

# Natural killer T cells and innate immune B cells from lupus-prone NZB/W mice interact to generate IgM and IgG autoantibodies

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Lupus-prone NZB/W F1 mice develop glomerulonephritis after T helper cell-dependent isotype switching of autoantibody secretion from IgM to IgG at about 6 months of age. We compared innate immune natural killer (NK) T cells and conventional T cells for their capacity to help spontaneous *in vitro* immunoglobulin and autoantibody secretion of innate immune (B-1 and marginal zone) and conventional (follicular) B cell subsets from NZB/W F1 mice. We found that purified NKT cells not only increased spontaneous secretion of IgM and IgM anti-double-stranded (ds)DNA antibodies by B-1 and marginal zone B cells, but also facilitated secretion of IgG anti-dsDNA antibodies predominantly by B-1 B cells. Few IgM or IgG anti-dsDNA antibodies were secreted by follicular B cells, and conventional T cells failed to provide potent helper activity to any B cell subset. All combinations of T and B cell subsets from normal C57BL/6 mice failed to generate vigorous IgM and IgG secretion. NZB/W NKT cell helper activity was blocked by anti-CD1 and anti-CD40L mAb. In conclusion, direct interactions between innate immune T and B cells form a pathway for the development of IgM and IgG lupus autoantibody secretion in NZB/W mice.

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## Introduction

Systemic lupus erythematosus is an autoantibody-mediated disease that causes injury to multiple organ systems [1, 2]. In the NZB/W F1 hybrid mouse model of lupus, spontaneous secretion of IgM and IgM anti-double-stranded (ds)DNA antibodies by B cells is observed in young mice, and isotype switching to IgG, especially pathogenic IgG2a autoantibodies, is associated with the onset of glomerulonephritis and proteinuria [3–5].

In NZB/W mice, the innate immune B-1 and marginal zone (MZ) B cells have been reported to

spontaneously secrete IgM and IgM autoantibodies *in vitro*, and conventional follicular (FO) B cells fail to spontaneously secrete these antibodies [6, 7]. In addition, the B-1 and MZ B cells are expanded in the spleen of NZB/W mice as they age, and their percentages are increased among all B cells as compared to non-autoimmune mice at the expense of the FO B cells [8, 9]. Absolute numbers of NKT cells in multiple organs and B-1 B cells in the peritoneum are also increased in NZB/W mice as compared to non-autoimmune strains [8, 10, 11]. In contrast, B-1 B cells are not expanded in lupus-prone MRL/*lpr* mice that fail to express the Fas receptor [8]. Initial reports that NKT cells were reduced in lupus-prone mouse strains including NZB/W mice as judged by PCR analysis of *V $\alpha$ 14* mRNA [12] were contradicted by subsequent reports that enumerated NKT cells by staining with NK1.1 markers and CD1d tetramers [10, 11, 13].

Since NKT cells can interact with B cells *via* the recognition of B cell surface CD1d by the invariant *V $\alpha$ 14* TCR, this interaction may contribute to helper activity for NZB/W B cells to secrete immunoglobulins and

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**Abbreviations:**  $\alpha$ -GalCer:  $\alpha$ -galactosylceramide · ds: double stranded · FO: follicular · MZ: marginal zone

autoantibodies [7, 10, 13–17]. Transfer of transgenic T cells that recognize CD1d receptors can induce lupus in BALB/c nu/nu mice, and treatment of lupus-prone NZB/W mice with anti-CD1d mAb ameliorated lupus disease activity [7, 13, 18]. The ability of freshly isolated CD4<sup>+</sup> T cells from NZB/W mice to help spontaneous IgM and IgM anti-dsDNA antibody secretion of NZB/W B cells *in vitro* was lost when NKT cells were depleted, even though the latter cells accounted for about 5% of the CD4<sup>+</sup> T cells [13]. On the other hand, NKT cells purified with a CD1d tetramer reagent provided potent T cell helper activity for the IgM and IgM anti-dsDNA secretion [13]. However, no helper activity was detected for isotype switching to total IgG1, total IgG2a or IgG anti-dsDNA antibody secretion in these 5-day cultures [13].

Switching to IgG isotypes is critical for development of glomerulonephritis [4, 5], and IgG2a anti-dsDNA antibodies have been reported to bind to the glomerular basement membrane and cause glomerular injury [2, 3, 5]. In view of the importance of the IgG antibodies, we modified the culture system in the current study and extended the duration of the culture period from 5 to 10 days, to determine whether NZB/W NKT cells and/or conventional T cells can provide help for IgG switching of NZB/W B cells. In addition, our previous studies [13] did not determine which subset of NZB/W B cells, innate immune B-1 and MZ B cells and/or adaptive immune FO B cells, can interact with NKT cells to secrete immunoglobulins and autoantibodies. In the current study, we found that by using the modified culture system, the NKT cells provided potent help for secretion of IgG1, IgG2a and IgG anti-dsDNA antibodies. Among the B-1, MZ, and FO B cell subsets, the B-1 B cells were induced by the NKT cells to secrete the highest levels of IgG anti-dsDNA antibodies. Surprisingly, conventional T cells failed to help IgM or IgG secretion of the B-1, MZ, and FO B cells. NKT cell help was blocked by anti-CD1d and anti-CD40L mAb.

## Results

### $\alpha$ -Galactosylceramide activation of NKT cells requires antigen-presenting cells in C57BL/6 but not NZB/W mice

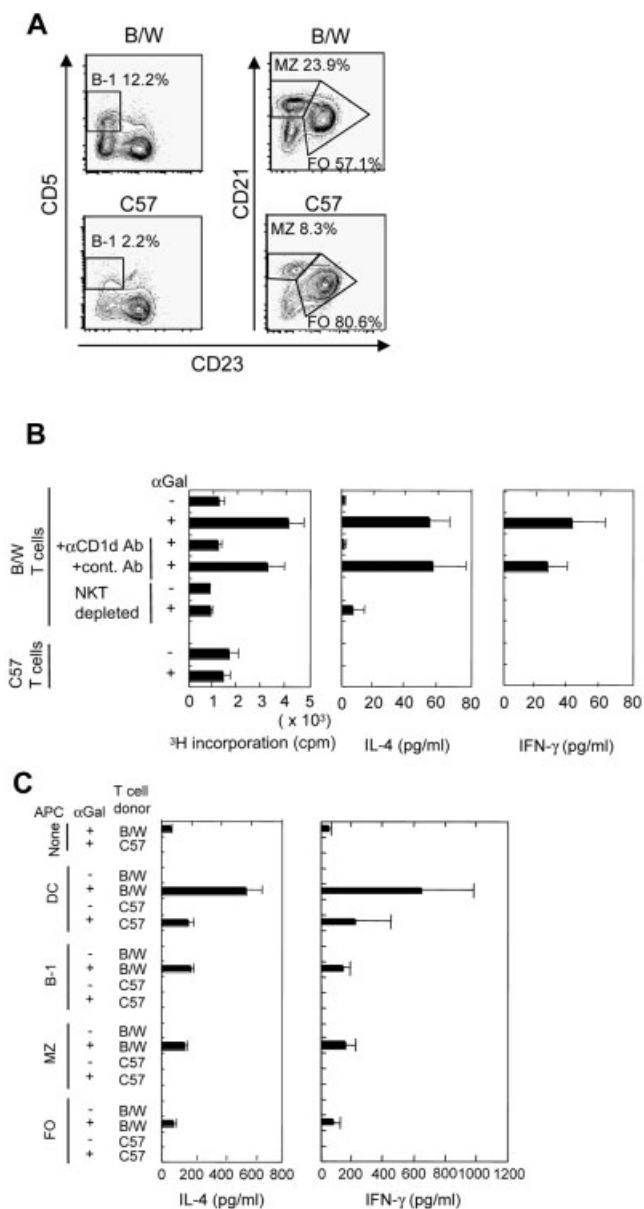
In non-autoimmune strains of mice, antigen-presenting cells (APC) such as dendritic cells or B cells are required to induce NKT cell activation *in vitro* with the stimulatory ligand,  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer), associated with CD1d [19]. In the current study, we compared the APC requirements for NKT cell activation in non-autoimmune C57BL/6 mice, and in lupus-prone NZB/W mice. Purified C57BL/6 or NZB/W T cells were stimulated with 100 ng/mL  $\alpha$ -GalCer, a concentration

previously determined to provide optimum activation of NKT cells in the presence of APC. Gated CD11c<sup>+</sup> TCR $\alpha\beta$ <sup>-</sup> B220<sup>-</sup> NK1.1<sup>-</sup> cells were used as the source of dendritic cells, and gated TCR $\alpha\beta$ <sup>+</sup> CD11c<sup>-</sup> B220<sup>-</sup> cells were used as the source of T cells. B cell subsets were separated by gating on B220<sup>+</sup> cells and identifying B-1 B cells as CD5<sup>+</sup>CD23<sup>lo</sup>, MZ B cells as CD23<sup>lo</sup>CD21<sup>hi</sup>, and FO B cells as CD23<sup>hi</sup>CD21<sup>lo</sup> cells (Fig. 1A).

NKT cell-dependent activation was measured by [<sup>3</sup>H]thymidine incorporation at 72 h, and by secretion of IL-4 and IFN- $\gamma$  in culture supernatants at 48 h. C57BL/6 T cells showed no significant difference in [<sup>3</sup>H]thymidine incorporation with or without  $\alpha$ -GalCer in the medium (Fig. 1B). Unexpectedly, NZB/W purified T cells increased [<sup>3</sup>H]thymidine incorporation fourfold ( $p = 0.004$ ) above background after stimulation with  $\alpha$ -GalCer. The proliferative response was reduced to background levels when the purified T cells were depleted of NKT cells using CD1d tetramer staining and sorting, or when anti-CD1d mAb was added to the purified T cell cultures (Fig. 1B). The results show that the proliferative response was dependent on NKT cell activation, but we did not determine the phenotype of the proliferating cells.

The ability of  $\alpha$ -GalCer to activate NKT cells from NZB/W but not C57BL/6 mice in the absence of APC was reflected also in the secretion of IL-4 and IFN- $\gamma$ . Purified C57BL/6 T cells secreted no detectable cytokines into the culture supernatant with or without  $\alpha$ -GalCer stimulation, and NZB/W T cells secreted about 50 pg/mL IL-4 ( $p < 0.0001$ ) and 40 pg/mL IFN- $\gamma$  ( $p = 0.04$ ) in cultures with  $\alpha$ -GalCer as compared to background (Fig. 1B). NZB/W T cell-dependent cytokine secretion was reduced to background when anti-CD1d mAb was added to the cultures or when NKT cells were depleted.

Addition of purified dendritic cells to cultures with T cells and  $\alpha$ -GalCer markedly increased cytokine secretion such that supernatants from C57BL/6 cells contained about 200 pg/mL IL-4 and IFN- $\gamma$ , and supernatants from NZB/W cells contained about 600 pg/mL of both cytokines (Fig. 1C). Addition of purified B-1, MZ, or FO B cells from C57BL/6 mice could not substitute for the dendritic cells in facilitating C57BL/6 NKT cell-dependent secretion of cytokines in response to  $\alpha$ -GalCer stimulation, but addition of B-1 and MZ B cells from NZB/W mice to the NZB/W T cell cultures significantly increased IL-4 cytokine secretion (IL-4: B-1,  $p < 0.0001$ , MZ B,  $p = 0.008$ ; IFN- $\gamma$ : B-1,  $p = 0.04$ , MZ B,  $p = 0.07$ ) (Fig. 1C). Thus, the requirements of NZB/W NKT cell-dependent activation differed from that of C57BL/6 NKT cells in that only the former was activated by  $\alpha$ -GalCer alone with augmentation in the presence of B cells.



**Figure 1.** Different requirements of APC for *in vitro* activation of NKT cells from NZB/W and C57BL/6 mice. (A) CD5 or CD21 versus CD23 expression on gated B220<sup>+</sup> spleen cells from NZB/W and C57BL/6 mice. Boxes in the left panels enclose CD5<sup>+</sup>CD23<sup>lo</sup> (B-1) B cells, and boxes in the right panels enclose CD21<sup>hi</sup>CD23<sup>lo</sup> (MZ) B cells or CD21<sup>lo</sup>CD23<sup>hi</sup> (FO) B cells. Percentages of cells in each box are shown. B cell subsets were sorted based on thresholds for each box. (B) [<sup>3</sup>H]Thymidine incorporation in supernatants of 72-h cultures, and concentrations of IL-4 and IFN- $\gamma$  in supernatants of 48-h cultures containing  $2 \times 10^5$  purified T cells with and without stimulation by 100 ng/mL  $\alpha$ -GalCer. Bars show means and brackets show standard errors of pooled data from two to three replicate experiments with triplicate wells in each experiment. In some cultures, anti-CD1d or control mAb were added for blocking, and in others T cells were depleted of CD1d tetramer<sup>+</sup> cells before culture. (C) IL-4 and IFN- $\gamma$  concentrations in supernatants from 48-h cultures of purified T cells alone, purified T cells and B cell subsets or dendritic cells with or without  $\alpha$ -GalCer stimulation. Means and standard errors are from pooled data as described above.

invariant NKT cells, since the CD1d tetramer reagent identifies the invariant V $\alpha$ 14 TCR [20]. About 95% of the sorted NKT cells from NZB/W mice were CD4<sup>+</sup>, and about 88% from C57BL/6 mice were CD4<sup>+</sup> (data not shown). Purified B cell subsets for these experiments were first enriched using anti-CD19 mAb beads, and then gated on IgM<sup>+</sup> cells instead of B220<sup>+</sup> cells in order to assess immunoglobulin isotype switching to IgG.

Co-culture of NZB/W NKT cells with B-1 B cells and MZ B cells increased IgM and IgM anti-dsDNA antibody secretion about 11–14-fold ( $p = <0.0001$ – $0.003$ ) and about 23-fold ( $p = 0.0004$ – $0.0005$ ), respectively (Fig. 2B). FO B cells secreted background levels of IgM and IgM anti-dsDNA antibodies after culture with NKT cells. Furthermore, all B cell subsets that were co-cultured with NKT cells secreted over 500 ng/mL of IgG1 and about 60–120 ng/mL IgG2a. The IgG levels were significantly increased ( $p = <0.0001$ – $0.03$ ), as compared to cultures with B cell subsets alone. These results indicated that NKT cells provide help for the B cell subsets to switch to IgG secretion (Fig. 2B). The lack of augmentation of IgM secretion of FO B cells by NKT cells may be due to either an inability to secrete IgM in the culture system, or a more rapid switching to IgG secretion induced by NKT cells.

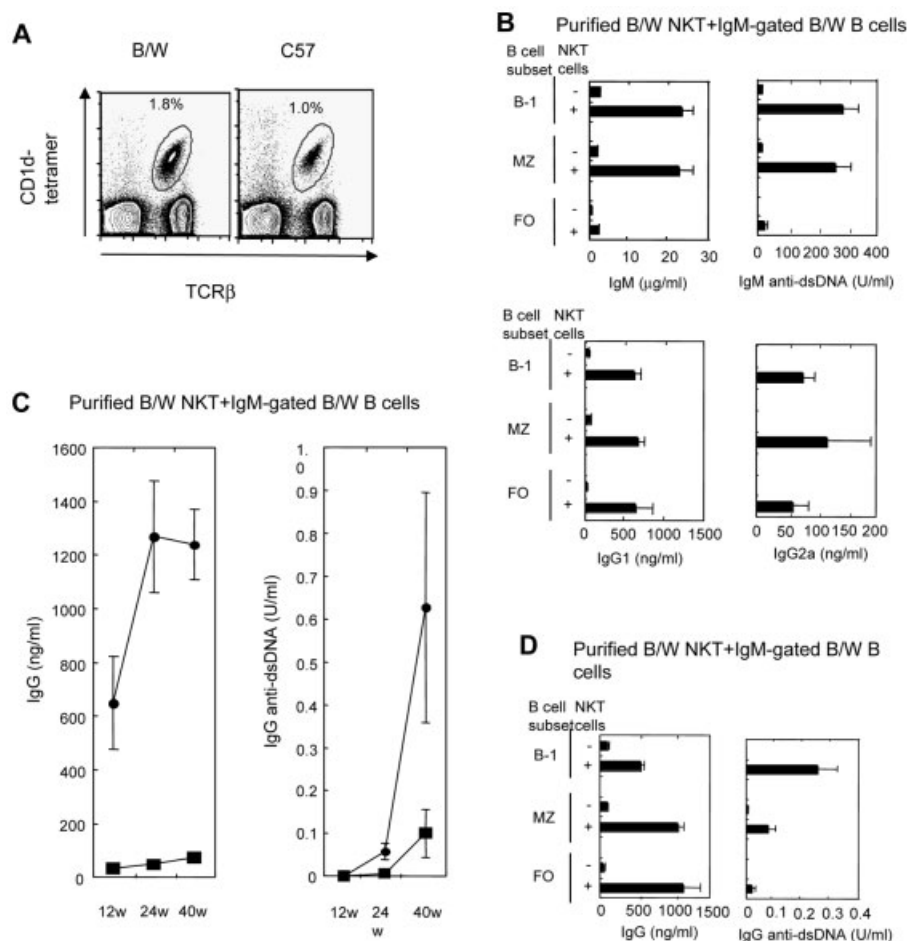
Since the concentrations of IgM in cultures of NKT cells and B-1 or MZ B cells from 12-wk-old NZB/W mice were considerably higher than that of IgG, and since IgG anti-dsDNA antibodies were not detected (less than 0.01 U/mL), we determined whether secretion of IgG antibodies would increase using cells from older NZB/W mice. Accordingly,  $2 \times 10^5$  sorted IgM<sup>+</sup> total B cells were cultured with  $5 \times 10^4$  NKT cells from 12-, 24-, or 40-wk-old mice. Fig. 2C shows the total IgG and IgG anti-dsDNA antibody concentrations of cultures of the sorted

### Purified NZB/W NKT cells help NZB/W B cells to spontaneously secrete IgM, IgM anti-dsDNA antibodies, IgG, and IgG anti-dsDNA antibodies

Since purified NZB/W NKT cells have been reported to augment the IgM but not IgG secretion of NZB/W total B cells *in vitro* in 5-day cultures [10, 13], we measured immunoglobulin secretion from purified NZB/W B cell subsets for both IgM and IgG after co-culture with or without purified NKT cells for 10 days. We reasoned that switching from IgM to IgG secretion *in vitro* may require an increase in the duration of the cultures. Splenic NKT cells were identified as TCR $\alpha\beta$ <sup>+</sup> CD1d tetramer<sup>+</sup> cells shown in Fig. 2A, and there was an increased percentage ( $\sim 2\%$ ) in the spleen of NZB/W as compared to C57BL/6 mice ( $\sim 1\%$ ). The TCR $\alpha\beta$ <sup>+</sup> CD1d tetramer<sup>+</sup> cells were

B cells with or without the sorted NKT cells. Supernatants from cultures of the B cells alone contained less than 80 ng/mL of total IgG using mice up to 40 wk old. When NKT cells were cultured with B cells, then there was an increase in IgG concentrations at 24 and 40 wk to about 1200 ng/mL. The increases at all time points were significant ( $p = <0.0001-0.001$ ). Although secretion of IgG anti-dsDNA antibodies was less than 0.01 U/mL in NKT and B cell cultures at 12 wk of age, these antibodies were detected at 24 wk of age (0.06 U/mL) and increased about tenfold to about 0.6 U/mL at 40 wk of age (Fig. 2C). Cultures of B cells alone from mice at 24 and 40 wk contained 0.01 and 0.1 U/mL of IgG anti-dsDNA antibodies, respectively. The increased antibody level after addition of NKT cells at 24 wk was significant ( $p = 0.03$ ).

In further studies, we examined the ability of NKT cells to augment IgG anti-dsDNA antibody secretion from purified B cell subsets using 24-wk-old NZB/W mice. As shown in Fig. 2D, NKT cells significantly increased total IgG secretion from all B cell subsets ( $p = <0.0001-0.001$ ). Interestingly, the IgG anti-dsDNA antibody secretion by the combination of NKT cells and B-1 B cells was more than tenfold higher than that of NKT cells and FO B cells ( $p = 0.006$ ), and significantly higher than that of NKT cells and MZ B cells ( $p = 0.04$ ).



**Figure 2.** NKT cells provide helper activity for spontaneous antibody secretion and isotype switching of NZB/W B cell subsets. (A) CD1d tetramer versus TCRαβ staining of spleen cells. NKT cells (CD1d tetramer<sup>+</sup> TCRαβ<sup>+</sup>) are enclosed in ellipses, and percentages of enclosed cells are shown. Sorting of NKT cells was based on thresholds for enclosed cells. (B) Mean concentrations of IgM, IgM anti-dsDNA, IgG1, and IgG2a antibodies in supernatants from 10-day cultures of  $1 \times 10^5$  sorted NZB/W B cell subsets with and without sorted  $5 \times 10^4$  NKT cells in six to ten experiments. (C) Mean concentrations of total IgG and IgG anti-dsDNA antibodies in cultures of sorted total IgM<sup>+</sup> B cells from 12-, 24-, and 40-wk-old NZB/W mice with or without the addition of sorted NKT cells. (■) B cells alone, (●) B and NKT cells. Data are from two to eight cultures at each time point. (D) Total IgG and IgG anti-dsDNA antibodies in cultures of sorted B cell subsets from 24-wk-old NZB/W mice with or without the addition of NKT cells.

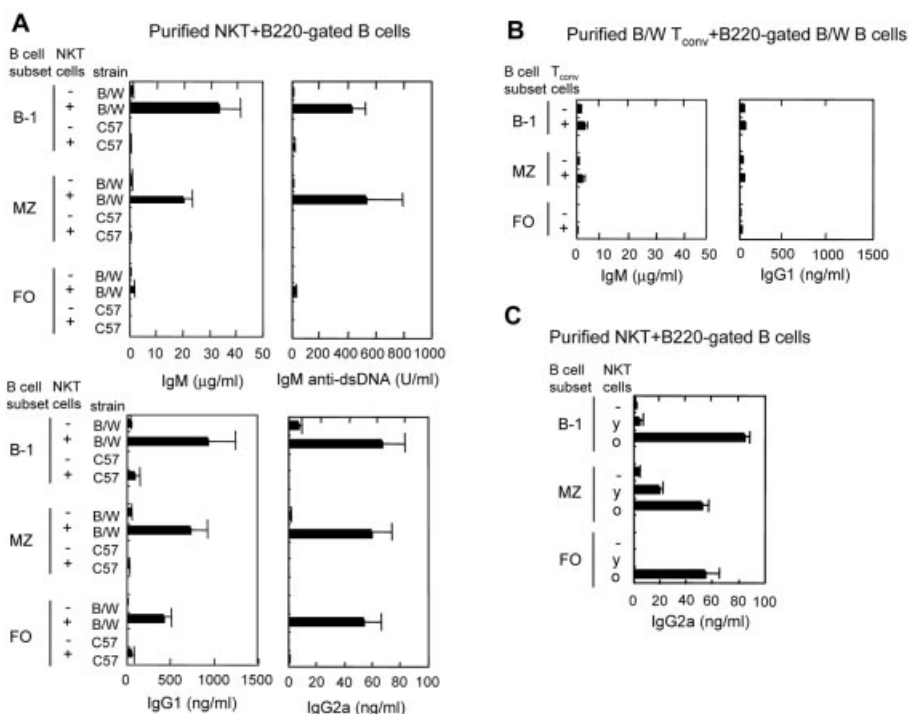


### Purified conventional T cells fail to help NZB/W B cells to secrete IgM, IgM anti-dsDNA antibodies, and IgG

In further studies, we compared the ability of conventional (non-NK) T cells and NKT cells from NZB/W and C57BL/6 mice to help B cell secretion of immunoglobulin and autoantibodies. In these experiments, B cell subsets were sorted using B220 gating instead of IgM gating in order to remove the possible contribution of cross-linking of IgM receptors to the spontaneous B cell activation. Purified NZB/W NKT cells co-cultured with NZB/W B-1 B cells or MZ B cells secreted markedly increased amounts of IgM ( $p = <0.0001$ – $0.001$ ) and IgM anti-dsDNA antibodies ( $p = 0.0007$ – $0.03$ ) (Fig. 3A) as compared to cultures of B cell subsets alone. Although FO B cells plus NKT cells secreted low levels of IgM and IgM anti-dsDNA antibodies as compared to the other subsets, all the NZB/W B cell subsets cultured with NKT cells secreted significantly increased amounts of IgG1 ( $p = 0.0003$ – $0.008$ ) and IgG2a ( $p = 0.0003$ – $0.002$ ) that were above 400 and 50 ng/mL, respectively (Fig. 3A). In contrast, cultures of purified NKT cells and B cell subsets from C57BL/6 mice contained less than 0.6  $\mu$ g/mL IgM, less than 15 U/mL IgM anti-dsDNA

antibodies, less than 80 ng/mL IgG1, and undetectable levels of IgG2a secretion (Fig. 3A). We did not assay for IgG2c, the predominant IgG2 isotype in C57BL/6 mice. In order to determine whether sorting C57BL/6 B cells with anti-IgM instead of anti-B220 mAb could enhance the ability of B cells to secrete IgM and IgG after interaction with C57BL/6 NKT cells, we repeated the above experiments using IgM<sup>+</sup> gated B cells. The results of the experiments were similar to those with B220<sup>+</sup> gated B cells, and secretion of IgM and IgG was minimal ( $\leq 1$   $\mu$ g/mL IgM,  $<10$  ng/mL IgG1,  $<3$  ng/mL IgG2a). The results indicate that autoimmune abnormalities in both NZB/W NKT cells and B cells are likely to contribute to the high levels of spontaneous immunoglobulin secretion.

We also determined whether NZB/W conventional T cells (non-NKT cells; TCR $\alpha\beta^+$  CD1d tetramer<sup>-</sup>) help NZB/W B cells to secrete immunoglobulins. Cultures of conventional T cells and B cell subsets did not show significantly increased secretion of IgM ( $p > 0.2$ ), or IgG isotypes (IgG1,  $p = >0.05$ – $0.5$ ) as compared to cultures of B cell subsets alone (Fig. 3B). IgG2a was not detected in supernatants from cultures of conventional T cells and B cell subsets, and IgM anti-dsDNA antibody levels were less than 20 U/mL (data not shown). In further



**Figure 3.** NKT cells but not conventional T cells provide help for spontaneous antibody secretion of B cells from NZB/W mice. (A) Concentrations of IgM, IgM anti-dsDNA, IgG1, and IgG2a antibodies in supernatants from 10-day cultures of  $1 \times 10^5$  C57BL/6 or NZB/W B cell subsets with or without  $5 \times 10^4$  NKT cells. Results are pooled data from three to four independent experiments. (B) Concentrations of antibodies from cultures of NZB/W B cell subsets with or without sorted conventional T cells. Results are pooled data from three independent experiments. (C) Concentrations of IgG2a from cultures of 4-wk-old NZB/W B cell subsets with or without 4-wk-old (y) or 24-wk-old (o) NZB/W NK T cells. Results are from three experiments.

experiments, we compared the NKT cell helper activity in young (4-wk-old) and old (24-wk-old) NZB/W mice using 4-wk-old B cells to determine whether the activity is related to age. Fig. 3C shows that the helper activity of the old NKT cells was markedly increased ( $p = 0.0002$ – $0.009$ ) as compared to young NKT cells for augmenting the secretion of IgG2a from all 4-wk-old B cell subsets. Interestingly, helper activity for IgM secretion was not significantly different between young and old NKT cells (data not shown).

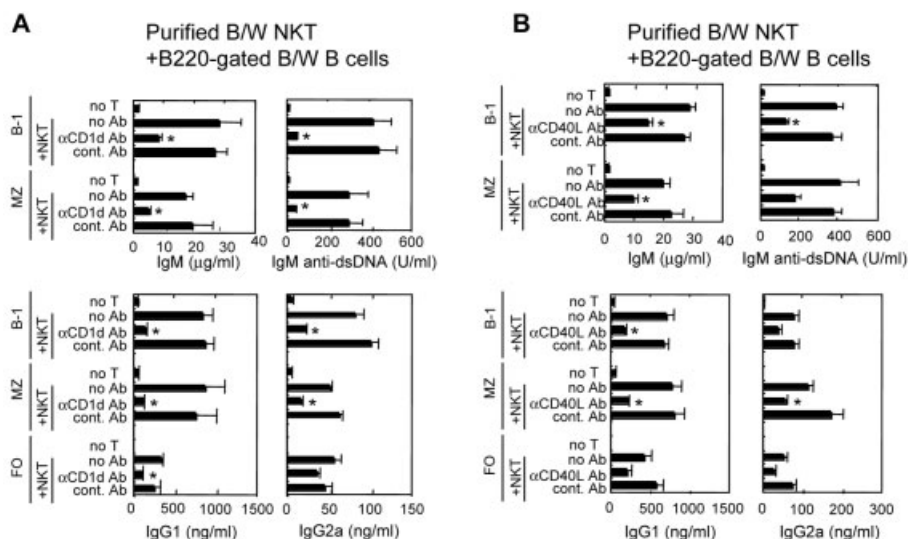
### NKT cell augmentation of antibody secretion by B cells is dependent on interactions with CD1d and CD40

Since the purified NZB/W NKT cells augmented antibody secretion, the dependence of the augmentation on cell-cell interactions *via* key surface receptors remained to be determined. Accordingly, we added anti-CD1d mAb to the cultures of purified NKT cells and B cell subsets obtained from 12-wk-old NZB/W mice to inhibit the interaction between the NKT cell TCR and CD1d. As shown in Fig. 4A, the addition of the anti-CD1d mAb significantly decreased the concentration of IgM, and IgM anti-dsDNA antibodies secreted by the B-1 B cells ( $p = 0.004$ – $0.03$ ), and by the MZ B cells ( $p = 0.003$ – $0.02$ ). Similarly, the addition of anti-CD1d mAb significantly reduced IgG1 secretion of all three subsets ( $p = 0.001$ – $0.01$ ), and IgG2a secretion of the B-1 and MZ B cells ( $p = 0.0001$ – $0.001$ ). No

significant reduction ( $p > 0.07$ ) of antibody secretion was observed when isotype-matched irrelevant mAb was used instead of anti-CD1d mAb (Fig. 4A).

The interactions between T cells and B cells or APC that result in disease activity in lupus-prone mice has been shown to be dependent upon the binding of the costimulatory ligand CD40L to CD40 [21, 22]. In order to determine the dependence of the NKT cell augmentation of antibody secretion *in vitro* on this interaction, anti-CD40L mAb was added to the NKT cell/B cell cultures from NZB/W mice. As shown in Fig. 4B, the addition of the anti-CD40L mAb significantly reduced IgM secretion of the B-1 and MZ B cells ( $p = 0.0009$ – $0.01$ ), as well as the IgM anti-dsDNA antibody secretion of the B-1 B cells ( $p = 0.0004$ ). Although, the MZ B cell secretion of IgM anti-dsDNA was reduced, statistical significance was not achieved ( $p = 0.07$ ). A similar pattern of reduction of IgG1 and IgG2a secretion was found for all B cell subsets, and statistically significant reductions were achieved for IgG1 secretion by B-1 and MZ B cells ( $p = 0.001$ – $0.003$ ), and for IgG2a secretion for MZ B cells ( $p = 0.01$ ) (Fig. 4B). Addition of control mAb failed to spontaneously reduce antibody secretion in any of the cultures ( $p > 0.2$ ).

In similar experiments, we attempted to inhibit the secretion of IgM and IgG by adding anti-IL-4, anti-IFN- $\gamma$ , or anti-IL-10 mAb to cultures of NZB/W NKT cells and B cell subsets. In contrast to the blocking activity observed with anti-CD1d and anti-CD40L mAb, we



**Figure 4.** Anti-CD1d and anti-CD40L mAb inhibit spontaneous antibody secretion in cultures of NZB/W B cell subsets and NKT cells. (A) Concentrations of antibodies in culture supernatants from  $1 \times 10^5$  sorted NZB/W B cell subsets alone or in combination with  $5 \times 10^4$  NKT cells with or without the addition of anti-CD1d or control mAb. Results are pooled data from three independent experiments. (B) Concentrations of antibodies in 10-day culture supernatants as above with or without the addition of anti-CD40L or control mAb. Results are pooled data from four independent experiments. Asterisks show cultures in which anti-CD1d or anti-CD40L mAb significantly reduced antibody concentrations compared to cultures with control mAb.

found no significant blocking activity with the anti-cytokine mAb in any of the culture combinations (data not shown).

## Discussion

The results of the present study show that there are abnormalities in the function of both innate immune NKT cells and innate immune B-1 and MZ B cells from lupus-prone NZB/W mice. Purified dendritic cells from C57BL/6 mice were required to activate the C57BL/6 NKT cells in the presence of the NKT cell TCR-specific ligand  $\alpha$ -GalCer, and all purified C57BL/6 B cell subsets failed to function as APC to induce activation with  $\alpha$ -GalCer. In contrast, NZB/W B-1 and MZ B cells significantly augmented NKT cell activation, and  $\alpha$ -GalCer still activated NZB/W NKT cells without B cells or dendritic cells. The results suggest that NZB/W NKT cells are abnormal and can be activated *via* ordinarily weak antigen presentation by CD1d on T and B cells.

NZB/W NKT cells markedly augmented the secretion of IgM, IgG, and anti-dsDNA antibodies by NZB/W B cells *in vitro* in the current study using 10-day cultures. The use of 10-day cultures to study *in vitro* IgG secretion has been reported before [10]. Our previous study using 5-day cultures showed augmentation of IgM secretion but not IgG [13], because switching to IgG secretion required a longer duration of culture. The ability of young (4-wk-old) NZB/W NKT cells to help young NZB/W B cells secrete IgG2a *in vitro* in the current study was minimal, and helper activity became considerably more potent for young B cells when NKT cells were obtained from 6-month-old NZB/W mice. This age-related change in the function of NZB/W NKT cells may help explain the reported inability of an NZB/W NKT cell line derived from 5-wk-old mice to help NZB/W B cells to secrete IgM but neither IgG2a nor IgG anti-dsDNA antibodies in a 10-day culture system [10]. In addition, the NKT cell line was irradiated (3000 rad) before addition to the culture, whereas the NKT cells used in the current study were fresh unirradiated cells. Secretion of IgG2a anti-dsDNA antibodies in cultures of NZB/W NKT cells and B-1 B cells was also markedly increased when cells from 6-month-old mice were compared to 1-month-old mice. We have not observed these age-related changes in NKT and B cells from non-autoimmune C57BL/6 mice. It is of interest that NKT cell lines from humans with systemic lupus show a pattern of helper activity for IgG and IgG anti-dsDNA antibody secretion by autologous B cells that is similar to that reported here for old NZB/W NKT cells (E. Engleman, Stanford University, personal communication).

Although the *in vitro* interaction between the NZB/W NKT cells and NZB/W B-1 B cells gave rise to the most vigorous secretion of IgG2a anti-dsDNA antibodies as compared to interactions of conventional T cells or NKT cells with other B cell subsets, further studies are required to show that the NKT cell/B-1 B cell interaction *in vivo* is a requirement for the *in vivo* generation of IgG2a anti-dsDNA antibodies and/or a requirement for the development of lupus kidney disease. Evidence that supports an important contribution of T cells that recognize CD1d to the development of lupus disease activity include the induction of lupus in nu/nu BALB/c mice injected with transgenic anti-CD1d T cells [18], the amelioration of lupus in NZB/W mice after treatment with anti-CD1d mAb [7, 13], the worsening of lupus in NZB/W mice after treatment with  $\alpha$ -GalCer [13], and the amelioration of lupus in NZB/W mice after treatment with glycolipid that binds to CD1d and antagonizes NKT cell cytokine secretion (S. Morshed *et al.*, unpublished data).

Additional evidence that B-1 B cells contribute to lupus in NZB/W mice include the amelioration of disease in NZB/W *xid* mice [23], and reduction of disease severity by relative depletion of peritoneal B-1 B cells [24]. The role of NKT cells in the development of lupus in mouse strains other than the NZB/W mice is variable. Although *in vivo* activation of NKT cells with  $\alpha$ -GalCer improved lupus induced by the injection of pristane in BALB/c mice, administration of  $\alpha$ -GalCer worsened lupus in B10.PLJ mice injected with pristane [25]. In addition, deficiency of NKT cells in CD1d<sup>-/-</sup> BALB/c mice worsened lupus nephritis compared to wild-type mice after injection of pristane [26], but did not affect lupus kidney or skin disease in MRL/*lpr* mice [27]. Reports of decreased NKT cells in humans with lupus and other autoimmune diseases have been limited to examination of the blood, and may not reflect the changes in the other lymphoid tissues [28–30]. Some of these studies have erroneously identified NKT cells as CD56<sup>+</sup> T cells [30], and our recent study showed that neither CD56<sup>+</sup> T cells nor CD161<sup>+</sup> T cells accurately reflect the number of invariant NKT cells in human blood samples [31]. In addition, decreased *in vitro* responses of NKT cells to  $\alpha$ -GalCer in lupus patients [28] may reflect the impact of immunosuppressive drugs, and decreased numbers of NKT cells may reflect *in vivo* activation and TCR down-regulation as has been shown in mice [32–34].

We are currently studying whether the adoptive transfer of purified NZB/W NKT cells and B-1 B cells to irradiated NZB/W mice will result in the rapid generation of IgG2a anti-dsDNA antibodies and the development of lupus kidney disease. A previous study showed that the transfer of an NZB/W T cell line that had been immunized to idiotypic segments of the anti-

dsDNA antibody molecule into young NZB/W hosts was able to generate anti-dsDNA antibodies *in vivo* [9]. Thus, T cells other than the NKT cells can contribute to the production of pathogenic autoantibodies in NZB/W mice. The helper activity of the NZB/W NKT cells in the current study was dependent on cellular interactions *via* CD1d and CD40/CD40L receptors, as judged by blocking experiments with receptor-specific and control mAb. The *in vitro* blocking with the anti-CD1d and anti-CD40L mAb can explain the ability of these mAb to ameliorate lupus disease activity *in vivo* [13, 21, 22]. Two recent papers indicate that NKT cells may have a protective role in the early development of lupus in NZB/W mice or in the late development of lupus in non-autoimmune-prone mice that are more than 24 months of age [35, 36]. CD1d<sup>-/-</sup> NZB/W mice lacking CD1d-dependent invariant and non-invariant NKT cells were reported to develop nephritis earlier than wild-type NZB/W mice [35]. In view of the age-dependent abnormalities of NZB/W NKT cells observed in the current study, it is possible that the NKT cells in young mice are protective, and change to pathogenic cells as the disease process accelerates at about 6 months of age. In non-autoimmune mice, the NKT cells may be protective even at very late time points because they do not develop the age-related abnormalities observed in the lupus-prone NZB/W mice [36].

The failure of freshly isolated NZB/W conventional T cells to provide helper activity in our *in vitro* system could have been explained by their lack of activation before culturing with B cells. Our previous studies showed that NKT cells are partially activated by staining with the CD1d tetramers [13]. However, NZB/W conventional T cells also failed to provide helper activity after polyclonal activation *in vitro* with anti-CD3 and anti-CD28 mAb (data not shown). Cultures of NZB/W total T cells augmented IgG secretion of B cells more than tenfold only after  $\alpha$ -GalCer was added to the cultures, and  $\alpha$ -GalCer augmentation was reversed by NKT cell depletion (data not shown). In conclusion, the spontaneous *in vitro* interaction between purified NKT cells and purified B-1 B cells results in the vigorous secretion of IgM, IgG, IgM and IgG anti-dsDNA antibodies in NZB/W mice. This interaction may play an important role in the development of lupus disease activity observed in older mice.

## Materials and methods

### Mice

NZB/W and C57BL/6 female mice were purchased from the Jackson Laboratories (Bar Harbor, ME), and were maintained in the Stanford University Department of Comparative Animal Medicine until they were used for experiments at 12 wk of age,

unless otherwise stated in the text. All animal protocols were reviewed and approved by the Stanford Administrative Panels on Laboratory Animal Care.

### Reagents

Anti-mouse CD19, IgG1 magnetic beads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). FITC-conjugated mAb recognizing mouse CD5 (53–7.3), CD11c (HL3), CD21 (7G6), B220 (RA3–6B2), I-A<sup>b</sup> (25-9-17), I-A<sup>d</sup> (39-10-8), PE-conjugated mAb recognizing CD1d (1B1), CD23 (B3B4), IgM (R6–60.2), APC-conjugated mAb recognizing B220 (RA3–6B2), TCR $\beta$  (H57–597), NK1.1 (PK136), streptavidin, and biotin-conjugated anti-CD23 antibody (B3B4) were purchased from BD Biosciences (San Diego, CA). PE-conjugated PBS-57 glycolipid-loaded CD1d tetramers were obtained from the NIH Tetramer Facility, Rockville, MD. CD1d dimers were purchased from BD Biosciences.  $\alpha$ -GalCer was kindly provided by Dr. Paul Savage (Brigham Young University, UT). Purified anti-mouse CD3 $\epsilon$  (145–2C11) and CD28 (37.51) antibodies for T cell stimulation and purified anti-mouse CD1d (1B1) and CD40L (MR1) antibodies for blocking experiments were purchased from BD Biosciences. Anti-IL-4 (11B11), anti-IL-10 (JES5–16E3), and anti-IFN- $\gamma$  (R4–6A2) mAb used for blocking experiments were purchased from BD Biosciences.

### Cell preparation

For B cell subset sorting, spleen cells were incubated with anti-mouse CD19 beads and CD19<sup>+</sup> cells were enriched on MACS columns (Miltenyi Biotec) according to the manufacturer's protocol. Then, cells were incubated with various combinations of mAb and sorted into subsets using a highly modified FACStar instrument (Becton Dickinson, Milpitas, CA). For dendritic cells, spleen cells were incubated with anti-mouse CD11c beads and CD11c<sup>+</sup> cells were enriched on MACS columns. Then, the cells were incubated with mAb and sorted. For NKT cells, spleen cells were first incubated with  $\alpha$ -GalCer-loaded CD1d dimers and PE-CD1d tetramers, washed, and then incubated with anti-mouse IgG1 beads. After enrichment on MACS columns, cells were incubated with PE-CD1d tetramers again and anti-TCR $\beta$  mAb, and sorted. Sorted conventional T cells were obtained by staining spleen cells for TCR $\beta$  *versus* CD1d tetramers, and sorting TCR $\beta$ <sup>+</sup> tetramer<sup>-</sup> cells. Flow cytometric data were analyzed with FlowJo software (Treestar, San Carlos, CA). All cell suspensions were stained with propidium iodide to exclude dead cells. The purity of sorted cell populations was always more than 95%, except B-1 B cells in C57BL/6 mice, which was 90–95%.

### Cell culture and ELISA

Combinations of  $1 \times 10^5$  sorted B cell subsets,  $5 \times 10^4$  dendritic cells,  $5 \times 10^4$  NKT or  $5 \times 10^4$  conventional T cells were cultured in 96-well round-bottom plates in RPMI medium supplemented with 10% FCS,  $1 \times 10^{-5}$  M  $\beta$ -mercaptoethanol, 2 mM glutamine, and 100  $\mu$ g/mL penicillin and streptomycin (hereafter referred to as cRPMI). Cells were incubated at 37°C with 5% CO<sub>2</sub>. Measurements of IL-4 or IFN- $\gamma$  in culture supernatants were performed using an ELISA kit according to



the manufacturer's protocol (IL-4: BioSource, Camarillo, CA; IFN- $\gamma$ : BD Biosciences). Measurements of IgM, IgG, IgG1, and IgG2a in culture supernatants were performed using an ELISA as described previously [18]. IgM or IgG anti-dsDNA antibodies were captured using deproteinized calf thymus DNA. Anti-dsDNA antibody titers are expressed as units per milliliter using a reference-positive standard of pooled sera from 9-month-old NZB/W mice. The details of the anti-dsDNA antibody assay have been described [18].

### In vitro proliferative response

Sorted splenic T cells (TCR $\beta^+$  CD11c $^-$  B220 $^-$  I-A $^-$ ) were incubated ( $2 \times 10^5$  cells) with or without 100 ng/mL  $\alpha$ -Gal in cRPMI with 10% FCS in 96-well round-bottom plates for 72 h at 37°C in 5% CO $_2$ . [ $^3$ H]Thymidine (1  $\mu$ Ci/well) was added 20 h before the cells were harvested. [ $^3$ H]Thymidine (New England Nuclear, Boston, MA) incorporation was measured in a liquid scintillation counter (Betaplate; Wallac, Turku, Finland). All assays were performed in triplicate wells.

### Pre-activation of T cell subset

To pre-activate NKT cells, spleen cells were cultured in the presence of  $\alpha$ -GalCer (100 ng/mL) for 2 h before purification. To pre-activate conventional T cells, sorted TCR $\alpha\beta^+$  CD1d tetramer $^-$  spleen cells were cultured in 24-well plates coated with anti-CD3 mAb (10  $\mu$ g/mL) with medium containing anti-CD28 mAb (5  $\mu$ g/mL), for 18 h in cRPMI with 10% FCS.

### Blockade experiments

In some cell cultures, mAb were added to the medium to inhibit immunoglobulin secretion. For CD1d blockade, 20  $\mu$ g/mL of rat anti-mouse CD1d mAb or irrelevant rat IgG2b mAb was used. For CD40L blockade, 20  $\mu$ g/mL of rat anti-mouse CD40L mAb or control rat IgG3 mAb was used.

### Statistical analyses

Differences in mean immunoglobulin secretion, cytokine secretion and proliferation were analyzed using the two-tailed Student's *t*-test. In experiments in which the majority of determinations in a given group were undetectable, Fisher's exact test was used. A value of *p* < 0.05 was considered statistically significant.

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