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Authors

Yang, Jun-Qi
Wen, Xiangshu
Kim, Peter J
et al.

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Invariant NKT Cells Inhibit Autoreactive B Cells in a Contact- and CD1d-Dependent Manner

Jun-Qi Yang,*,†,‡,[1] Xiangshu Wen,*,[1] Peter J. Kim,* and Ram Raj Singh*,†,§,¶

Autoantibody production is a hallmark of autoimmune diseases, such as lupus and rheumatoid arthritis. Accumulating evidence suggests a role of invariant NKT (iNKT) cells in their pathogenesis. Mechanisms underlying the role of iNKT cells in these diseases, however, remain unclear. In this study, we show that iNKT cells suppress IgG anti-DNA Ab and rheumatoid factor production and reduce IL-10–secreting B cells in a contact-dependent manner, but increase total IgG production and enhance activation markers on B cells via soluble factors. In vivo reconstitution with iNKT cells also reduces autoantibody production in iNKT-deficient mice and in SCID mice implanted with B cells. Using an anti-DNA transgenic model, we found that autoreactive B cells spontaneously produce IL-10 and are activated in vivo. In the presence of activated iNKT cells, these autoreactive B cells are selectively reduced, whereas nonautoreactive B cells are markedly activated. Because iNKTs recognize CD1d, we reasoned that CD1d might play a role in the differential regulation of autoreactive versus nonautoreactive B cells by iNKT cells. Indeed, autoreactive B cells express more CD1d than nonautoreactive B cells, and CD1d deficiency in lupus mice exacerbates autoantibody production and enhances Ab response to a self-peptide but not to a foreign peptide. Importantly, iNKT cells fail to inhibit autoantibody production by CD1d-deficient B cells. Thus, iNKT cells inhibit autoreactive B cells in a contact- and CD1d-dependent manner but activate nonautoreactive B cells via cytokines. Such ability of iNKTs to suppress autoantibody production, without causing global suppression of B cells, has important implications for the development of iNKT-based therapy for autoimmune diseases. ***The Journal of Immunology*****, 2011, 186: 1512–1520.**

Invariant NKT (iNKT) cells are CD1d-restricted, lipid Ag-reactive T cells that express an invariant TCR Vα14Jα18 in mice (1–3). Upon activation by glycolipid Ag, such as α-galactosylceramide (αGalCer) (4), these cells transactivate a variety of other cells, including NK cells, T cells, B cells, and dendritic cells (5–9). Activated iNKT cells have been reported to enhance Ab responses against T-dependent and T-independent Ag and pathogens (10–12). These observations suggest a supportive role of iNKT cells on B cell functions.

B cell depletion is emerging as an effective treatment for inflammatory diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (13). Ablation of autoreactive B cells suppresses disease (14) in the New Zealand Black (NZB) × New Zealand White (NZW) F_1 (BWF_1) model of SLE (15). These studies as well as reports showing prevention of autoimmune disease in B cell-deficient mice (16) emphasize a pivotal role of B cells in these diseases. B cells also promote Ag presentation and produce cytokines such as IL-10, which contribute to the development of these diseases (17, 18).

We have previously reported that CD1d deficiency worsens autoantibody production and nephritis in BWF_1 (19) and pristane-injected BALB/c mice (20), suggesting a role of iNKT cells in regulating autoantibody production. Hence, we investigated the role of iNKT cells on autoantibody production and explored mechanisms underlying iNKT cell effects on autoreactive B cells in this study. Our results show that iNKT cells inhibit IgG autoantibody production in a contact- and CD1d-dependent manner but leave normal polyclonal IgG responses intact and increase the expression of activation markers on B cells via cytokines. Thus, iNKT cells selectively suppress autoreactive B cells, without impairing normal B cell responses.

Materials and Methods

Mice

NZB, NZW, and SCID BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BWF_1 mice that develop T cell-dependent autoantibody-mediated lupus nephritis (15) were bred locally. In some experiments, lupus-like autoantibodies and nephritis were induced in BALB/c mice by pristane injection (20). $Vα14^{Tg}$ BALB/c (21) and $Jα18^{-/-}$ BALB/c (22) mice were provided by Dr. A. Bendelac (Chicago, IL) and Dr. M. Taniguchi (Yokohama, Japan), respectively. R4A-$γ2b^{Tg}$ mice that have increased numbers of IgG2b anti-dsDNA Ab B cells (23) were provided by Dr. B. Diamond. $CD1d^{-/-}$ BWF_1 mice were generated by introgressing CD1d-null allele onto NZB and NZW backgrounds (19). All mice were handled in accordance with institutional guidelines.

Reagents

For detection of iNKT cells, recombinant soluble dimeric mouse CD1d:Ig was loaded with αGalCer (Kyowa Hakko Kirin, Tokyo, Japan), and labeled with PE following the manufacturer's instructions. Synthetic immuno-

*Autoimmunity and Tolerance Laboratory, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA 90095; †University of Cincinnati College of Medicine, Cincinnati, OH 45267; ‡Jiangsu Institute of Parasitic Diseases, Wuxi, Jiangsu, China 214064; §Department of Pathology and Laboratory Medicine, University of California, Los Angeles, Los Angeles, CA 90095; and ¶Jonsson Comprehensive Cancer Center, University of California, Los Angeles, Los Angeles, CA 90095

[1]J.-Q.Y. and X.W. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Ram Raj Singh, Division of Rheumatology, David Geffen School of Medicine at the University of California, Los Angeles, 1000 Veteran Avenue, Room 32-59, Los Angeles, CA 90095-1670. E-mail address: rrsingh@mednet.ucla.edu

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Abbreviations used in this article: αGalCer, α-galactosylceramide; iNKT, invariant NKT; NZB, New Zealand Black; NZW, New Zealand White; RF, rheumatoid factor; SLE, systemic lupus erythematosus; Tg, transgenic; WT, wild-type.

stimulatory phosphorothioate-modified oligodeoxynuleotide (CpG-ODN 1826: 5′-TCCATGACGTTCCTGACGTT-3′) was synthesized at the local core facility (underlined letters represent CpG motifs). LPS and anti-mouse IgM were purchased from Sigma-Aldrich (St. Louis, MO). Peptides A6.1 V_H31–45 (p31) (GYFMNWVKQSHGKSL) and hen egg lysozyme 106–116 (p106) (NAWVAWRNRCK) were synthesized at the Chiron Laboratories (Clayton, Victoria, Australia), using F-moc chemistry. The synthetic peptides were analyzed for purity by HPLC and by mass spectrometry, as described previously (24). Peptide p31, an autoantigenic peptide derived from the V_H of a pathogenic anti-dsDNA mAb, stimulates T cells that promote anti-DNA Ab production and accelerate lupus nephritis (24, 25). Both p31 and p106 bind MHC class II ($I\text{-}E^d$) and elicit strong immune response in BWF_1 mice (25, 26). Animals were immunized with the peptide in CFA s.c. and challenged i.p. with the respective peptide in IFA.

Purification of T, B, and iNKT cells

Splenic T cells and B cells were purified using anti-CD90 (Thy1.2) and anti-CD45R (B220) microbeads, respectively, on an autoMACS magnetic cell separator (Miltenyi Biotec, Auburn, CA). In some experiments, T cells were enriched using mouse T cell enrichment columns (R&D Systems, Minneapolis, MN). To purify iNKT cells, spleen cells were incubated with PE-labeled CD1d–αGalCer dimer on ice for 20 min. After washing, spleen cells were incubated with anti-PE microbeads (Miltenyi Biotec), and iNKT cells were positively selected using autoMACS. Purity of T and B cells was >97%, and purity of iNKT cells was >92%.

Flow cytometry

Cells were incubated with anti-CD16/32 (2.4G2) to block FcRγII/III, followed by staining with conjugated mAbs, as described previously (19). iNKT cells were detected using CD1d-αGalCer dimer and anti-TCRβ (27). Stained cells were analyzed using FACSCalibur (BD Biosciences, San Diego, CA) and CellQuest (BD Biosciences, San Jose, CA) or FlowJo softwares (Ashland, OR).

Reconstitution of $J\alpha18^{-/-}$ mice

Recipient $J\alpha18^{-/-}$ mice were injected i.p. with 5μg LPS, followed by i.v. transfer of CFSE-labeled purified T cells (5×10^6) from donor $V\alpha14^{Tg}$ or $J\alpha18^{-/-}$ mice. Donor mice were injected i.v. with 4 μg αGalCer or vehicle 90 min prior to harvesting cells. Reconstitution was assessed by detection of $CFSE^+$ cells in peripheral blood cells after 7 d (Supplemental Fig. 1). Animals transferred with T cells from αGalCer-injected $V\alpha14^{Tg}$ mice had higher numbers (≥2-fold) of $CFSE^+$ cells than recipients of control T cells (from αGalCer-injected $J\alpha18^{-/-}$ mice or vehicle-injected $V\alpha14^{Tg}$ mice), suggesting an expansion of activated iNKT cells. Spleen cells harvested from these mice on day 7 were cultured for 6 d, and supernatants were tested for autoantibodies.

Reconstitution of SCID mice

BALB/c SCID mice were injected i.p. with 5 μg LPS and 6 μg αGalCer separately and then transferred i.v. with CFSE-labeled B cells (1×10^7) isolated from 10-mo-old $J\alpha18^{-/-}$ mice. SCID mice were then transferred i.v. with 6×10^6 T ($TCR\beta^+$) or iNKT ($TCR\beta^+$CD1d-αGalCer dimer$^+$) cells from donor 10-wk-old $V\alpha14^{Tg}$ mice. Control animals received the same numbers of $B220^+$ cell-depleted spleen cells from $J\alpha18^{-/-}$ mice. Four days after the transfer, spleen cells harvested from these mice were analyzed for TCRβ and CD1d-αGalCer dimer-positive cells to verify the reconstitution of SCID mice with iNKT cells. As expected, the recipients of $J\alpha18^{-/-}$ T cells had no iNKT cells; and recipients of purified iNKT cells had higher numbers of iNKT cells in their spleens than recipients of purified T cells from $V\alpha14^{Tg}$ mice (Supplemental Fig. 2). Spleen cells from these mice were cultured in complete medium without any stimulant or with LPS or CpG for 6 d, and supernatants were assayed for autoantibodies.

Detection of autoantibodies, Ig isotypes, and cytokines

Spleen cells (1.5×10^6/ml) were incubated in complete medium with LPS (10 μg/ml), CpG-ODN (4 μg/ml), anti-mouse IgM (10 μg/ml) or αGalCer (50 ng/ml). Supernatants were collected after 5-6 d. IgG anti-DNA Abs were measured by ELISA, as described previously (14). Rheumatoid factor (RF) (anti-$IgG2a^a$) was determined by ELISA, as described previously (20). Total Ig and its isotypes were measured by a standard sandwich ELISA (14) using appropriate Ab pairs (Southern Biotechnology Associates, Birmingham, AL). Cytokines were assayed in culture supernatants by ELISA using appropriate mAb pairs and standards, as described previously (14).

Transwell experiments

$V\alpha14^{Tg}$ BALB/c mice (2–4 mo old, five mice per group) were injected i.v. with vehicle or αGalCer 90 min before harvesting their spleens. T cells were purified from these spleens using T cell enrichment columns (R&D Systems). These purified T cells were cultured in RPMI 1640 medium (Sigma-Aldrich) with 10% FCS (Life Technologies) with B cells that were purified from the spleens of BALB/c mice 6–12 mo after pristane injection (20) using anti-B220 microbeads and autoMACS (Miltenyi Biotec). In Transwell experiments, T cells (1×10^6) in upper wells and the same numbers of B cells in lower wells were cultured using HTS Transwell-24 Plate (Corning, Acton, MA). For the mixed cultures, the same numbers of T cells and B cells were cultured in regular 24-well plates (Corning). B cell stimulants LPS (10 μg/ml) or CpG-ODN (2 μg/ml) were added to cultures, as indicated. Culture supernatants collected after 5–7 d were tested for Abs. Activation marker (CD86) and IL-10 production were assessed in 18-h cultures.

Cytokine secretion assay

Cellular sources of cytokines were identified using MACS cytokine secretion assay kit (Miltenyi Biotec), following the manufacturer's protocol with some modifications (20). Briefly, spleen cells (1×10^6) were incubated at 37°C for 45 min with the cytokine catch reagent that attaches to all leukocytes via CD45 and binds to the specific cytokine that is secreted by the cell. After washing, cells were stained with PE-conjugated cytokine detection Ab, counterstained with anti-B220 or anti-CD19 and anti-TCRβ Abs, and analyzed by FACSCalibur. To enrich IL-10–secreting B cells, T cell-depleted spleen cells were incubated at 37°C for 45 min with the IL-10 catch reagent. After washing, cells were stained with PE-conjugated anti–IL-10 Ab, followed by incubation with anti-PE microbeads. IL-10–secreting cells were then positively selected using AutoMACS (Miltenyi Biotec). More than 90% of such IL-10–enriched cells were B220 positive.

Statistical analysis

Levels of Abs and cytokines, lymphocyte percentage, and numbers were compared using Student *t* or Mann–Whitney *U* test.

Results

iNKT cell ligand αGalCer suppresses autoantibody production in vitro

To determine the effects of iNKT cells on autoreactive B cells, we added αGalCer to spleen cell cultures from 6-mo-old BWF_1 mice that spontaneously produce IgG autoantibodies (Fig. 1*A*). Strikingly, although total IgG levels increased, levels of IgG anti-DNA Ab were significantly reduced in the presence of αGalCer. Inhibition of autoantibody production, but not of total IgG, by αGalCer might reflect a selective suppression of in vivo activated autoreactive B cells in diseased BWF_1 mice. Hence, we investigated the effect of αGalCer on activated (LPS-stimulated) BWF_1 spleen cells. αGalCer still did not reduce total IgG levels, but significantly reduced IgG anti-DNA Ab production in a dose-dependent manner (Fig. 1*B*). Inhibition of autoantibody production by αGalCer is not limited to anti-DNA, as another autoantibody, RF, is also reduced. These data further confirm the suppressive effect of αGalCer on autoreactive B cells.

αGalCer-mediated suppression of autoantibody production is not limited to BWF_1 mice, as addition of αGalCer to spleen cell cultures from IgG2b anti-dsDNA transgenic ($R4A\text{-}\gamma2b^{Tg}$) (23) NZW mice reduced autoantibody levels but increased total IgG levels (Fig. 1*C*). Thus, the suppressive effects of αGalCer on autoreactive B cells must be quite robust.

αGalCer does not "directly" act on autoantibody-producing B cells

αGalCer-induced suppression of autoantibody production in spleen cell cultures (Fig. 1*A–C*) could be due to αGalCer-mediated direct signaling via CD1d on B cells. However, addition of αGalCer to cultures of purified B cells without (Fig. 1*D*) or with LPS (Fig. 1*E*) from BWF_1 mice or from $R4A\text{-}\gamma2b^{Tg}$ mice (Fig.

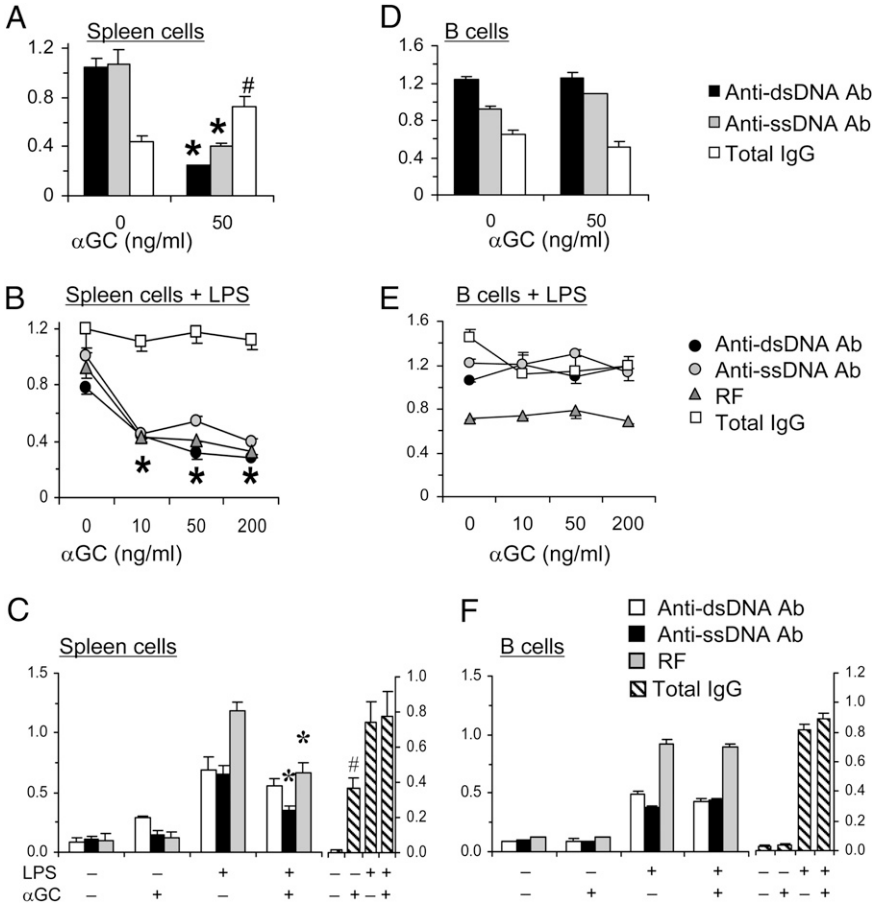

FIGURE 1. αGalCer suppresses production of IgG autoantibodies. Whole spleen cells (*A–C*) or purified B220$^+$ cells (*D–F*) were cultured with αGalCer (*A*, *D*), LPS plus varying concentrations of αGalCer (*B, E*), or LPS with or without 50 ng/ml αGalCer (*C*, *F*). Supernatants collected on day 5 were assayed for autoantibodies or total IgG, which are expressed as the mean ± SD OD or micrograms per milliliter, respectively. Results from 6-mo- (*A*, *D*) or 3-mo-old BWF$_1$ mice (*B*, *E*) or 4-mo-old R4A-γ2b^{Tg} NZW mice (*C*, *F*) are shown. Addition of αGalCer to whole spleen cells, but not to B cells alone, results in reduction in autoantibodies. *$p < 0.05–0.01$; $n = 5$. Total IgG levels, however, are unaffected or increased in these cultures. #$p < 0.01–0.06$. Results are representative of three to five experiments, each using three to seven animals per group.

1*F*) had no effect on autoantibody production. Thus, αGalCer confers suppression of autoantibody production through its effects on non-B cells.

αGalCer-mediated regulation of autoantibody production requires the presence of iNKT cells

Next, we investigated the role of iNKT cells in αGalCer-mediated suppression of autoantibody production. Spleen cells from iNKT cell-deficient Jα18$^{-/-}$ mice (22) or iNKT cell overexpressing Vα14^{Tg} mice (21) were cultured with B cell stimulants LPS or CpG to induce autoantibody production. Addition of αGalCer to these cultures reduced autoantibody production by spleen cells from Vα14^{Tg} mice (Fig. 2*B*) but not by spleen cells from Jα18$^{-/-}$ (Fig. 2*A*) or CD1d$^{-/-}$ mice (data not shown). To determine whether Jα18$^{-/-}$ B cells that have never been exposed to iNKT cells are amenable to suppression by αGalCer-activated T cells,

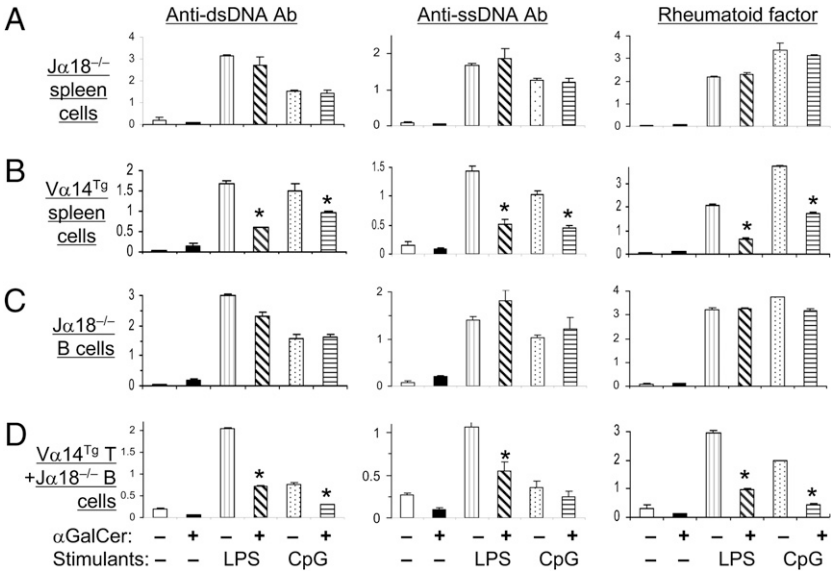

FIGURE 2. iNKT cells regulate IgG autoantibody production. *A* and *B*, Spleen cells from Jα18$^{-/-}$ (*A*) or Vα14^{Tg} (*B*) mice (both 3 mo old) were cultured without or with LPS or CpG in the presence or absence of αGalCer. *C* and *D*, Purified B cells from Jα18$^{-/-}$ mice were cultured alone (*C*) or with T cells from Vα14^{Tg} mice (*D*) in the presence of LPS, CpG, and/or αGalCer. Supernatants collected on day 5 were tested for autoantibodies. Results are shown as the mean ± SD of the mean triplicate OD from three mice per group. *$p < 0.05–0.01$. Results represent four independent experiments.

we cultured Jα18$^{-/-}$ B cells with αGalCer or αGalCer plus Vα14^{Tg} mouse T cells (Fig. 2*C*, 2*D*). Results show that Vα14^{Tg} T cells efficiently suppress Jα18$^{-/-}$ B cells. Thus, suppression of autoantibody production by αGalCer requires the presence of iNKT cells.

In vivo regulation of autoantibody production by iNKT cells in reconstituted Jα18$^{-/-}$ and SCID mice

To more directly test the role of iNKT cells on autoantibody production in vivo, Jα18$^{-/-}$ mice were injected with LPS to stimulate B cells and then reconstituted with T cells from vehicle- or αGalCer-treated Vα14^{Tg} mice or with T cells from control (αGalCer-treated Jα18$^{-/-}$) mice (Supplemental Fig. 1). Anti-DNA Ab and RF production was reduced in Jα18$^{-/-}$ mice that were reconstituted with αGalCer-stimulated Vα14^{Tg} T cells as compared with control mice (Jα18$^{-/-}$ mice that received T cells from αGalCer-treated Jα18$^{-/-}$ mice) (Fig. 3).

Activation of iNKT cells results in *trans* activation of other immune cell types (5, 6, 20). To investigate whether iNKT cell itself or whether *trans*-activated conventional T cells suppress autoreactive B cells in vivo, we conducted an adoptive transfer experiment in SCID mice that lack B and T cells. SCID mice were injected with B cells from Jα18$^{-/-}$ mice and with purified T (TCRβ^{+}) or iNKT (CD1d-αGalCer dimer^{+}TCRβ^{+}) cells from Vα14^{Tg} mice (all BALB/c background) (Supplemental Fig. 2). Spleen cells from the reconstituted SCID mice were then tested for autoantibody production. As shown in Fig. 4*A*, anti-DNA Ab levels are lower in SCID mice reconstituted with Vα14^{Tg} mouse T cells or iNKT cells than in SCID mice reconstituted with Jα18$^{-/-}$ T cells. Spleen cells from the iNKT cell-reconstituted mice continue to produce lower levels of autoantibodies after further in vitro stimulation with LPS or CpG (Fig. 4*B*). These data further confirm that activated iNKT cells alone can suppress autoantibody production in the absence of conventional T cells.

iNKT cells do not cause global suppression of B cells

In contrast to suppressive effects of αGalCer on autoantibody production, addition of αGalCer to whole spleen cell cultures increases the expression of activation markers CD69 and CD86 on B cells in BWF$_1$ or R4A-γ2b^{Tg} mice (Fig. 5*A*, 5*B*). αGalCer also enhances these markers on Jα18$^{-/-}$ B cells when cocultured with Vα14^{Tg} T cells (Fig. 5*C*). αGalCer further upregulates the expression of these markers on spleen cells stimulated with LPS

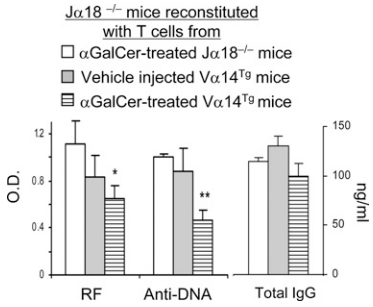

FIGURE 3. Reconstitution of Jα18$^{-/-}$ mice with activated iNKT cells suppresses autoantibody production. Jα18$^{-/-}$ mice were injected with T cells from αGalCer or vehicle-treated Vα14^{Tg} or Jα18$^{-/-}$ mice, as described in *Materials and Methods*. Spleen cells from the reconstituted mice were cultured for 6 d, and supernatants were tested for autoantibodies and total IgG. *$p < 0.05$, **$p < 0.01$; mean ± SE; n = 3/group. Results represent two similar experiments.

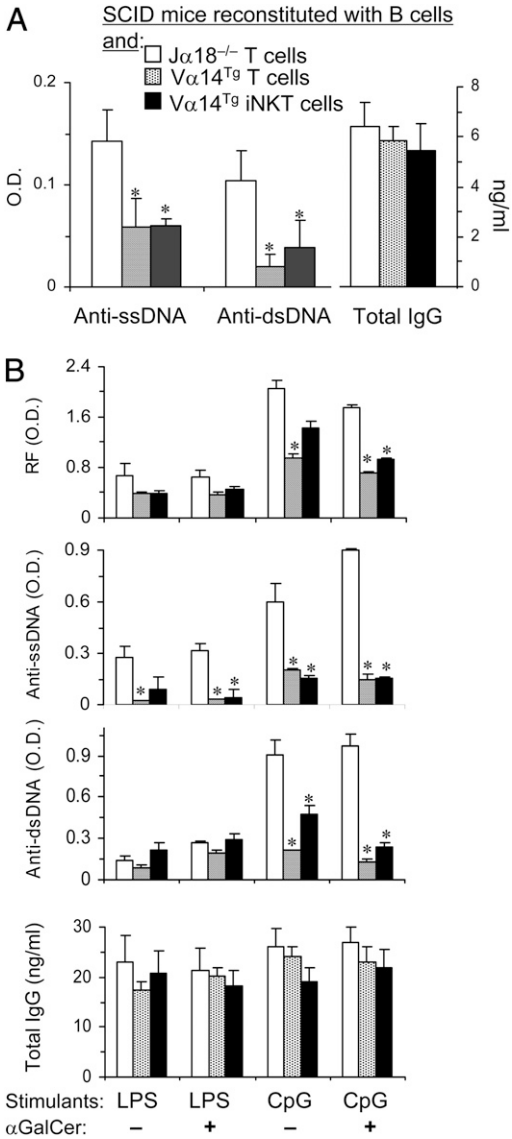

FIGURE 4. iNKT cells suppress autoantibody production in reconstituted SCID mice. BALB/c SCID mice were injected i.p. with LPS and αGalCer and then reconstituted with B cells (Jα18$^{-/-}$ B cells were used to avoid any iNKT cells) and with purified T (*Vα14^{Tg} T cells*) or CD1d-αGalCer dimer^{+} T cells (*Vα14^{Tg} iNKT cells*) from Vα14^{Tg} mice or Jα18$^{-/-}$ spleen cells depleted of B220^{+} cells (*Jα18$^{-/-}$ T cells*), as described in *Materials and Methods*. Spleen cells from the reconstituted animals were cultured without any stimulant (*A*) or with LPS or CPG in the presence or absence of αGalCer (*B*) for 6 d, and supernatants were assayed for autoantibodies and total IgG. *$p < 0.05$–0.01; mean ± SE; n = 3–5 mice/group. Results represent two similar experiments.

(Fig. 5*A–C*) or CpG (Fig. 5*D*). Again, αGalCer-induced upregulation of activation markers requires the presence of T cells in culture, because there is no effect on activation markers when αGalCer is added to pure B cells alone. Thus, αGalCer-activated T cells generally activate B cells, whereas at the same time, they suppress autoantibody production.

iNKT cell-mediated suppression of autoantibody production requires cell–cell contact but increase in B cell activation markers occurs in a contact-independent manner

To begin to understand mechanisms underlying iNKT cell-mediated effects on B cells, we performed a Transwell culture experiment where T cells from Vα14^{Tg} mice were cultured together or sep-

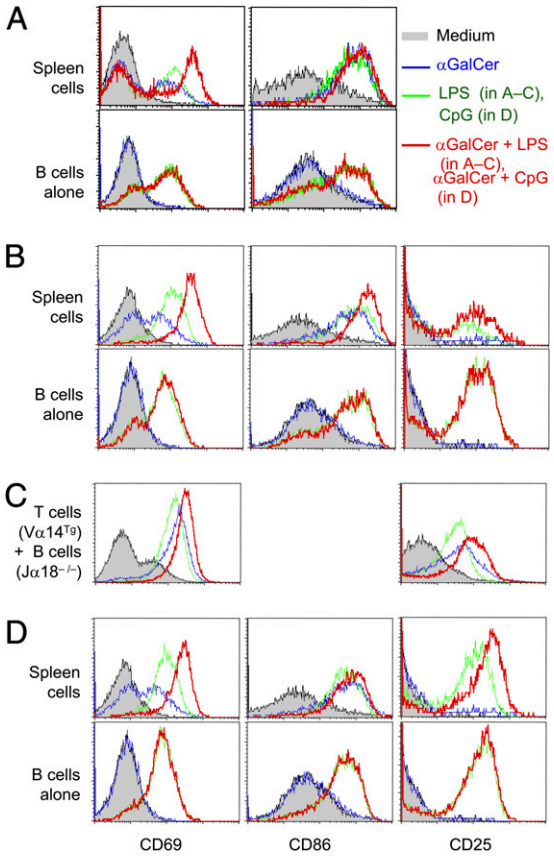

FIGURE 5. Effect of activated iNKT cells on B cell activation markers in autoimmune mice. Whole spleen cells or purified B cells were stimulated overnight with LPS (*A–C*) or CpG (*D*) in the presence or absence of αGalCer. Cells from 5 mo-old BWF1 mice (*A*) or from 4 mo-old R4A anti-DNA transgenic NZW mice (*B*, *D*), and mixed Vα14^{Tg} T cells plus Jα18$^{-/-}$ B cells from 2- to 3-mo-old mice (*C*) were analyzed. Expression of activation markers is shown on gated B220^{+} cells. Results are representative of at least five similar experiments.

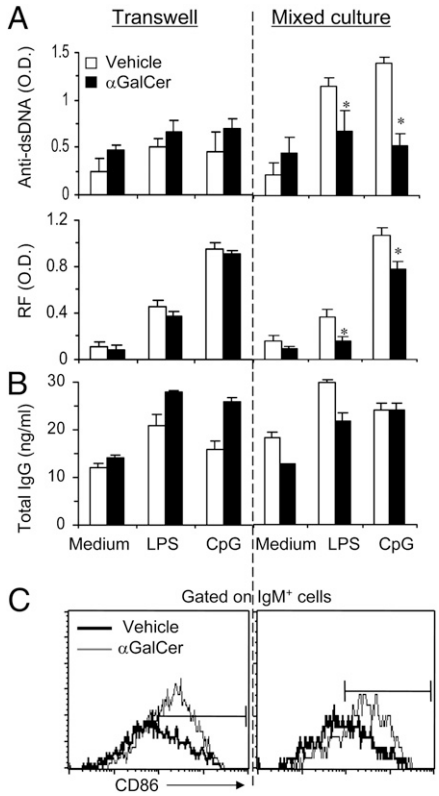

FIGURE 6. Activated iNKT cells suppress autoantibody production in a contact-dependent manner, but upregulate activation markers on B cells in a contact-independent manner. Purified T cells from vehicle- or αGalCer-treated Vα14^{Tg} BALB/c mice were cultured with B cells from BALB/c mice (6 mo post-pristane injection; as a source of autoimmune B cells (20)) separately in Transwells or together in mixed cultures, as described in *Materials and Methods*. Fifth day culture supernatants were tested for autoantibodies (*A*, shown as OD) or total IgG (*B*, shown as nanograms per milliliter). The expression of activation marker (CD86) was assessed on B cells after 18 h in culture (*C*). *$p < 0.05$; anti-DNA and RF levels were reduced in LPS- or CpG-stimulated mixed cultures but not in Transwell cultures. Results from one representative of three experiments are shown as the mean ± SD of mean triplicate values.

arated in Transwells with B cells from pristane-injected mice (Fig. 6). Results show that inhibition of autoantibody production requires direct contact between αGalCer-activated T cells and B cells, whereas increase in the expression of activation markers on B cells occurs both in Transwell and mixed cultures. Thus, different mechanisms (i.e., cognate interaction versus cytokine-dependent effects) mediate diverse influences of iNKT cells on B cells.

iNKT cells reduce IL-10–producing B cells from autoimmune mice

Next, we measured cytokines, IL-2, IL-4, IL-10, IL-13, TGFβ1, and IFN-γ, in supernatants of Vα14^{Tg} T cells and B cells (from pristane-injected BALB/c mice) cultured together or separately in Transwells, as described above in Fig. 6. We found that IL-10 levels were significantly reduced when Vα14^{Tg} T cells and B cells were together in mixed cultures as compared with when the two cell types were separated in Transwells (Fig. 7*A*). Other cytokines were increased or unchanged (data not shown). To determine whether iNKT cells inhibit IL-10 production specifically by B cells, we enumerated IL-10–secreting B cells using the cytokine secretion assay (Fig. 7*B*). Results show that IL-10–secreting B cells are reduced in the mixed cultures, but not in Transwells, when LPS-stimulated B cells from autoimmune pristane-injected mice are cultured with αGalCer-primed T cells compared with when they are cultured with T cells from control (vehicle-injected) mice. Taken together, data in Figs. 6 and 7 show that iNKT cells inhibit the production of autoantibodies and IL-10 by B cells in a contact-dependent manner.

Autoreactive B cells spontaneously produce IL-10 and are selectively inhibited by iNKT cells

The above findings led us to ask whether autoreactive B cells preferentially secrete IL-10. To address this, we used anti-DNA transgenic mice, R4A γ2b, where anti-DNA B cells can be detected using flow cytometry (23, 28). Intracellular staining of freshly isolated spleen cells for IL-10 showed that anti-DNA B cells (IgG2b^{+} cells from R4A mice) expressed more IL-10 than nonanti-DNA B cells (IgG2b^{-} cells from R4A mice or IgG2b^{+} cells from nontransgenic mice) (Fig. 8*A*). In addition, co-culture of R4A spleen cells with iNKT cells along with LPS and αGalCer significantly reduced the proportion and number of anti-DNA (IgG2b^{+}) B cells, without affecting the number of non-autoreactive B cells – R4A mouse IgG2b^{-} cells (Fig. 8*B*) and IgG2b^{+} B cells from nontransgenic mice (data not shown in figure). Thus, anti-DNA B cells that spontaneously produce IL-10 are selectively inhibited by iNKT cells, whereas nonautoreactive B cells are not reduced in presence of iNKT cells.

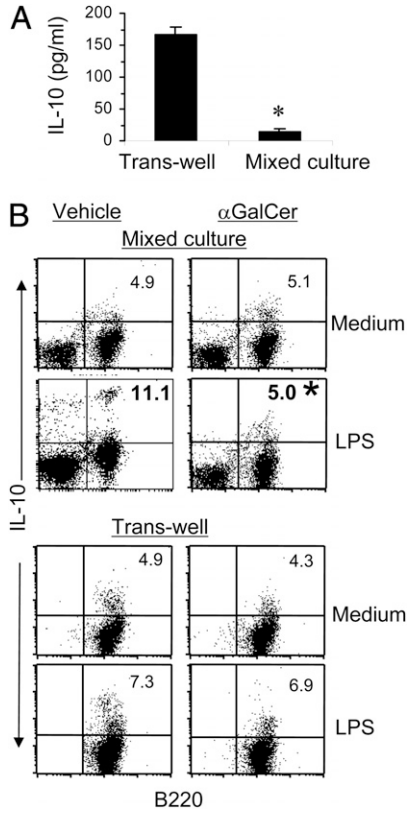

FIGURE 7. Activated iNKT cells suppress IL-10–secreting autoimmune B cells. *A*, T cells from αGalCer-treated Vα14^{Tg} mice and B cells from pristane-injected mice were cultured separately in Transwells or together in mixed cultures. Supernatants collected at 18 h were assayed by ELISA for IL-10 (mean ± SD of mean triplicate values). *B*, B cells from pristane-injected mice and T cells from vehicle- or αGalCer-treated Vα14^{Tg} mice were cultured separately in Transwells or together in mixed cultures, as described in Fig. 6. B cells from Transwell cultures and all cells from mixed cultures were collected and analyzed for IL-10 production using the cytokine secretion assay. IL-10–secreting cells are shown as the percent of B cells. Results are representative of three independent experiments, each using cells pooled from three to four mice per group. *$p < 0.05$.

Autoreactive and nonautoreactive B cells differ in terms of in vivo activation status and response to in vitro stimuli

The above data showing IL-10 production by freshly isolated anti-DNA B cells, but not by nonautoreactive B cells, suggest that autoreactive B cells might be activated in vivo. Indeed, CD86 expression was higher on R4A IgG2b$^+$ cells than on R4A IgG2b$^-$ cells or nontransgenic IgG2b$^+$ cells in freshly isolated spleen cells (Fig. 8*A*). Intriguingly, however, the addition of αGalCer or LPS to R4A spleen cells did not further increase CD86 expression on anti-DNA B cells, whereas both stimuli markedly increased CD86 expression on nonautoreactive B cells (Fig. 8*C*). Autoreactive B cells, however, are not completely unresponsive, as the addition of LPS$^+$ αGalCer and purified iNKT cells to R4A mouse B cells at 1:2 ratio can increase CD86 expression on both IgG2b$^+$ and IgG2b$^-$ cells (data not shown). Thus, iNKT cell stimulation reduces the numbers of autoreactive B cells and does not activate them further, whereas it markedly increases the numbers of activated nonautoreactive B cells.

Autoreactive B cells express more CD1d than nonautoreactive B cells

We have thus far shown that iNKT cells reduce autoantibody production in a contact-dependent manner, and they seem to differentially interact with autoreactive versus nonautoreactive B cells. Because iNKT cells recognize CD1d (1–3), we reasoned that differences in CD1d expression between autoreactive and nonautoreactive B cells might explain their differential regulation. Indeed, CD1d expression was higher on anti-DNA B cells than on nonautoreactive B cells (Fig. 8*A*). Addition of αGalCer to R4A spleen cells or coculture of R4A B cells with iNKT cells isolated from αGalCer-treated mice further increased CD1d expression on anti-DNA B cells but had no effect on CD1d expression on nonautoreactive B cells (data not shown). LPS, however, had little effect on CD1d expression on both autoreactive and nonautoreactive B cells.

CD1d deficiency increases anti–self-Ab responses but does not affect Ab response to a foreign Ag peptide

If CD1d expression on autoreactive B cells plays a regulatory role in autoantibody production, CD1d deficiency might increase autoantibody production. In fact, we have reported that CD1d$^{-/-}$ BWF$_1$ mice have increased levels of circulating IgG anti-DNA Abs and experience more severe lupus compared with their wild-type (WT) littermates (19). In this study, we show that production of RF by spleen cells was higher in CD1d$^{-/-}$ BWF$_1$ mice than in their WT littermates (Fig. 9*A*). Total IgG levels in these cultures, however, were not different between CD1d$^{-/-}$ and CD1d$^{+/+}$ BWF$_1$ mice (Fig. 9*A*).

To further examine whether CD1d deficiency selectively increases autoimmune responses while leaving normal IgG responses intact, young CD1d$^{-/-}$ BWF$_1$ mice and their WT littermates were immunized with self (p31)- or foreign (p106) peptides that are known to induce strong immune responses in BWF$_1$ mice (24, 26). Immunized animals were monitored for serum anti-peptide Abs (Fig. 9*B*). Interestingly, whereas CD1d$^{-/-}$ and CD1d$^{+/+}$ mice mounted equivalent responses to the foreign peptide, the Ab response against the self-peptide was higher in CD1d$^{-/-}$ mice than in WT littermates. Thus, in vivo autoreactive B cell responses are heightened in the absence of CD1d in autoimmune-prone conditions.

iNKT cells inhibit autoantibody production in CD1d-dependent manner, but total IgG production is unaffected by CD1d expression on B cells

To directly test the role of CD1d on iNKT cell-mediated inhibition of autoantibody production, we cocultured CD1d$^{-/-}$ or WT B cells without or with iNKT cells (T cells from CD1d$^{-/-}$ or Vα14^{Tg} mice, respectively). As shown in Fig. 10, the addition of activated iNKT cells significantly reduced the production of IgG anti-DNA autoantibodies by WT B cells but had no effect on anti-DNA Ab production by CD1d-deficient B cells. As shown above, iNKT cells did not reduce total IgG levels in cocultures with CD1d$^{-/-}$ or WT B cells. Thus, inhibition of autoantibody production by iNKT cells requires CD1d expression on B cells.

Discussion

In this study, we report that iNKT cell ligand αGalCer suppresses IgG autoantibody production in vitro and in vivo. In contrast, αGalCer exposure does not reduce or, in some instances, increases total polyclonal IgG production, and upregulates activation markers on B cells. These αGalCer-induced effects on B cells are mediated by iNKT cells, because: 1) addition of αGalCer to purified B cells alone has no effect on IgG production or activation markers; 2) addition of αGalCer to spleen cells from Vα14^{Tg} mice, but not those from Jα18$^{-/-}$ mice, suppresses autoantibody production; 3) αGalCer-activated T cells suppress autoantibody production by B cells from Jα18$^{-/-}$ mice in vitro as well as

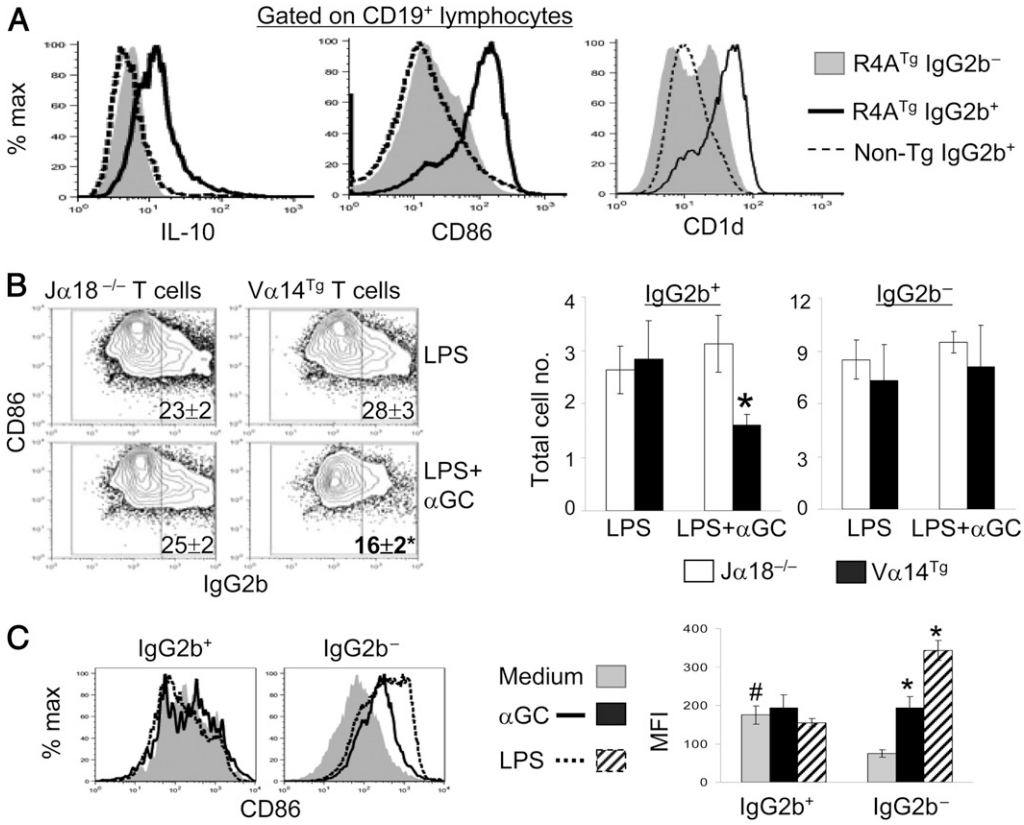

FIGURE 8. Differences between autoreactive and nonautoreactive B cells in cytokine production, surface markers, and response to in vitro stimuli. *A*, Analysis of freshly isolated spleen cells from anti-DNA^{Tg} mice. Freshly isolated spleen cells from R4A $\gamma 2b^{Tg}$ or WT control mice were stained for CD19, CD86, and CD1d and intracellular IgG2b and IL-10. Histograms show IL-10, CD86, and CD1d expression on gated CD19$^+$IgG2b$^+$ or CD19$^+$IgG2b$^-$ cells. Anti-DNA B cells (R4A IgG2b$^+$) expressed more IL-10, CD86, and CD1d than nonautoreactive B cells (R4A IgG2b$^-$ and normal mouse IgG2b$^+$ cells) [mean fluorescence intensity (MFI)]. $p < 0.05$–0.001). *B*, Effect of iNKT cells on anti-DNA B cells. R4A spleen cells were cocultured without or with iNKT cells (sorted T cells from J$\alpha 18^{-/-}$ or V$\alpha 14^{Tg}$ mice, respectively) with LPS or LPS+αGalCer for 3 d. Cells were analyzed for the expression of CD86 and intracellular IgG2b on gated CD19$^+$ B cells. Numbers on dot plots indicate the percentages of IgG2b$^+$ cells. Total numbers of IgG2b$^+$ and IgG2b$^-$ cells are shown in bar diagrams. *$p < 0.05$–0.005 compared with various controls. *C*, Response of anti-DNA B cells to in vitro stimuli. R4A spleen cells were cultured with medium alone, αGalCer, or LPS for 3 d. Lymphocytes were analyzed for CD86 expression on gated CD19$^+$IgG2b$^+$ and CD19$^+$IgG2b$^-$ B cells. CD86 expression was higher on anti-DNA (IgG2b$^+$) than on nonautoreactive (IgG2b$^-$) B cells in unstimulated cultures. #$p < 0.00002$. In vitro stimulation with αGalCer or LPS did not significantly increase CD86 expression on anti-DNA B cells but significantly increased CD86 expression on nonautoreactive B cells. *$p < 0.00001$; mean ± SE of mean triplicates; $n = 3$ mice each group. Results are representative of two independent experiments. αGC, αGalCer.

in vivo upon adoptive transfer; and 4) transfer of purified iNKT cells into SCID mice reconstituted with B cells from J$\alpha 18^{-/-}$ mice suppresses autoantibody production. Using anti-DNA^{Tg} mice, we show that iNKT cells reduce the numbers of autoreactive B cells but activate nonautoreactive B cells. Consistently, CD1d deficiency enhances IgG autoantibody and anti–self-peptide responses but leaves total IgG and antiforeign peptide IgG responses unaffected. Finally, iNKT cells reduce autoantibody production in a contact-

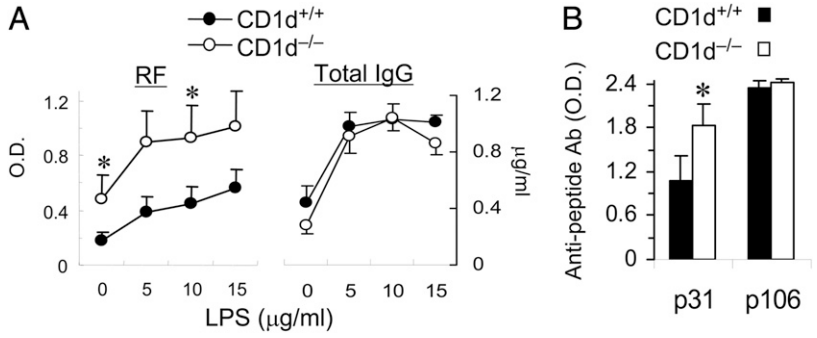

FIGURE 9. Effect of CD1d deficiency on self versus nonself-Ab responses in autoimmune-prone mice. *A*, Spleen cells from CD1d$^{-/-}$ BWF$_1$ mice or control littermates (10 mo old) were cultured with or without LPS. Supernatants collected on day 5 were assayed for autoantibodies and total IgG. *B*, CD1d$^{-/-}$ BWF$_1$ mice and control littermates (10 wk old) were immunized with a self (p31) or a foreign (p106) peptide, in CFA and challenged with the peptide in IFA, as described in *Materials and Methods*. Sera collected on day 14 postchallenge were assayed for IgG anti-peptide Abs. Results are shown as the mean ± SD of the mean triplicate OD from six mice per group. *$p < 0.05$. Results are from one representative of two independent experiments.

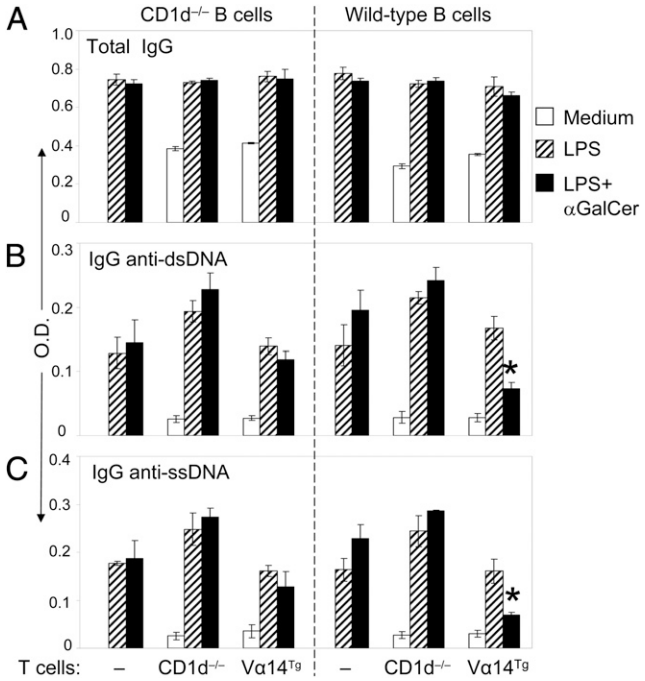

FIGURE 10. iNKT cells inhibit autoantibody production in a CD1d-dependent manner. Purified B cells from 5-mo-old CD1d$^{-/-}$ or WT mice were cocultured with purified T cells from CD1d$^{-/-}$ or Vα14^{Tg} mice (all BALB/c) in the presence of medium alone, LPS, or LPS+αGalCer for 6 d (B cells alone were not cultured with medium alone). Supernatants were assayed for total IgG (*A*) and IgG anti-dsDNA and anti-ssDNA autoantibodies (*B*, *C*). Results are expressed as the mean ± SE of mean triplicate OD. *$p < 0.01$–0.0001, anti-dsDNA; $p < 0.05$–0.001, anti-ssDNA.

and CD1d-dependent manner. These data strongly indicate a regulatory role of CD1d and CD1d-reactive iNKT cells on autoantibody production.

Several studies have shown that iNKT cells can potentiate Ab responses (10–12). For example, human iNKT cells stimulate Ab production by B cells in the presence of αGalCer (11). CD1d/iNKT cell dependence is also reported in contact allergen-induced IgM production by murine B cells (29). However, others have found Ab production to be independent of iNKT cells (30). In this study, we show that whereas CD1d-deficiency does not impair the production of total polyclonal IgG in vitro or IgG Ab against a foreign peptide in vivo, αGalCer-stimulated iNKT cells enhance total IgG levels in cultures. Addition of an anti–CD3-stimulated iNKT cell line to BWF$_1$ B cells also increases total IgG levels (31). In addition, cocultures of anti–CD3-stimulated iNKT cells and B cells increases the levels of total IgM and IgM anti-DNA Abs but not IgG anti-DNA Abs (31, 32). Such an increase in IgM Abs is not blocked by an anti-CD1d mAb (32), suggesting that direct CD1d-dependent interactions between iNKT cells and B cells do not mediate such iNKT cell-induced B cell stimulatory effects. Indeed, our data show that activated iNKT cells increase total IgG levels and B cell activation markers even when the two cells are separated in Transwell cultures. Collectively, iNKT cells seem to increase total polyclonal IgM and IgG levels and B cell activation markers via soluble factor(s), such as IL-4 (8).

In contrast to the above B cell stimulatory effects, activated iNKT cells suppress IgG anti-DNA Ab and RF production (Figs. 1–4, 6, 10) when the iNKT cells and B cells are cultured together (Fig. 6). Our data in this study show suppressive effects of iNKT cells on spontaneous autoantibody production in old BWF$_1$ mice as well as on induced autoantibody responses in young BWF$_1$, anti-DNA^{Tg} NZW, pristane-injected BALB/c, Vα14^{Tg} BALB/c, and Jα18$^{-/-}$ BALB/c mice in vitro or in reconstituted Jα18$^{-/-}$ and SCID mice in vivo. In resonance with these findings, CD1d$^{-/-}$ BWF$_1$ mice exhibit increased autoantibody levels (19) and mount a strong Ab response to a self-peptide, p31 (Fig. 9), that has been shown to promote anti-DNA Ab production (24). Consistently, in a model where injections of syngeneic apoptotic cells transiently trigger autoantibody production (33), the absence or reduction of iNKT cells as well as absence of CD1d expression on B cells leads to increased autoreactive B cell activation but does not affect the activation of B cells reactive to 4-hydroxy-3-nitrophenyl-OVA (33). The regulatory effects of CD1d-reactive iNKT cells on autoreactive B cells must be important in the development of lupus, as BWF$_1$ and BALB/c mice rendered deficient in CD1d experience more severe spontaneous or induced lupus, respectively, than their WT littermates (19, 20).

Our results show that addition of αGalCer to purified B cells alone has no direct effects on IgG production (Figs. 1*D–F*, 2*C*), activation markers on B cells (Fig. 5), and B cell proliferation (J. Yang and R.R. Singh, unpublished observations). Thus, αGalCer-mediated B cell effects must be conducted by iNKT cells themselves or by another cell type enlisted by iNKT cells. That addition of Vα14^{Tg} T cells to purified B cells can modulate B cell functions (Figs. 2*D*, 5*C*) suggests that iNKT cells or *trans*-activated conventional T cells can mediate αGalCer effects on B cells and that NK or other cells are not required in this process. Furthermore, ability of iNKT cells alone to confer autoantibody suppression upon transfer into reconstituted SCID mice (Fig. 4) suggests that the presence of conventional T cells is also not required for iNKT cell-mediated suppression of autoantibody production. Thus, iNKT cells can directly suppress autoreactive B cells. These results, however, do not exclude the synergistic or additive roles of *trans*-activated NK, T or dendritic cells in conferring protection from autoimmune disease in vivo.

We have explored several mechanisms whereby iNKT cells might regulate autoreactive B cells. First, we found a significant reduction in IL-10–producing B cells in presence of iNKT cells (Fig. 7). This finding is important, because autoreactive B cells from patients with SLE produce increased amounts of IL-10 (18), and autoreactive B cells in anti-DNA^{Tg} mice spontaneously produce more IL-10 than nonautoreactive B cells (Fig. 8*A*). These anti-DNA B cells are selectively reduced in the presence of iNKT cells (Fig. 8*B*). In vivo neutralization of IL-10 has been shown to reduce autoantibody production in reconstituted hu-SCID mice (18) and to suppress lupus nephritis in BWF$_1$ mice (34). A pilot trial of an anti–IL-10 mAb also showed reduced disease activity in SLE patients (35). Thus, ability of iNKT cells to inhibit IL-10–producing B cells may underlie their capacity to suppress autoantibody production.

iNKT cells recognize CD1d-bound lipid Ags (2, 4). Increased expression of CD1d on autoreactive B cells compared with nonautoreactive B cells (Fig. 8*A*) suggests a mechanism whereby iNKT cells might discriminate between autoreactive versus nonautoreactive B cells. In fact, iNKT cell-mediated inhibition of autoantibody production requires CD1d expression on B cells (Fig. 10). Suppression of autoantibodies by iNKT cells also requires contact between iNKT and B cells (Fig. 6). These data suggest that iNKT cells directly interact with autoreactive B cells via high levels of CD1d on their surface. In previous studies, CD1d on B cells has been suggested to promote iNKT cell-dependent tolerance (36) and protection from inflammation (37) by facilitating iNKT–B cell interactions. Ongoing studies will determine whether such interaction might further activate and cause activation-induced cell death of autoreactive B cells that are

already activated in vivo (Fig. 8*A*). In contrast, iNKT cells induce a marked activation of naive nonautoreactive B cells (Fig. 8*C*) and elicit a prompt polyclonal response (10, 31, 38).

In summary, we report that iNKT cells inhibit IL-10–secreting, $CD1d^+$ autoreactive B cells in a contact- and CD1d-dependent manner, whereas they activate nonautoreactive B cells via cytokines. Further elucidation of mechanisms underlying discriminate regulation of autoreactive versus normal B cells by iNKT cells will help pave the way for iNKT cell-based therapies for human autoantibody-associated diseases, such as SLE, where reduced numbers of iNKT cells is considered to be a primary defect (39). The iNKT cell-based therapy is particularly appealing, given the limited polymorphism in *CD1* genes (2), which obviates one of the major hurdles of therapies aimed at MHC class I and II system. Enhancing this appeal is our finding that iNKT cells suppress harmful autoreactivity without causing global suppression of B cells.

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Disclosure

The authors have no financial conflicts of interest.

References

1. Bendelac, A., M. N. Rivera, S. H. Park, and J. H. Roark. 1997. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 15: 535–562.
2. Porcelli, S. A., and R. L. Modlin. 1999. The CD1 system: antigen-presenting molecules for T cell recognition of lipids and glycolipids. *Annu. Rev. Immunol.* 17: 297–329.
3. Godfrey, D. I., H. R. MacDonald, M. Kronenberg, M. J. Smyth, and L. Van Kaer. 2004. NKT cells: what's in a name? *Nat. Rev. Immunol.* 4: 231–237.
4. Kawano, T., J. Cui, Y. Koezuka, I. Toura, Y. Kaneko, K. Motoki, H. Ueno, R. Nakagawa, H. Sato, E. Kondo, et al. 1997. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 278: 1626–1629.
5. Carnaud, C., D. Lee, O. Donnars, S. H. Park, A. Beavis, Y. Koezuka, and A. Bendelac. 1999. Cutting edge: cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J. Immunol.* 163: 4647–4650.
6. Singh, N., S. Hong, D. C. Scherer, I. Serizawa, N. Burdin, M. Kronenberg, Y. Koezuka, and L. Van Kaer. 1999. Cutting edge: activation of NKT cells by CD1d and α-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 163: 2373–2377.
7. Fujii, S., K. Shimizu, C. Smith, L. Bonifaz, and R. M. Steinman. 2003. Activation of natural killer T cells by α-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J. Exp. Med.* 198: 267–279.
8. Kitamura, H., A. Ohta, M. Sekimoto, M. Sato, K. Iwakabe, M. Nakui, T. Yahata, H. Meng, T. Koda, S. Nishimura, et al. 2000. α-Galactosylceramide induces early B-cell activation through IL-4 production by NKT cells. *Cell. Immunol.* 199: 37–42.
9. Kronenberg, M. 2005. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 23: 877–900.
10. Lang, G. A., M. A. Exley, and M. L. Lang. 2006. The CD1d-binding glycolipid α-galactosylceramide enhances humoral immunity to T-dependent and T-independent antigen in a CD1d-dependent manner. *Immunology* 119: 116–125.
11. Galli, G., S. Nuti, S. Tavarini, L. Galli-Stampino, C. De Lalla, G. Casorati, P. Dellabona, and S. Abrignani. 2003. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J. Exp. Med.* 197: 1051–1057.
12. Schofield, L., M. J. McConville, D. Hansen, A. S. Campbell, B. Fraser-Reid, M. J. Grusby, and S. D. Tachado. 1999. CD1d-restricted immunoglobulin G formation to GPI-anchored antigens mediated by NKT cells. *Science* 283: 225–229.
13. Eisenberg, R., and D. Albert. 2006. B-cell targeted therapies in rheumatoid arthritis and systemic lupus erythematosus. *Nat. Clin. Pract. Rheumatol.* 2: 20–27.
14. Fan, G. C., and R. R. Singh. 2002. Vaccination with minigenes encoding V(H)-derived major histocompatibility complex class I-binding epitopes activates cytotoxic T cells that ablate autoantibody-producing B cells and inhibit lupus. *J. Exp. Med.* 196: 731–741.
15. Hahn, B. H., and R. R. Singh. 2007. Animal models of systemic lupus erythematosus. In *Dubois' Lupus Erythematosus*, 7th Ed., D. J. Wallace, and B. H. Hahn, eds. Lippincott, Williams and Wilkins, Philadelphia, p. 299–355.
16. Chan, O. T., M. P. Madaio, and M. J. Shlomchik. 1999. B cells are required for lupus nephritis in the polygenic, Fas-intact MRL model of systemic autoimmunity. *J. Immunol.* 163: 3592–3596.
17. Shlomchik, M. J., J. E. Craft, and M. J. Mamula. 2001. From T to B and back again: positive feedback in systemic autoimmune disease. *Nat. Rev. Immunol.* 1: 147–153.
18. Llorente, L., W. Zou, Y. Levy, Y. Richaud-Patin, J. Wijdenes, J. Alcocer-Varela, B. Morel-Fourrier, J. C. Brouet, D. Alarcon-Segovia, P. Galanaud, and D. Emilie. 1995. Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus. *J. Exp. Med.* 181: 839–844.
19. Yang, J. Q., X. Wen, H. Liu, G. Folayan, X. Dong, M. Zhou, L. Van Kaer, and R. R. Singh. 2007. Examining the role of CD1d and natural killer T cells in the development of nephritis in a genetically susceptible lupus model. *Arthritis Rheum.* 56: 1219–1233.
20. Yang, J. Q., A. K. Singh, M. T. Wilson, M. Satoh, A. K. Stanic, J. J. Park, S. Hong, S. D. Gadola, A. Mizutani, S. R. Kakumanu, et al. 2003. Immunoregulatory role of CD1d in the hydrocarbon oil-induced model of lupus nephritis. *J. Immunol.* 171: 2142–2153.
21. Bendelac, A., R. D. Hunziker, and O. Lantz. 1996. Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1 T cells. *J. Exp. Med.* 184: 1285–1293.
22. Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno, and M. Taniguchi. 1997. Requirement for Vα14 NKT cells in IL-12–mediated rejection of tumors. *Science* 278: 1623–1626.
23. Grimaldi, C. M., D. J. Michael, and B. Diamond. 2001. Cutting edge: expansion and activation of a population of autoreactive marginal zone B cells in a model of estrogen-induced lupus. *J. Immunol.* 167: 1886–1890.
24. Singh, R. R., B. H. Hahn, B. P. Tsao, and F. M. Ebling. 1998. Evidence for multiple mechanisms of polyclonal T cell activation in murine lupus. *J. Clin. Invest.* 102: 1841–1849.
25. Singh, R. R., V. Kumar, F. M. Ebling, S. Southwood, A. Sette, E. E. Sercarz, and B. H. Hahn. 1995. T cell determinants from autoantibodies to DNA can up-regulate autoimmunity in murine systemic lupus erythematosus. *J. Exp. Med.* 181: 2017–2027.
26. Singh, R. R., B. H. Hahn, and E. E. Sercarz. 1996. Neonatal peptide exposure can prime T cells and, upon subsequent immunization, induce their immune deviation: implications for antibody vs. T cell-mediated autoimmunity. *J. Exp. Med.* 183: 1613–1621.
27. Yang, J. Q., V. Saxena, H. Xu, L. Van Kaer, C. R. Wang, and R. R. Singh. 2003. Repeated α-galactosylceramide administration results in expansion of NKT cells and alleviates inflammatory dermatitis in MRL-lpr/lpr mice. *J. Immunol.* 171: 4439–4446.
28. Kawabata, D., J. Venkatesh, M. Ramanujam, A. Davidson, C. M. Grimaldi, and B. Diamond. 2010. Enhanced selection of high affinity DNA-reactive B cells following cyclophosphamide treatment in mice. *PLoS One* 5: e8418.
29. Campos, R. A., M. Szczepanik, A. Itakura, M. Akahira-Azuma, S. Sidobre, M. Kronenberg, and P. W. Askenase. 2003. Cutaneous immunization rapidly activates liver invariant Vα14 NKT cells stimulating B-1 B cells to initiate T cell recruitment for elicitation of contact sensitivity. *J. Exp. Med.* 198: 1785–1796.
30. Molano, A., S. H. Park, Y. H. Chiu, S. Nosseir, A. Bendelac, and M. Tsuji. 2000. Cutting edge: the IgG response to the circumsporozoite protein is MHC class II-dependent and CD1d-independent: exploring the role of GPIs in NKT cell activation and antimalarial responses. *J. Immunol.* 164: 5005–5009.
31. Forestier, C., A. Molano, J. S. Im, Y. Dutronc, B. Diamond, A. Davidson, P. A. Illarionov, G. S. Besra, and S. A. Porcelli. 2005. Expansion and hyperactivity of CD1d-restricted NKT cells during the progression of systemic lupus erythematosus in (New Zealand Black × New Zealand White)F_1 mice. *J. Immunol.* 175: 763–770.
32. Zeng, D., Y. Liu, S. Sidobre, M. Kronenberg, and S. Strober. 2003. Activation of natural killer T cells in NZB/W mice induces Th1-type immune responses exacerbating lupus. *J. Clin. Invest.* 112: 1211–1222.
33. Wermeling, F., S. M. Lind, E. D. Jordö, S. L. Cardell, and M. C. Karlsson. 2010. Invariant NKT cells limit activation of autoreactive CD1d-positive B cells. *J. Exp. Med.* 207: 943–952.
34. Ishida, H., T. Muchamuel, S. Sakaguchi, S. Andrade, S. Menon, and M. Howard. 1994. Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F_1 mice. *J. Exp. Med.* 179: 305–310.
35. Llorente, L., Y. Richaud-Patin, C. García-Padilla, E. Claret, J. Jakez-Ocampo, M. H. Cardiel, J. Alcocer-Varela, L. Grangeot-Keros, D. Alarcón-Segovia, J. Wijdenes, et al. 2000. Clinical and biologic effects of anti-interleukin-10 monoclonal antibody administration in systemic lupus erythematosus. *Arthritis Rheum.* 43: 1790–1800.
36. Sonoda, K. H., and J. Stein-Streilein. 2002. CD1d on antigen-transporting APC and splenic marginal zone B cells promotes NKT cell-dependent tolerance. *Eur. J. Immunol.* 32: 848–857.
37. Wei, B., P. Velazquez, O. Turovskaya, K. Spricher, R. Aranda, M. Kronenberg, L. Birnbaumer, and J. Braun. 2005. Mesenteric B cells centrally inhibit $CD4^+$ T cell colitis through interaction with regulatory T cell subsets. *Proc. Natl. Acad. Sci. USA* 102: 2010–2015.
38. Zeng, D., M. K. Lee, J. Tung, A. Brendolan, and S. Strober. 2000. Cutting edge: a role for CD1 in the pathogenesis of lupus in NZB/NZW mice. *J. Immunol.* 164: 5000–5004.
39. Wither, J., Y. C. Cai, S. Lim, T. McKenzie, N. Roslin, J. O. Claudio, G. S. Cooper, T. J. Hudson, A. D. Paterson, C. M. Greenwood, et al. 2008. Reduced proportions of natural killer T cells are present in the relatives of lupus patients and are associated with autoimmunity. *Arthritis Res. Ther.* 10: R108.

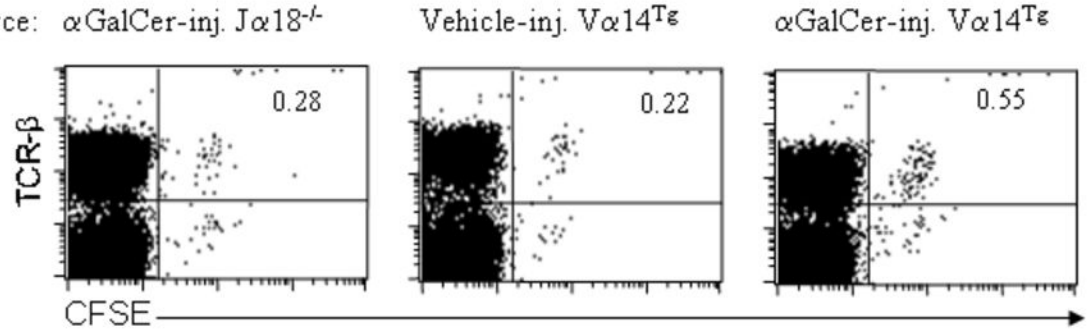
Supplemental information

Invariant Natural Killer T Cells Inhibit Autoreactive B Cells in a Contact- and CD1d-dependent Manner

Jun-Qi Yang, Xiangshu Wen, Peter J. Kim, Ram Raj Singh

Supplemental Figure S1. Reconstitution of BALB/c Jα18$^{-/-}$ mice with iNKT cells

Jα18$^{-/-}$ mice were injected i.v. with CFSE-labeled 5 x 10^6 purified T cells from αGalCer-injected Jα18$^{-/-}$ mice (left panel), vehicle-injected Vα14^{Tg} mice (middle panel) or αGalCer-injected Vα14^{Tg} (right panel) mice, all BALB/c strain. Seven days later, their peripheral blood leukocytes were assessed for the presence of CFSE$^+$ cells. While animals in all groups had reduced CFSE expression, there was more than two-fold increase in CFSE$^+$ cells in animals transferred with T cells from αGalCer-injected Va14Tg mice, suggesting an expansion of activated iNKT cells in these mice.



Supplemental Figure S2. Reconstitution of SCID mice with iNKT cells

BALB/c SCID mice were injected i.p. with LPS and αGalCer, followed by i.v. transfer of B cells isolated from Jα18$^{-/-}$ mice (WT mouse B cells were not used to avoid any contamination with or prior exposure to iNKT cells). Animals were then adoptively transferred i.v. with Jα18$^{-/-}$ T cells (spleen cells depleted of B220$^+$ cells), purified T cells from Vα14^{Tg} mice or purified CD1d-αGalCer dimer$^+$ TCRβ$^+$ cells from Vα14^{Tg} mice. Four days later, SCID mice were euthanized and their spleen cells stained for TCR-β and CD1d-αGalCer dimer. SCID mice reconstituted with purified dimer$^+$ T cells had a more efficient reconstitution with iNKT cells than mice reconstituted with whole T cells from Vα14 mice.