [1–3], suggesting different mechanisms of tolerance are in play.

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Anti-chromatin B cells in BALB/c mice are primarily excluded from B cell follicles but migrate into the B cell follicle upon receipt of T cell help [1]. In contrast, in autoimmune *lpr/lpr* or *gld/gld* mice these cells are found in the B cell follicle in a CD4 dependent manner [2–4]. One possibility to account for this data is that B cells from young Fas/FasL-deficient mice are partially defective in their response to T cell help, responding by migrating into the follicles but not differentiating into AFCs. Alternatively, CD4 T cell help may be partially lacking in young *lpr/lpr gld/gld* mice, leading to the abortive phenotype of the anti-chromatin B cells. We have hypothesized that Treg cells maintain B cell tolerance and consistent with this have demonstrated that Treg cells inhibit autoreactive B cell response to Th cells in an *in vivo* transfer model [5].

The status of Treg cells in lupus is controversial. Lupus patients appear to have a reduced frequency of Treg cells [6,7]. Likewise, in some murine models of SLE Treg cells are diminished and/or have reduced Foxp3 protein expression [8–10]. In contrast, 16–20 week old C3H *gld/gld* mice have an increased frequency of $Foxp3^+$ T cells with Foxp3 protein expressed at levels similar to normal mice [11]. The functional capacity of Treg cells to block T cell proliferation *in vitro* also varies according to strain. In the C3H *gld/gld* model, Treg cells inhibited Th cell proliferation while in NZM2410 mice the Treg cells were less effective [10, 11]. The ability of Treg cells to block lupus *in vivo* has also depended upon the symptoms examined. We have demonstrated that Treg cells can block the production of anti-chromatin Abs induced by the provision of T cell help [4,5]. Likewise, in the NZM2328 or SNF1 lupus models, the presence of Treg cells decreases anti-dsDNA Abs; however, glomerulonephritis is not improved [12,13].

Here, we show that Fas/FasL-deficient anti-chromatin B cells readily respond to T helper cell infusion *in vivo* by differentiating into AFCs. Thus, the delay in autoantibody production until 10–12 weeks of age is not due to an intrinsic defect in the anti-chromatin B cells in young *lpr/lpr gld/gld* mice. To account for this delay, we have analyzed the phenotype and functional abilities of $Foxp3^+$ Treg cells before (< 8 week old) and after (> 12 week old) autoAbs are detected in *lpr/lpr gld/gld* mice. Immunofluorescence analyses reveal that the majority of $Foxp3^+$ cells are located in the PALS of both BALB/c, young (serum VH3H9/λ_1 $autoAb^-$) *lpr/lpr gld/gld*, and old *lpr/lpr gld/gld* (serum VH3H9/λ_1 $autoAb^+$) mice. However, as we have previously reported [2,14], the splenic architecture (separation of B and T cell areas) becomes progressively disrupted in older *lpr/lpr gld/gld* mice.

We further tested the responsiveness of *lpr/lpr gld/gld* T cells to suppression by Treg cells in terms of proliferation and cytokine production. Here we report a deviance in the dampening of IFN-γ potential by Treg cells upon young versus old *lpr/lpr gld/gld* T cells. This phenomenon, along with other factors such as the disruption of splenic architecture, may account for the breakdown of tolerance in older *lpr/lpr gld/gld* mice.

Materials and Methods

Mice

Male and female mice between 6–24 weeks of age were maintained in specific-pathogen-free conditions at the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited Wistar Institute under the supervision of the Institutional Animal Care and Use Committee (IACUC). All mice are on the BALB/c background. TS1 TCR (anti-hemagglutinin) Tg, TS1xHA28 Tg, *lpr/lpr gld/gld*, VH3H9 HACII *lpr/lpr gld/gld*, and VH3H9/HACII/Ig $\kappa^{-/-}$ mice were bred at the Wistar Animal Facility. BALB/c and CB17 [a congenic strain carrying the Ig heavy-chain allele (*Igh-1b*) from a C57BL/Ka on a BALB/c background] mice were purchased from the National Cancer Institute and Charles River Laboratory, respectively.

T and B cell injections into CB17 mice

2 million anti-HA Th ($CD4^+$ $CD25^-$) cells were sorted from TS1 TCR Tg BALB/c or TS1 TCR Tg BALB/c-*lpr/lpr gld/gld* mice (the latter were <8 weeks old), and injected *i.v.* with 1000 hemagglutinating units of purified PR8 influenza virus. Splenocytes from VH3H9/HACII/Ig $\kappa^{-/-}$ mice were depleted of RBC, and an aliquot was stained by flow cytometry to determine the frequency of anti-chromatin B cells. CB17 recipient mice were injected *i.v.* with splenocytes containing 5×10^6 anti-chromatin B cells. Mice were killed eight days later.

Flow cytometry

$1–4 \times 10^6$ cells were prepared and surface stained as per standard protocol [15]. The following Abs were used: anti-B220-FITC, -PerCP-Cy5.5, or -APCCy7 (RA3-6B2), anti-CD3-FITC or -PE-Cy7 (145-2C11), anti-CD25-biotin (7D4), anti-CD95-biotin (Jo2), anti-CXCR5-biotin (2G8), anti-CD4-PerCP-Cy5.5 (RM4-5), streptavidin-PE, -PerCP-Cy5.5, or -PerCP (BD Biosciences), anti-CD4-APC (RM4-5), anti-Thy1.2-APC (53-2.1), anti-Foxp3-FITC or -PE (FJK-16s), isotype control Rat IgG_{2a}-FITC or -PE (EBR2a), anti-IFN-γ-PE (XMG1.2), and anti-IL-4-PE (11B11) (Ebioscience). Intracellular cytokine and Foxp3 staining were performed as previously described [5]. Stained cells were run on a FACScan, FACSCalibur, LSRII (Becton Dickinson), or Cyan ADP machine (Dako) and analyzed using FlowJo software (Treestar). All plots show $\log_{10}$ fluorescence.

Localization of $CD4^+$, $CD4^+$ $B220^+$, and DN T cells

Lymph nodes from *lpr/lpr gld/gld* mice aged >12 weeks of age were stained with CD19-biotin (1D3) (BD Biosciences)/ streptavidin-PE, anti-CD4-APC and anti-B220-PerCP-Cy5.5. Cells were sorted into three groups, $CD19^-$ $CD4^+$ $B220^-$ ($CD4^+$), $CD19^-$ $CD4^+$ $B220^+$, and $CD19^-$ $CD4^-$ $B220^+$ (DN), and were subsequently CFSE labeled and transferred *i.v.* into BALB/c mice. The following day mice were killed and spleens were taken for Immunofluorescence analysis.

Immunostaining

Spleens were frozen, sectioned, and stained [1]. Immunohistochemistry protocols used anti-CD22-FITC (Cy34.1) and anti-IgM^a-biotin (DS-1) (BD Biosciences). Secondary reagents were anti-FITC-horseradish peroxidase (HRP) and streptavidin-AP (Southern Biotech). Immunofluorescence protocols used anti-B220-FITC and anti-Foxp3-PE.

ANAs

The presence of anti-nuclear Abs (ANAs) in serum was detected as previously described using permeabilized HEp-2 cells as the substrate [2]. Serum was considered ANA^+ if homogeneous nuclear staining was observed [16].

Chromatin ELISAs

ELISA plates (ThermoLabSystems) were coated with 2 μg/ml of chromatin (a generous gift of M. Monestier, Temple University, Philadelphia, PA) overnight at 4°C. The remaining steps were performed at room temperature. All washes were conducted at least eight times in 1X PBS/0.05% Tween 20. Following the coating step, plates were washed, blocked with 1% BSA/PBS/azide for at least 1h, and washed again. Sera were then added at increasing dilutions, as indicated in figure, and incubated for a minimum of 1h. Plates were washed and incubated with developing Ab (anti-IgM^a biotin; BD Biosciences), for at least 1h. Finally plates were washed, incubated with streptavidin-AP (Southern Biotechnology Associates) for at least 1h, washed and developed for 14–18 h. The plates were developed with Immunopure *p*-nitro-phenyl

phosphate (Pierce) as the substrate. Absorbances were read at dual wavelength, 405/650 nm using a microplate reader.

Treg inhibition of proliferation or cytokine assay

$CD4^+$ $CD25^-$ (BALB/c) and $B220^-$ $CD4^+$ $CD25^-$ (BALB-*lpr/lpr gld/gld*) Th cells were sorted from lymph nodes. Treg cells were sorted from lymph nodes of BALB/c or TS1xHA28 mice ($CD4^+$ $CD25^+$), and *lpr/lpr gld/gld* mice ($B220^-$ $CD4^+$ $CD25^+$). Sorted cells were plated at a starting ratio of 2.5 Th cells : 1 Treg cell to a final ratio of 20 Th : 1 Treg cell with anti-CD3 (2C11) (33 ng/ml) (BD Biosciences) and irradiated BALB/c spleen cells as APCs. After three days of stimulation, cells were pulsed with ^3H thymidine and 16–18 hours later cpm were recorded. The percent inhibition is determined by the following formula: ((cpm of Th alone culture – cpm of Th+Treg culture) / cpm of Th alone culture) × 100 = % inhibition.

Responder T cells for *in vitro* proliferation or cytokine assays were either obtained by sorting $CD4^+$ $CD25^-$ cells from BALB/c, TS1 TCR Tg mice, or $B220^-$ $CD4^+$ $CD25^-$ cells from *lpr/lpr gld/gld* or mice. The Th1 and Th2 lines were established as described previously [5]. 5×10^4 $CD4^+$ $CD25^-$ cells were stimulated with 0.125 μg/ml of anti-CD3 (2C11), 5×10^5 CD3 depleted BALB/c splenocytes and with 5×10^4 Treg cells where indicated. After three days cells were stimulated with PMA, Ionomycin, and Brefeldin A as previously described [5]. The cells were then stained for CD4, Thy1.2 and IFN-γ or IL-4.

CD25 depletion protocol

>BALB/c-*gld/gld* or *lpr/lpr gld/gld* mice were injected *i.p.* weekly from two to four weeks of age with 1 mg of PC61 (bio-express.com) dissolved in 0.5 ml of PBS. Control mice were given 0.5 ml of PBS or 1 mg of Rat IgG, respectively. Beginning at week 5 of age, mice were bled weekly and anti-chromatin ELISAs were performed to detect anti-chromatin IgM, IgG_1, and IgG_{2a} Abs in sera. The level of CD25 depletion was determined weekly starting at week 4 by staining peripheral lymphocytes for CD4 and CD25. The frequency of $CD25^+$ cells within the $CD4^+$ population was: week 4, PC61 treated = 0.76% +/− 0.12%, control = 6.8% +/− 0.5%; week 5, PC61 treated = 1.12% +/− 0.18%, control = 6.68% +/− 0.51%; week 6, PC61 treated = 2.97% +/− 0.68%, control = 8.03% +/− 2.17%; week 7, PC61 treated = 2.63% +/− 0.71%, control = 8.28% +/− 1.96% (n=5). All mice in the PC61 group had a significantly lower frequency of $CD4^+$ $CD25^+$ cells at all time points compared to control mice.

Statistical Analysis

Statistical significance was determined via the Student's t test provided by Microsoft Excel software unless otherwise noted. Significance was ascribed when $p < 0.05$.

Results

In single mutant *lpr/lpr* mice, FasL mRNA is upregulated [17,18]. Consistent with this finding, a high level of non-specific cell death and/or graft versus host disease is observed when *lpr/lpr* T cells are adoptively transferred. These outcomes are avoided when *lpr/lpr gld/gld* T cells are used [19,20]. Therefore, to bypass these complications as well as to prevent the transferred cells from being killed by host cells bearing FasL, we used double mutant *lpr/lpr gld/gld* mice in our studies.

Anti-chromatin B cells from young VH3H9 Tg *lpr/lpr gld/gld* mice are responsive to T cell help

In order to address whether the abortive response of anti-chromatin B cells in young *lpr/lpr gld/gld* mice is due to a defect in their response to T cell help, we engineered a means to provide them with defined T cell help. This system previously allowed us to dissect the effects of Th

and Treg cells on anti-chromatin B cells in Fas-sufficient BALB/c mice. TS1 transgenic T cells specific for the site 1 peptide from HA of the PR8 influenza virus were injected into VH3H9 Tg *lpr/lpr gld/gld* mice that expressed HA on all class II^+ cells (Fig 1A). Ten days after transferring lymph node cells from TS1 mice into VH3H9/HACII Tg *lpr/lpr gld/gld* mice, serum was collected and assayed for anti-nuclear antibodies (ANAs). Mice that received no T cells were ANA^-, however when mice received T cell help they were ANA^+ (Fig 1B). Age-matched uninjected mice remained ANA^-. Darkly staining extrafollicular anti-chromatin B cells were observed in the spleens of mice that had received T cell help; in contrast, these B cells were found in the B cell follicles in mice that did not receive T cells (Fig 1C). The extrafollicular localization of anti-chromatin cells is remarkably similar to that previously observed with VH3H9/HACII Tg Fas-sufficient BALB/c mice as recipients [4]. Therefore, anti-chromatin B cells from *lpr/lpr gld/gld* mice are capable of terminal ASC (antibody secreting cell) differentiation in the presence of sufficient T cell help. The abortive response seen in young *lpr/lpr gld/gld* mice must therefore be due to another factor – perhaps the quantity or nature of available T cell help.

Treg cells can suppress *lpr/lpr gld/gld* T cell help for B cells

In order to test the ability of Fas/FasL-deficient T cells to help anti-chromatin B cells, we made use of an *in vivo* transfer model in which anti-chromatin B cells engineered to express HA are transferred along with anti-HA TS1 TCR Tg T cells, and mice monitored for development of anti-chromatin Abs. This system allowed us to demonstrate previously that Th cells can induce the differentiation of anti-chromatin B cells into plasma cells, and that Treg cells can block this maturation [5]. We bred the TS1 Tg onto BALB/c-*lpr/lpr gld/gld* mice in order to test the ability of Fas/FasL-deficient T cells to help anti-chromatin B cells and the susceptibility of these cells to suppression by Treg cells. Anti-HA $CD4^+$ $CD25^-$ cells, with or without Fas and FasL, were injected into CB17 mice along with PR8 influenza virus. In some cases, purified Treg cells were coinjected with the Th cells (Fig 2A). The following day, HA-expressing anti-chromatin B cells (Ig^a) were injected into the Ig^b-expressing CB17 mice. This system allows anti-chromatin antibody production to be tracked using anti-Ig^a reagents. On day 8 anti-chromatin antibodies in the sera and plasma cell localization in the spleen was analyzed (Fig. 2A).

As described previously, anti-HA $CD4^+$ $CD25^-$ T cells induced autoAbs which were inhibited by the co-administration of Treg cells (Fig. 2B, C)[5]. When *lpr/lpr gld/gld* anti-HA T cells from young (≤8 week old) mice were transferred with anti-chromatin B cells, anti-chromatin Abs were likewise produced, but at lower titers than recipients of Fas/FasL-sufficient anti-HA $CD4^+$ $CD25^-$ cells (Fig. 2B). This difference may be due to the fact that Fas and FasL can also act as costimulators for $CD4^+$ T cells [21]. Nevertheless, when Treg cells were added in addition to the young anti-HA *lpr/lpr gld/gld* T cells, antibody production was dramatically curtailed and anti-chromatin B cells were not detected in the spleen by immunohistochemistry (Fig. 2B, C). Thus, T cells from young Fas/FasL-deficient mice can provide help to anti-chromatin B cells, and this help can be suppressed by Treg cells.

Characterization of T cell subsets in *lpr/lpr gld/gld* mice

Multiple studies of *lpr* and *gld* mice on various strains (including MRL, C3H, C57BL/6, and BALB/c) have documented an increased number of $CD4^+$ T cells in the periphery, as well as an increase in the unusual $B220^+$ $CD4^+$ and $B220^+$ $CD4^-$ $CD8^-$ (double negative, DN) T cells compared to Fas/FasL-wildtype mice [22–26]. Indeed, in older Fas/FasL-deficient mice, the DN cells account for much of the lymphoproliferation [22–26]. The BALB/c-*lpr/lpr gld/gld* double mutant mice studied here duplicated this trend, with increased numbers of $CD4^+$, $B220^+$ $CD4^+$, and DN T cells (Fig. 3A, B).

In addition to rising T cell numbers, multiple publications have described an increase in the activation level of T cells in Fas/FasL-deficient mice [27–30]. We have previously documented a rise in the frequency of $CD4^+$ T cells found in splenic B cell follicles in *lpr/lpr* or *gld/gld* mice [2,3]. However, because as *lpr/lpr gld/gld* mice age, their splenic architecture becomes increasingly disrupted [14], it is difficult to ascertain where the $CD4^+$, $B220^+$ $CD4^+$, and DN T cells localize. To determine where these T cells home in the spleen, CFSE labeled T cell subsets from *lpr/lpr gld/gld* mice were transferred into BALB/c recipients. Sixteen hours after transfer, the majority of $CD4^+$ T cells localized in the splenic PALS, but with a minority clearly present in the follicle (Fig 3C, left). DN T cells were located near the marginal zones, confirming a previous publication (Fig 3C, right) [31]. Like the DN T cells, the $CD4^+$ $B220^+$ T cells were also found on the outer edges of the B cell follicle (Fig 3C, center).

Flow cytometry showed that the $CD4^+$ T cell population was predominantly negative for CXCR5 expression but did include a small $CXCR5^+$ population consistent with the presence of $CD4^+$ T cells in the follicle (Fig 3C, D, left). The DN and $CD4^+$ $B220^+$ cells were uniformly $CXCR5^+$, but with a slightly lower mean fluorescence than follicular B cells (Fig 3D, right and center). The vast majority of $CD4^+$ T cells from the recipient mouse were $CXCR5^-$ (data not shown) [5]. Therefore, CXCR5 expression on *lpr/lpr gld/gld* T cell subsets correlated with splenic localization in recipient BALB/c mice.

Foxp3 expression in T cells from *lpr/lpr gld/gld* mice is primarily in the $CD4^+$ T cell subset

An important factor in the potential for T cells to help B cells is the counterbalancing influence of Treg cells. To characterize the resident Treg cell population in *lpr/lpr gld/gld* mice, Foxp3 expression was evaluated in the $CD4^+$, $B220^+$ $CD4^+$, and DN T cell populations (Fig. 4A, B). As described previously, $Foxp3^+$ cells comprise about 10% of CD4 T cells in BALB/c mice [32]. Strikingly, the frequency and absolute number of $CD4^+$ $Foxp3^+$ cells in the spleen and lymph nodes of both the young and old *lpr/lpr gld/gld* mice was significantly higher than in the BALB/c mice (Fig. 4A,B).

In the *lpr/lpr gld/gld* mice, a decrease in the frequency of Foxp3 staining was observed in the $B220^+$ $CD4^+$ compartment compared to $B220^-$ $CD4^+$ cells, with virtually no Foxp3 staining in the DN compartment (Fig. 4A). The rare $B220^+$ $CD4^+$ cells in BALB/c mice contain a similar fraction of $Foxp3^+$ cells as the $B220^-$ $CD4^+$ cells (~12% vs. ~11%, respectively), whereas no Foxp3 staining in the miniscule DN compartment was observed (Fig. 4A). Analysis of absolute numbers of $Foxp3^+$ cells shows that the vast majority of $Foxp3^+$ cells reside in the $B220^-$ $CD4^+$ population in *lpr/lpr gld/gld* mice, as in the Fas-sufficient BALB/c mice (Fig. 4B).

Finally, immunofluorescence was performed to determine where the $Foxp3^+$ population is located in the spleen. In the white pulp of BALB/c and young *lpr/lpr gld/gld* mice the $Foxp3^+$ population is located in the PALS and so is co-localized with $CD4^+$ T cells (Fig. 4C). While the lymphoid architecture gets progressively disrupted in *lpr/lpr*, *gld/gld*, or *lpr/lpr gld/gld* mice as they age [2,14], the $Foxp3^+$ cells were still predominately found in the PALS of the old *lpr/lpr gld/gld* mice (Fig 4C).

Fas/FasL deficiency does not diminish the suppressive effects of Treg cells nor does it lessen the susceptibility of Th proliferation to suppression by Treg cells

Although there is an increase in the frequency and number of $Foxp3^+$ cells in the CD4 compartment in Fas/FasL-deficient mice, it was possible that these cells could become functionally unable to suppress the proliferation of target T cells. If Treg cells in *lpr/lpr gld/gld* mice were to lose their ability to suppress proliferation as the mice aged, this could account for the onset of autoantibody production. Likewise, if Th cells developed a resistance to suppression by Treg cells, this could trigger autoAb production.

We investigated whether B220$^-$ CD4$^+$ CD25$^-$ cells from young or old *lpr/lpr gld/gld* mice were susceptible to suppression by Treg cells. TS1xHA28 BALB/c mice were used as donors for the Treg cells because greater numbers of cells could be obtained [33]. Consistent with previous data, Treg cells inhibited CD4$^+$ CD25$^-$ cell proliferation from BALB/c mice by > 80% (Fig. 5A). Likewise, the proliferation of B220$^-$ CD4$^+$ CD25$^-$ cells isolated from either young or old *lpr/lpr gld/gld* mice was equally inhibited by the Treg cells (Fig. 5A).

In similar experiments, Treg cells were isolated from both young and old *lpr/lpr gld/gld* mice and tested for their ability to inhibit CD4$^+$ CD25$^-$ cell proliferation *in vitro*. Treg cells from both young and old *lpr/lpr gld/gld* mice were equally capable of suppressing BALB/c CD4$^+$ CD25$^-$ cell proliferation at a 2.5 or 5 to 1 ratio of CD4$^+$ CD25$^-$ cells to Treg cells (Fig 5B and data not shown).

IFN-γpotential is not reduced in *lpr/lpr gld/gld* B220$^-$ CD4$^+$ CD25$^-$ T cells in the presence of Treg cells

As another function of CD4$^+$ CD25$^-$ T cells is their ability to produce cytokines that direct other cells in the immune system, we investigated whether the production of cytokines by *lpr/lpr gld/gld* T cells was affected by the presence of Treg cells. Previously, it was shown that CD4$^+$ T cells from 4–6 month old C3H *gld/gld* mice stimulated *in vitro* had secreted high levels of IFN-γ and low amounts of IL-4 [27]. We confirmed this observation after *ex vivo* stimulation with PMA and ionomycin from BALB/c-*lpr/lpr gld/gld* mice (Fig. 6A). Thus we focused on whether Treg cells can inhibit IFN-γ production from BALB/c, young, and old *lpr/lpr gld/gld* B220$^-$ CD4$^+$ CD25$^-$ T cells after stimulation *in vitro*.

When CD4$^+$ CD25$^-$ T cells from BALB/c mice or B220$^-$ CD4$^+$ CD25$^-$ T cells from *lpr/lpr gld/gld* mice were stimulated with soluble anti-CD3 and CD3 depleted BALB/c splenocytes for three days, in the absence of Treg cells, all populations produced a similar frequency of IFN-γ$^+$ cells upon restimulation with PMA and Ionomycin (Fig 6B). In contrast, when CD4$^+$ CD25$^-$ T cells from BALB/c mice were initially stimulated with Treg cells from one of three different sources (BALB/c, young, or old *lpr/lpr gld/gld* mice) the frequency of IFN-γ producing cells was reduced to nearly baseline (Fig 6B, C, *top row*). B220$^-$ CD4$^+$ CD25$^-$ T cells from young *lpr/lpr gld/gld* mice stimulated with Treg cells from either TS1xHA28 BALB/c mice or young *lpr/lpr gld/gld* mice decreased their IFN-γ production, although a small IFN-γ$^+$population remained (Fig. 6B, C, *middle row*). In contrast, when the B220$^-$ CD4$^+$ CD25$^-$ T cells from old *lpr/lpr gld/gld* mice were stimulated with Treg cells from either TS1xHA28 BALB/c or old *lpr/lpr gld/gld* mice, there was no significant reduction in the percentage of IFN-γ cells observed upon restimulation (Fig 6B, C, *bottom row*, and D). These data suggest that Treg cells, regardless of their source, can not diminish the potential IFN-γ production that is already present in B220$^-$ CD4$^+$ CD25$^-$ T cells from old *lpr/lpr gld/gld* mice.

Treg cells do not diminish the potential of IFN-γ production from *in vitro* generated Th1 cells but modestly affect IL-4 levels from Th2 cells

Treg cells have been shown to block the *in vitro* proliferation of naïve T cells, which is required for Th1/Th2 differentiation [34,35]. However, their ability to alter the status of effector T cells is less clear. It has been previously demonstrated that *lpr/lpr* or *gld/gld* mice have a greater percentage of effector T cells [27]. In order to test whether Treg cells can influence the potential of Th1 or Th2 cells to make their signature cytokine, Th1 and Th2 cells were generated *in vitro* from CD4$^+$ CD25$^-$ BALB/c TS1 TCR Tg cells, and IFN-γ or IL-4 production after culture with or without Treg cells was measured. Naïve TS1 cells stimulated with anti-CD3 had a substantial number of cells producing IFN-γ after PMA and Ionomycin stimulation that was inhibited when Treg cells were added to the initial anti-CD3 cultures (Fig. 7). As expected, the Th1 cultures also had a higher number of cells that produced IFN-γ and had a higher MFI

compared to naïve T cells (Fig. 7). However unlike naïve T cells, the number of IFN-γ cells did not decrease when Treg cells were present in the same culture (Fig. 7). The Th2 cells in the absence of Treg cells had a majority of cells producing IL-4 and this frequency was modestly decreased in the presence of Treg cells (31%) (Fig 7). This data suggests that regardless of the Fas or FasL status on the $CD4^+$ $CD25^-$ T cell, Treg cells have only a limited effect, at best, on the potential of effector T cells to make cytokines.

CD25 depletion in *gld/gld* or *lpr/lpr gld/gld* deficient mice augments anti-chromatin IgG_1 antibodies

In order to further address the role of Treg cells in delaying autoimmunity in autoimmune-prone mice, $CD25^+$ cells were depleted from *gld/gld* or *lpr/lpr gld/gld* mice, beginning at a young age, and anti-chromatin IgM, and IgG_1 and IgG_{2a} responses assayed. It has been previously shown that IgG_1 and IgG_{2a} are associated with IL-4 (Th2) and IFN-γ (Th1) production, respectively [36]. Anti-CD25 depletion compared to PBS treatment had no effect on anti-chromatin IgM or IgG_{2a} production at weeks 5 and 6 in *gld/gld* mice (Fig. 8A). However, CD25 depletion did result in significantly greater anti-chromatin IgG_1 responses compared to the control mice at weeks 5 and 6 (Fig. 8A). This affect on IgG_1 was even more prominent in anti-CD25 treated *lpr/lpr gld/gld* mice (Fig. 8B). The anti-CD25 treated *lpr/lpr gld/gld* mice also exhibited slightly higher levels of anti-chromatin IgM compared to control treated mice which may correspond to a slight increase in total IgM (Fig. 8B).

Discussion

Fas/FasL-deficient mice have proven to be a useful model for studying the breakdown of B and T cell tolerance en route to the development of anti-chromatin antibodies [14]. We have tracked the phenotype and fate of VH3H9/λ_1 anti-chromatin B cells in these mice before and after anti-chromatin autoantibodies become detectable [2,3]. Although anti-chromatin B cells show certain phenotypic changes even in young mice, their differentiation into ASCs is not observed until 10 weeks of age, coinciding with the detection of serum autoantibodies [2,3]. We have hypothesized that the apparent incomplete activation of the anti-chromatin B cells in young *lpr/lpr* or *gld/gld* mice may be due to one or more factors, including an intrinsic defect in the B cells themselves at the younger ages, a limiting quantity or quality of available T cell help at early time points, or the suppressive effects of T regulatory cells [4,14].

Here, we show that the absence of autoAbs in young *lpr/lpr* or *gld/gld* mice is not due to an inherent defect in the B cells themselves, as anti-chromatin autoAbs are quickly produced when a defined, plentiful source of T cell help is provided. We further show that Th cells from young *lpr/lpr gld/gld* mice, when transferred into a third party mouse, are capable of inducing the production of anti-chromatin Abs. Why, then, do the Th cells in the young *lpr/lpr gld/gld* mouse not induce autoAbs until at least 10 weeks of age? One possibility is that the process of transferring the *lpr/lpr gld/gld* Th cells into a third-party recipient mouse may improve the apparent helper ability of the Th cells by concentrating their numbers (this transfer system uses a TCR Tg and purifies a defined number of Th cells for injection). Alternatively, the process of purification and transfer may remove the Th cells from a non-permissive environment in the young *lpr/lpr gld/gld* mouse, perhaps including the dampening effects of Treg cells. Indeed, we show here that the co-transfer of an equal number of Treg and Th cells effectively aborts autoantibody production.

To better understand the T cell environment in the young versus old *lpr/lpr gld/gld* mice, we studied $CD4^+$ Th and Treg cells as well as further characterizing the $B220^+$ $CD4^+$ and DN T cells. CD4 cell numbers increase as *lpr/lpr gld/gld* mice age compared to BALB/c mice. Although the majority of $CD4^+$ T cells expresses baseline levels of CXCR5 and localizes in the PALS, a small but distinct minority expresses CXCR5 and appears to co-mingle with B

cells in the follicle. This may be relevant as T cells with a follicular localization (T_{FH}) have been shown to be important in promoting germinal center development and Ab production [37].

We found that the frequency and number of $Foxp3^+$ $CD4^+$ Treg cells is also elevated in *lpr/lpr gld/gld* mice compared to BALB/c mice, and this number increases with age. Thus, even as the CD4 Th population is expanded in Fas/FasL-deficient mice, the number of Treg cells appears to keep pace with this expansion. Foxp3 expression was observed in both the typical $B220^-$ $CD4^+$ population [38] as well as in the $B220^+$ $CD4^+$ subset in all mice examined. It is worth noting however that while the Foxp3 population increases in the $B220^-$ $CD4^+$ compartment, the population of $Foxp3^+$ $B220^+$ $CD4^+$ cells appears to decline as the *lpr/lpr gld/gld* mice age, coinciding with the onset of autoAb production. As this population comprises only a small population in total number, however, the significance of these cells is unclear.

To examine the functionality of Treg cells in *lpr/lpr gld/gld* mice, we tested their ability to suppress proliferation and IFN-γ production *in vitro*. We also examined the susceptibility of *lpr/lpr gld/gld* Th cells to Treg cells in similar assays. We found that Treg cells derived from both young and old *lpr/lpr gld/gld* mice ably suppress T cell proliferation *in vitro*. These data are consistent with other studies that have shown that blocking with an anti-FasL antibody has no effect on Treg cell suppression [39,40], and that Treg cells from *gld/gld* mice can inhibit *gld/gld* Th cell proliferation [11]. Conversely, we found that Treg cells suppress the proliferation of Th cells derived from either young or old *lpr/lpr gld/gld* mice.

Many studies have shown that Treg cells can inhibit naïve T cell proliferation *in vitro* and hence this would block potential Th1 and Th2 cell development [34,35,41]. However, we have shown *in vivo* that naïve T cell proliferation at the beginning of an immune response is minimally impaired, yet the autoantibody response is inhibited [5]. Furthermore, Treg cells can suppress different ongoing autoimmune responses including gastritis and colitis [42,43], but the mechanism for stopping autoimmunity in these models is still unknown. These data suggest that blocking of Th cell proliferation may not be the only, or even the main, mechanism of inhibition *in vivo*.

As one possible mechanism for the suppression of an ongoing autoimmune response would be to alter the cytokine potential of established effector T cells, we tested whether cytokine production from *lpr/lpr gld/gld* Th cells or *in vitro*-generated Th1 and Th2 cell lines was altered by Treg cells. We found that Treg cells effectively blocked IFN-γ production by Th cells from BALB/c mice and young *lpr/lpr* mice. This data suggests that Treg cells can block naïve T cells from differentiating into effector Th1 cells. However, if the Th cells were derived from older *lpr/lpr gld/gld* mice, co-culture with Treg cells did not diminish the frequency of IFN-γ-producing cells upon restimulation *in vitro*. Thus, although Treg cells suppress the proliferation of a majority of Th cells from old *lpr/lpr gld/gld* mice, a pre-existing population of cells appears resistant to this suppression and maintains the potential to produce IFN-γ. This supports the hypothesis that as *lpr/lpr gld/gld* mice age, they accumulate dedicated effector T cells that produce IFN-γ and are resistant to suppression by Treg cells. The assays we present here demonstrating the resistance of an established Th1 cell line to suppression of cytokine production by Treg cells bolster this hypothesis. Established Th2 cells appear less resistant than Th1 cells to the effects of Treg cells on their cytokine potential. We and others have shown that IFN-γ-producing Th cells are effective at promoting Abs *in vivo*, including anti-chromatin Abs [44,45]. Furthermore, many studies have shown a preponderance of a Th1-type environment in Fas or FasL-deficient mice [27,46,47].

To test the ability of Treg cells to suppress Th1 or Th2-type T cells *in vivo*, we used the anti-CD25 Ab (PC61) to deplete Treg cells from young *gld/gld* or *lpr/lpr gld/gld* mice and tracked

the appearance of Th1-and Th2-associated Ab isotypes. Young *lpr/lpr gld/gld* mice were used to avoid the complications of the dramatic serum hypergammaglobulinemia and other disease-associated problems (such as scabby skin, lymphoproliferation, and enlarged lymph nodes and spleens) in older *lpr/lpr gld/gld* mice. We demonstrated in figure 6 that even young *lpr/lpr gld/gld* mice have a small population of pre-existing IFN-γ cells and postulated that the IFN-γ potential of these cells would be resistant to Treg cells *in vivo* as *in vitro*. In an environment depleted of Treg cells, IgG_1 (a Th2-associated isotype) but not IgG_{2a} (a Th1-associated isotype) was significantly elevated. Thus, as *in vitro*, Treg cells *in vivo* do not affect IFN-γ secretion, thereby leaving IgG_{2a} production unchanged.

We have shown here and in previous publications that Treg cells can block anti-chromatin Ab production in an *in vivo* transfer model [4,5,45]. Why, then do older *lpr/lpr gld/gld* mice develop anti-chromatin Abs even though they have high numbers of Treg cells? In other transgenic models we have suggested that high numbers of Th cells can lead to autoantibody production or autoimmunity even in the presence of Treg cells [48,49]. Furthermore, in the *in vivo* transfer model that we used to test Th and Treg function, the recipient mice received a single dose of Treg cells at the same time they received Th cells. We have shown that if the injection of Treg cells is delayed by even one day after administration of Th cells, the Treg cells lose their effectiveness at blocking autoAb production [5]. Thus, in the aging *lpr/lpr gld/gld* mice, Treg cells may be simply overwhelmed by the continual expansion of Th cells. Finally, another possibility is that Treg cells lose their effectiveness as the Fas/FasL-deficient mice age or T effectors become resistant to regulation, thereby allowing Th cells to promote the induction of anti-chromatin Abs. Strong support of the idea that T cells can become resistant to Treg cell suppression comes from a recent study which showed that during experimental autoimmune encephalomyelitis, a population of Treg cells from the CNS could suppress naïve responder T cells but not responder T cells from the CNS [50].

Likely, other factors in the aging *lpr/lpr gld/gld* mice also play a role in the development of autoAbs. For instance, older Fas/FasL-deficient mice lose normal compartmentalization of the B and T cells in the spleen, which may alter how and when Th, Treg, and B cells interact [14]. Another difference in the autoimmune and non-autoimmune environment may be that the dendritic cells secrete inflammatory cytokines that block Treg cell suppression. Cytokines such as IL-6 and IL-12, known to be secreted by dendritic cells, inhibit Treg cell suppression [51,52]. As dendritic cells in at least one murine lupus model overexpress IL-6 compared to control C57BL/6 mice [53], the status of these cells in lupus models merits further attention.

Taken together, the data presented here suggests that the interplay between Th and Treg cells is crucial to preventing autoAb production in young Fas/FasL-deficient mice. However, as the mice age and accumulate ever-increasing numbers of Th cells, some Th cells acquire resistance to Treg cells. Our data suggest that IFN-γ-producing cells may be crucial in the process of developing anti-chromatin Abs even in the face of a numerous Treg cell population.

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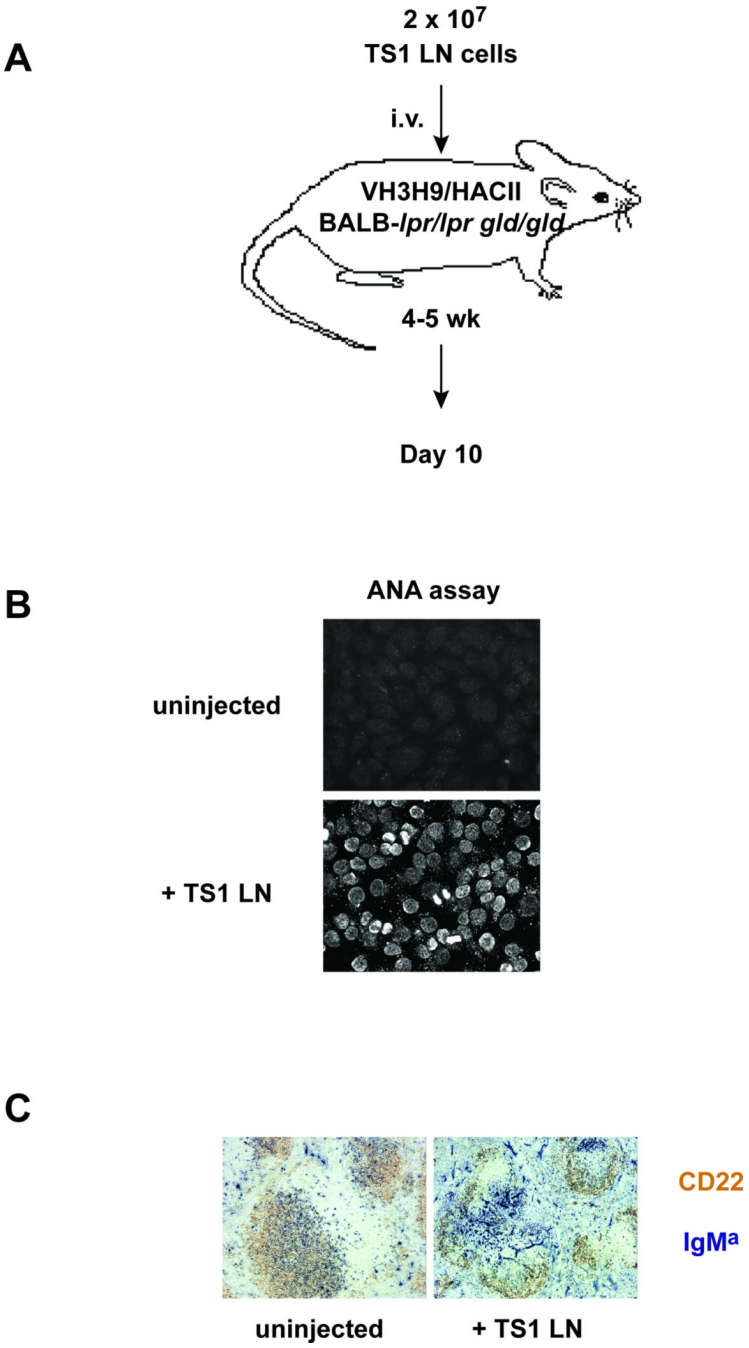

Figure 1. *lpr/lpr gld/gld* anti-chromatin B cells do produce autoAbs in the presence of Th cells

A) 20 ×10^6 TS1 lymph node cells were transferred into 4–5 week old VH3H9/HACII *lpr/lpr gld/gld* mice for 10 days. At day 10, B) serum or C) spleen was taken and assayed for ANAs and splenic localization, respectively (n=3).

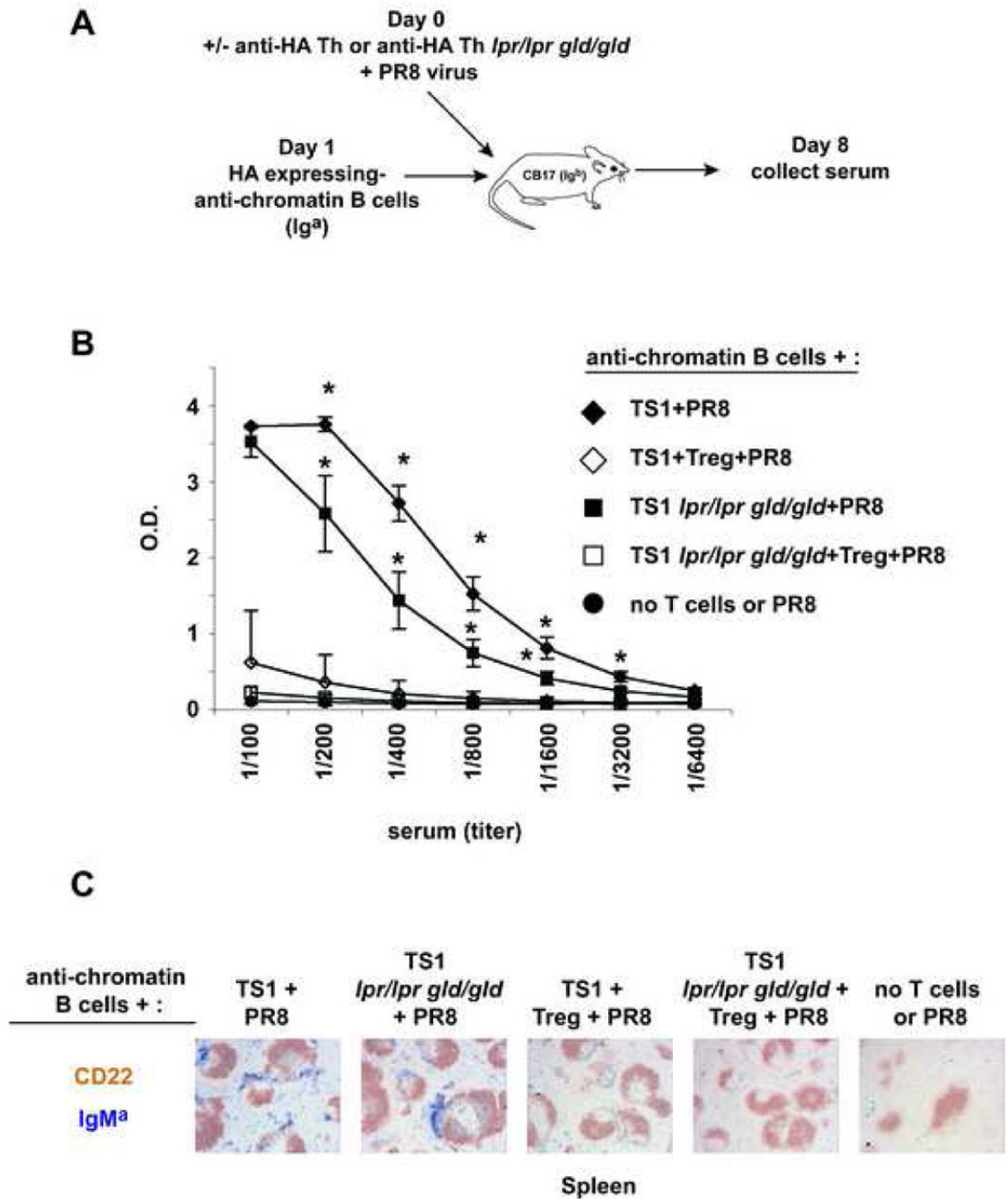

Figure 2. Treg cells block *in vivo* autoAb production promoted by *lpr/lpr gld/gld* Th cells

A) *In vivo* protocol for determining whether young *lpr/lpr gld/gld* Th cells can be blocked from inducing autoreactive anti-chromatin antibody production. B) Anti-chromatin Abs from the serum of day 8 CB17 mice that received B cells alone (black circles), TS1 Th cells (black diamonds), TS1 Th + Treg cells (white diamonds), TS1 *lpr/lpr gld/gld* Th (black squares), and TS1 *lpr/lpr gld/gld* Th + Treg cells (white squares). Data are the average of three experiments. (*) indicates $p < 0.05$ and is different compared to all groups. C) Localization of IgM^a cells in the spleen of the indicated groups by immunohistochemistry (n=3).

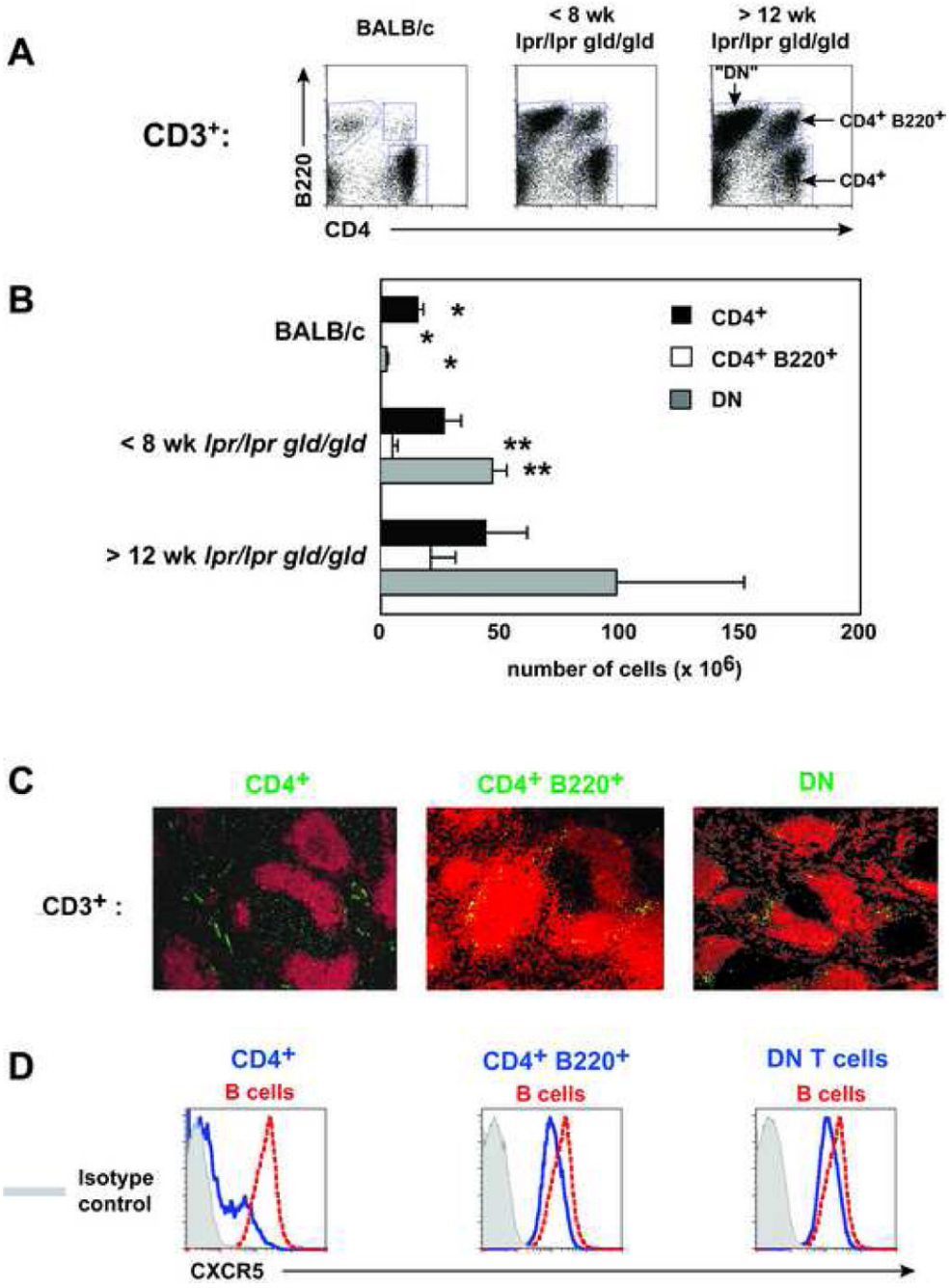

Figure 3. T cell subsets in the lymph node and spleen

A) Representative plots of $CD3^+$ cells from the spleens of BALB/c and young and old *lpr/lpr gld/gld* mice (n=4). B) Total cell numbers from the spleen of BALB/c and *lpr/lpr gld/gld* mice (n=4). (*) indicates significantly different compared to all group of mice except for those labeled. (**) indicateds are significantly different than old *lpr/lpr gld/gld* mice only. C) The indicated T cell populations were sorted from >12 week old *lpr/lpr gld/gld* spleens, CFSE labeled, and transferred into BALB/c mice overnight. Recipient mice were stained with anti-B220 (red) and transferred cells were visualized by immunofluorescence (n=3). D) Histograms of CXCR5 expression on the T cell population indicated in C) (n=3).

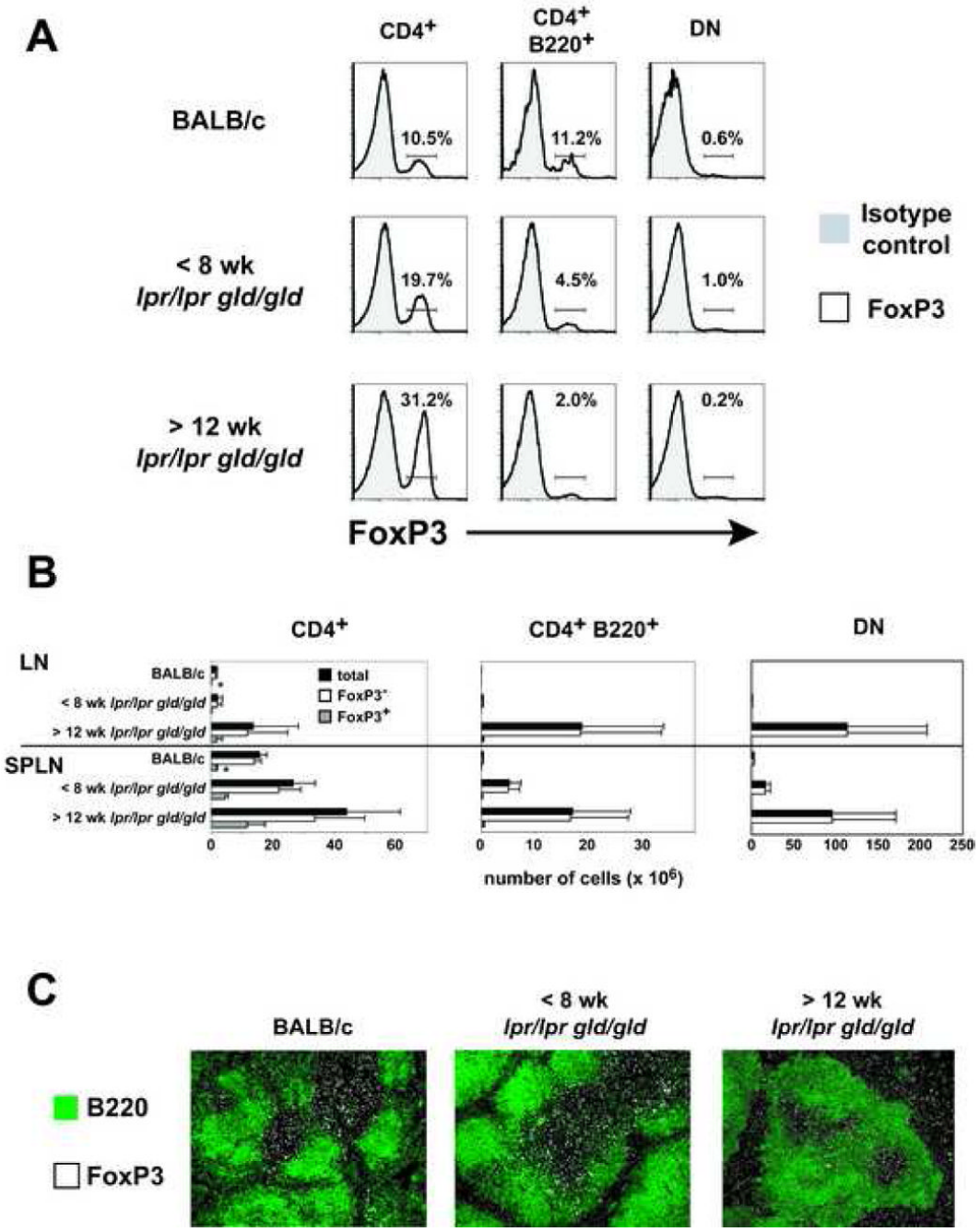

Figure 4. Foxp3 expression in *lpr/lpr gld/gld* mice

A) Representative plots of Foxp3 in the spleens of the indicated T cell population. The average percentage of Foxp3$^+$ cells in BALB/c mice: CD4$^+$, inguinal LN = 10.9% +/− 2.3%, spleen = 6.8% +/− 2.6% (n=4); CD4$^+$ B220$^+$, inguinal LN = 12.0% +/− 3.0%, spleen = 12.1% +/− 1.7% (n=3). The average percentage of Foxp3$^+$ cells in young *lpr/lpr gld/gld* mice: CD4$^+$, inguinal LN = 11.9% +/− 6.4%, spleen = 18.0% +/− 5.0% (n=3–4); CD4$^+$ B220$^+$, inguinal LN = 4.0% +/− 1.6%, spleen = 3.9% +/− 0.8% (n=3). The average percentage of Foxp3$^+$ cells in old *lpr/lpr gld/gld* mice: CD4$^+$, inguinal LN = 14.7% +/− 3.7%, spleen = 25.8% +/− 5.0% (n=3–4); CD4$^+$ B220$^+$, inguinal LN = 1.1% +/− 0.5%, spleen = 2.5% +/− 0.7% (n=2–3). B) Absolute cell number of Foxp3$^+$ and Foxp3$^-$ cells in the CD4$^+$, CD4$^+$ B220$^+$, and DN T cell subsets

(n=3). C) Foxp3 localization in the spleens of BALB/c and young and old *lpr/lpr gld/gld* mice (n≥3).

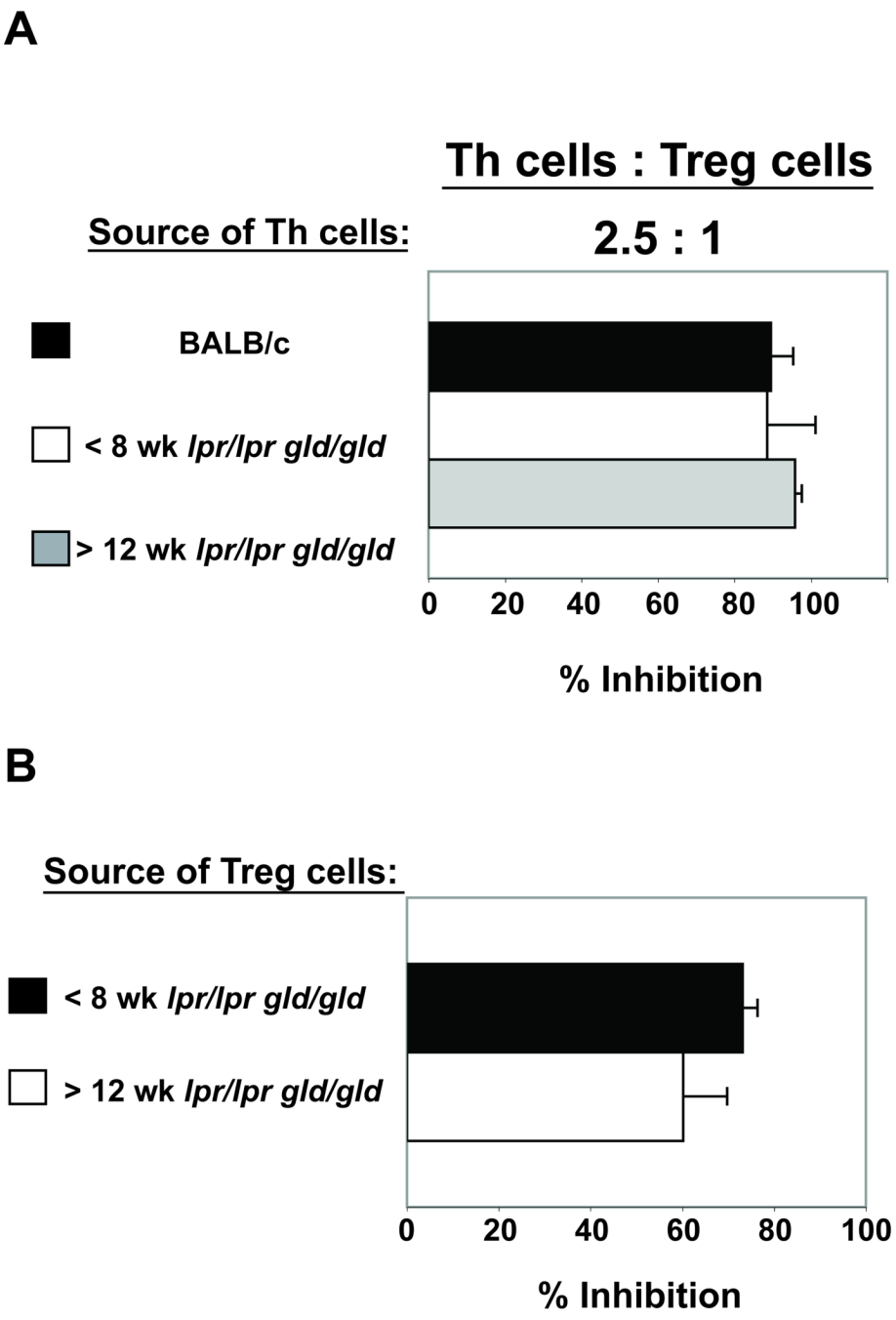

Figure 5. Treg and Th cells from *lpr/lpr gld/gld* mice can inhibit proliferation and be inhibited *in vitro*

A) 5×10^4 Treg cells ($CD4^+$ $CD25^+$) from TS1xHA28 BALB/c mice were cultured with 5×10^4 Th cells from young or old *lpr/lpr gld/gld* mice and stimulated as in B). The data is the average of three separate experiments. A) 5×10^4 Treg ($B220^-$ $CD4^+$ $CD25^+$) cells from young and old *lpr/lpr gld/gld* mice were cultured with 5×10^4 Th cells from BALB/c mice, 5×10^5 irradiated BALB/c spleen cells and stimulated with anti-CD3. After 72h stimulation the cultures were pulsed with ^3H thymidine and then harvested 16h later. The data is the average of three separate experiments.

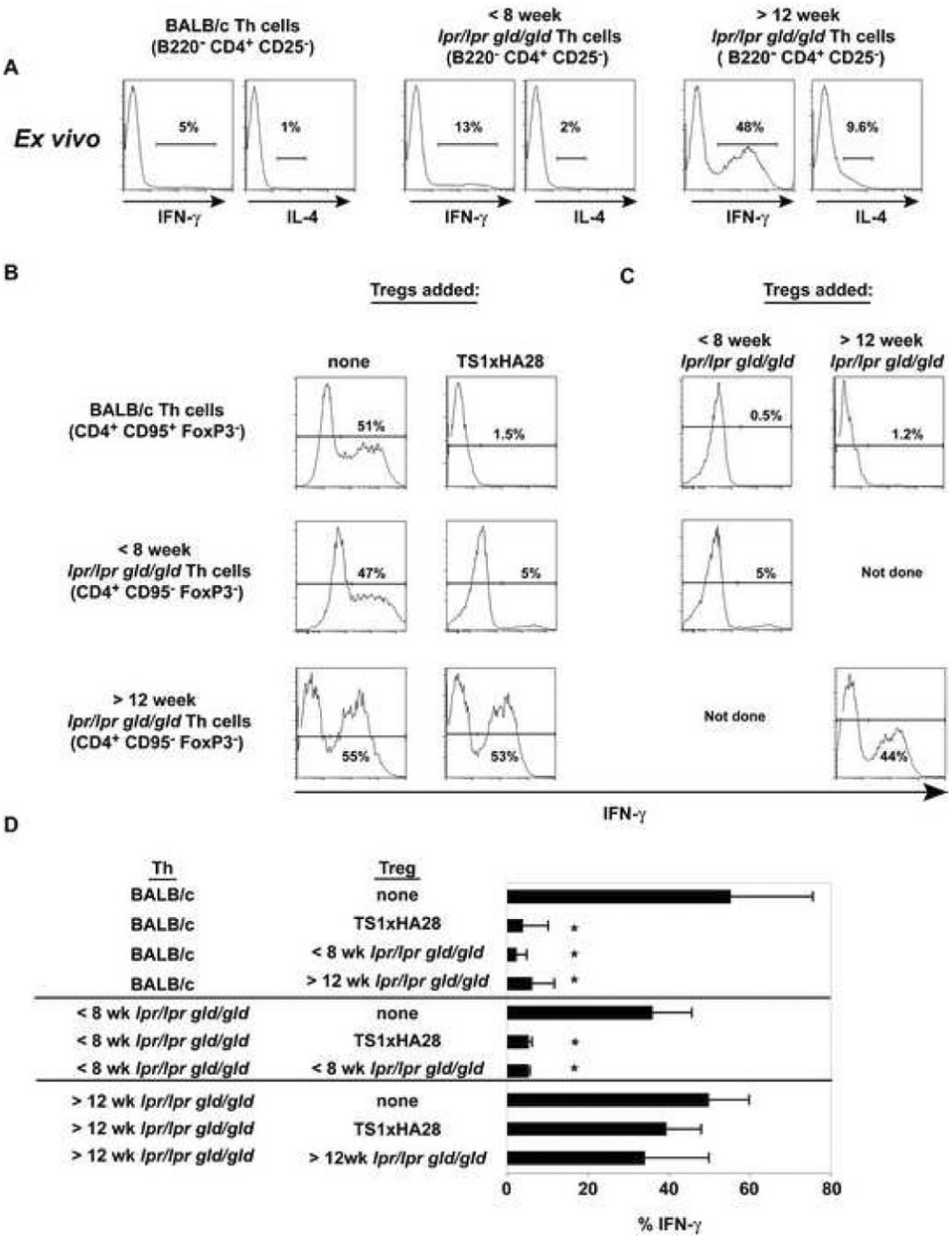

Figure 6. The effect of Treg cells on the IFN-γ potential of Th cells

A) Th cells from the indicated mice were stimulated with PMA and Ionomycin *ex vivo* and 5 hours later were stained for IFN-γ and IL-4 (n=2). B) and C) Th cells and Treg cells were sorted from BALB/c and *lpr/lpr gld/gld* mice and stimulated with anti-CD3 for three days in the presence or absence of different Treg cell populations. On day 3 the cultures were stimulated with PMA and Ionomycin and stained for IFN-γ. The representative histograms are gated on $CD4^+$ $Foxp3^-$ cells and the percentage of IFN-γ^+ cells are shown (n=3). D) The bar graph shows the average frequency of cells making IFN-γ from all three experiments. * denotes $p < 0.05$ between Th cells with and without Treg cells.

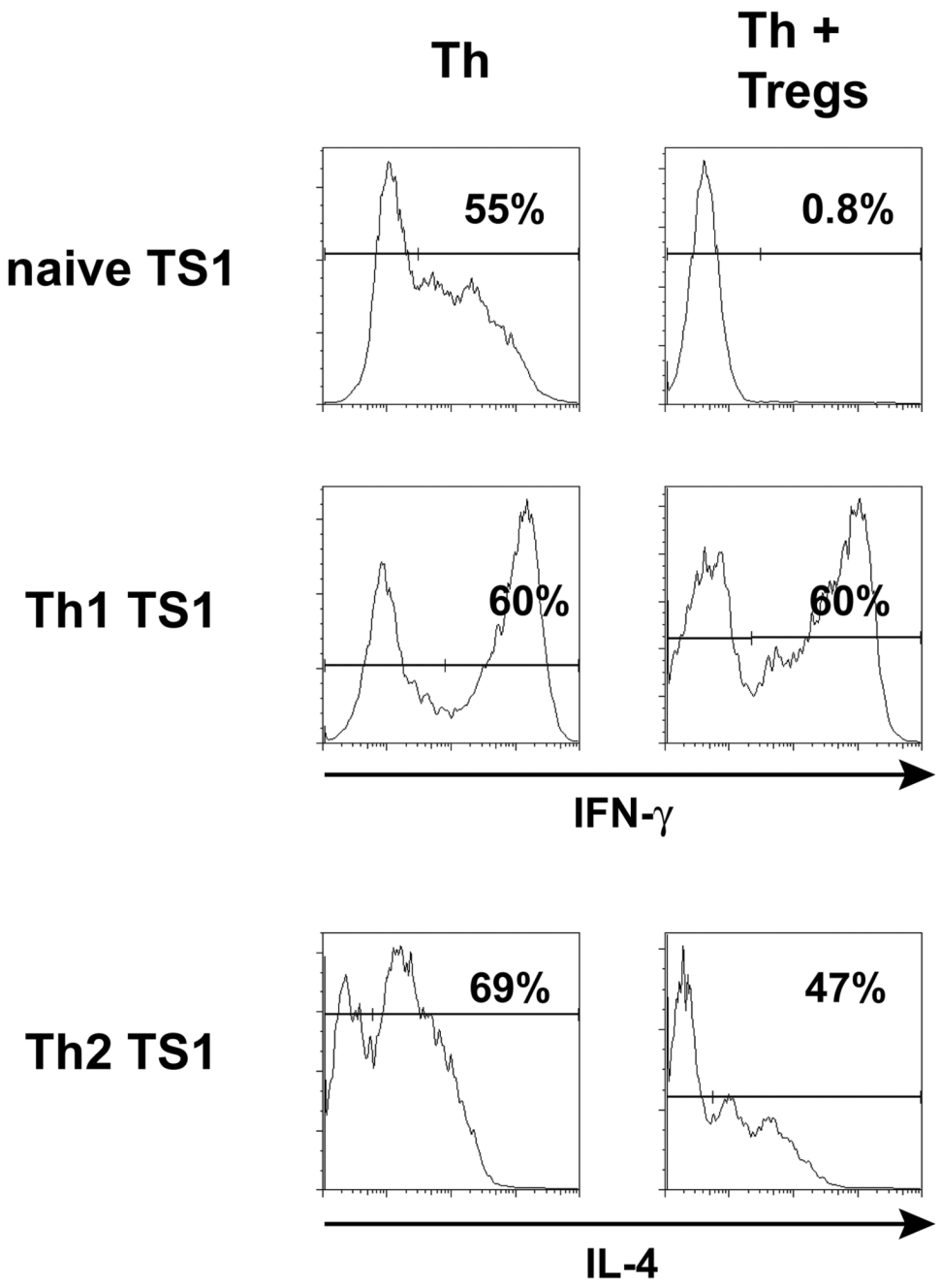

Figure 7. The response of Th1 and Th2 cells to Treg cells

Using BALB/c TS1 TCR Tg mice, naïve, Th1, or Th2 cells were stimulated as in Figure 6 and stained for the number of cells producing IFN-γ or IL-4 (n=2).

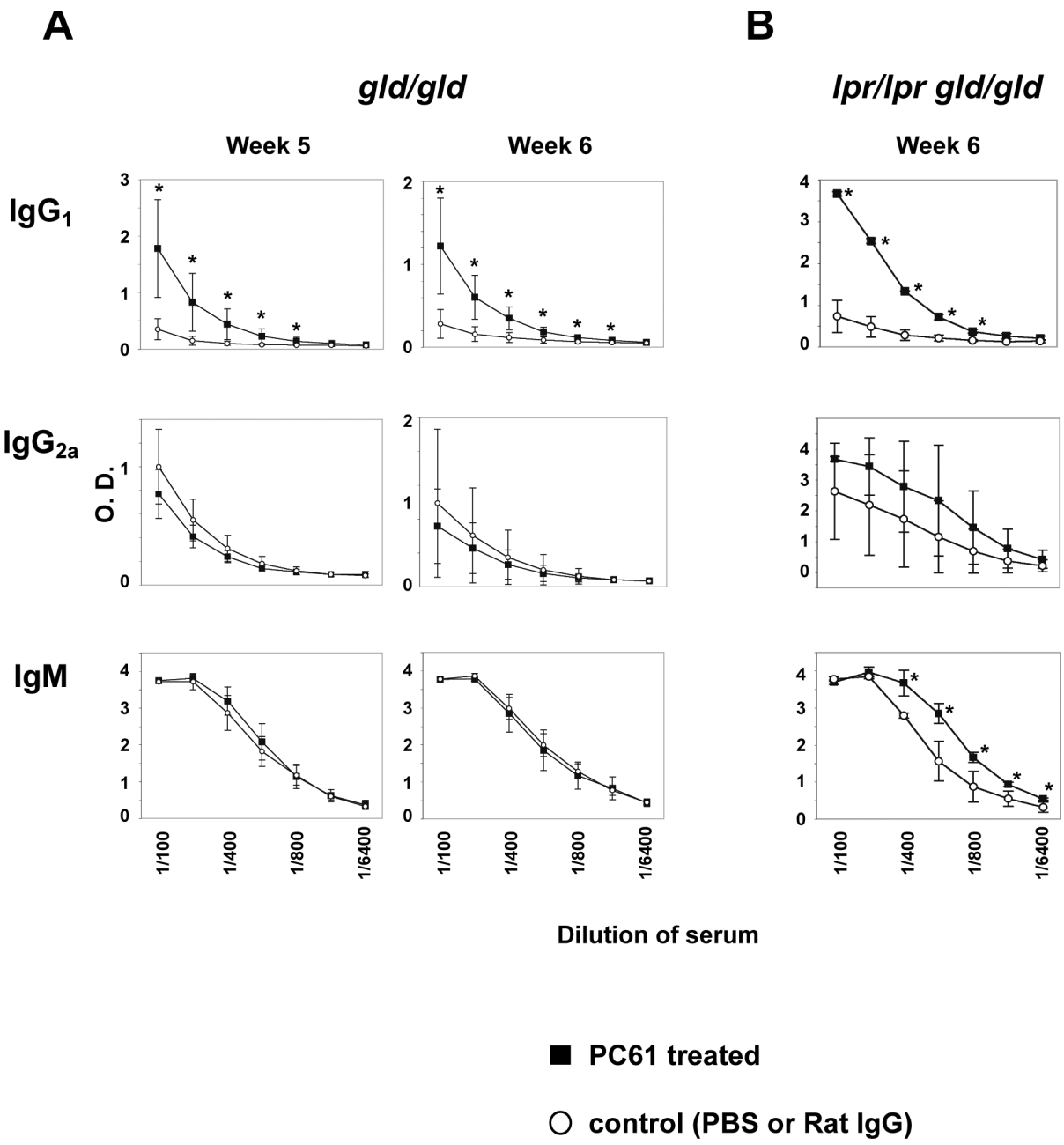

Figure 8. Anti-chromatin Ab responses from anti-CD25 depleted *gld/gld* or *lpr/lpr gld/gld* mice

Weekly at 2–4 weeks of life 1 mg of PC61 (anti-CD25) was administered *i.p.* to A) *gld/gld* (n=3) or B) *lpr/lpr gld/gld* (n=2). Simultaneously control *gld/gld* or *lpr/lpr gld/gld* mice were treated with A) PBS (n=5) or B) Rat IgG (n=4), respectively. At weeks A) 5 and 6 or B) week 6, mice were bled and the isotype of anti-chromatin antibodies were determined. (*) denotes $p<0.05$ between PC61-treated and control treated mice.