Invariant NKT cells limit activation of autoreactive CD1d-positive B cells

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Faulty activation of autoreactive B cells is a hallmark of autoimmune diseases like systemic lupus erythematosus (SLE). An important feature restricting activation of autoreactive B cells is efficient removal of apoptotic material. Mounting evidence also connects a primary defect in invariant natural killer T (iNKT) cells to autoimmune disease development. However, exactly how this unconventional T cell subset is involved remains to be defined. Here, we identify a suppressive role for iNKT cells in a model where autoantibody production is triggered by an increased load of circulating apoptotic cells, resembling the situation in SLE patients. Absence or reduction of iNKT cells as well as absence of CD1d-expression on B cells, needed for direct iNKT–B cell interaction, leads to increased autoreactive B cell activation and symptoms of disease. The suppression mediated by the iNKT cells is observed before B cell entry into germinal centers and can be rescued by transferring iNKT cells to deficient mice. This links iNKT cells to handling of dying cells and identifies a novel peripheral tolerance checkpoint relevant for autoimmune disease. Thus, these observations connect two clinical observations in SLE patients previously considered to be unrelated and define a new target for immunotherapy.

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Abbreviations: αGalCer, α-galactosylceramide; FoB, follicular B cell; GC, germinal center; IC, immune complex; iNKT, invariant natural killer T cell; MZB, marginal zone B cell; PI, preimmune; SLE, systemic lupus erythematosus.

It has been estimated that as many as one million cells die each second by apoptosis in the human body as part of normal tissue turnover (Green et al., 2009). The remnants of the dying cells are cleared by phagocytic cells, like macrophages and DCs, with potent immunomodulatory abilities. The end result is normally an antiinflammatory milieu and homeostasis (Savill et al., 2002). Nevertheless, both exogenous (Torchinsky et al., 2009) and endogenous factors (Green et al., 2009) can shift the response to a more proinflammatory situation. Because an apoptotic cell can be described as a bag of modified self-antigens, the connection to autoreactive activation is quite plausible (Utz et al., 2000). Examples of this are seen in SLE patients where defects in clearing apoptotic cells are linked to development of autoimmune disease with autoantibody production and systemic organ manifestations (Gaipl et al., 2006). It has been shown that these patients have increased numbers of apoptotic cells in the circulation and that their macrophages have defects in clearing apoptotic cells (Herrmann et al., 1998; Perniok et al., 1998). Animal studies further solidify these theories by demonstrating that repeated injections of syngenic apoptotic cells, without adjuvant, lead to a transient SLE-like phenotype (Mevorach et al., 1998). Collectively, these observations highlight the importance of proper removal of apoptotic cells to limit inflammation and activation of autoreactive lymphocytes. This also suggests the existence of a threshold for autoreactive activation dependent on factors involved in the removal of apoptotic cells. As the etiology of SLE is complex, many factors and cell types can be involved in setting this threshold, a threshold that is needed to limit autoreactivity, while allowing activation against microbes in the vicinity of apoptosis.

The role of the CD1d-restricted NKT cell population has been widely studied since its discovery more than two decades ago (Godfrey and Kronenberg, 2004). This unconventional T cell expresses NK cell markers and a semi-invariant T cell receptor that recognize lipid

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derivatives presented by the MHC class I–like molecule CD1d (Brutkiewicz, 2006). The NKT cell population can be further divided into the most studied type I/invariant (from now on called iNKT) and type II NKT cells, where the former expresses an invariant Vα14-Jα18 TCRα chain and the latter uses diverse TCR, resulting in different ligand recognition (Rolf et al., 2008). Studies in several murine models for autoimmune disease have identified an important regulatory role for the NKT cells (Wu and Van Kaer, 2009). For example, SLE and diabetes susceptibility loci have been shown to negatively control iNKT cell numbers (Esteban et al., 2003), and introduction of CD1d deficiency, and thereby iNKT cell deficiency, does worsen nephritis in the SLE prone (NZBxNZW)F1 mice (Yang et al., 2007). Controversy does, however, exist as some studies show evidence that iNKT cells could also be involved in driving the disease development (Forestier et al., 2005; Takahashi and Strober, 2008). Human studies describe reduced numbers of iNKT cells in several autoimmune patient cohorts and this is, at least in SLE, considered a primary defect as it is seen also in family members without clinical symptoms (Wither et al., 2008).

Here, using a model where injections of syngenic apoptotic cells trigger autoantibody production, we identify a novel mechanism linking iNKT cells to autoreactivity. We demonstrate, in vivo, a protective role for iNKT cells and that CD1d expression on B cells regulates autoreactive B cell activation. This regulatory CD1d-dependent checkpoint is seen at the pre–germinal center (GC) stage as B cells dramatically down-regulate CD1d when they enter GCs. Furthermore, we identify that iNKT cells are rapidly affected by apoptotic cell death of surrounding cells, shown by their decreased ability to produce IFN-γ. Collectively, this identifies the involvement of an iNKT cell–$CD1d^+$ B cell axis in the antiinflammatory response to apoptotic cell death with potential relevance for autoimmune disease.

RESULTS AND DISCUSSION

Absence of iNKT cells exaggerates the activation of autoreactive B cells triggered by an increased load of apoptotic cells without affecting B cell development or general B cell activation

During the process of apoptosis, major changes occur in the lipid bilayer that makes up the cell membrane. This sends a multitude of signals to surrounding cells (Huynh et al., 2002). Because NKT cells scan the body for lipid derivatives presented by CD1d (Brutkiewicz, 2006), we hypothesized that NKT cells could be involved in the immunological silence mediated by apoptotic cell death. To examine if a link exists between the handling of apoptotic cells and NKT cells in the development of autoimmune disease, WT and two different NKT cell-deficient mice ($NKT^{-/-}$) were injected with syngenic apoptotic cells (Mevorach et al., 1998). Both $CD1d^{-/-}$ (needed for selection of both iNKT and type II NKT cells; Chen et al., 1997), and $Jα18^{-/-}$ mice (lacking the J segment used by the semiinvariant iNKT cell receptor; Cui et al., 1997) showed a significantly higher IgG anti-DNA response compared with WT mice (Fig. 1 A). No difference in preimmune (PI) levels were observed, although spontaneous SLE-like disease has been observed in old $Jα18^{-/-}$ mice (Sireci et al., 2007). These data suggest that the predisposition for autoimmune disease development in these mice could be triggered by challenging the response to apoptotic cell death. An increase in IgG3 anti-DNA was also seen (Fig. 1 B), indicating a role for the splenic marginal zone B (MZB) cell population, a B cell pool known to rapidly produce IgG3 and to contain autoreactive clones (Enzler et al., 2006; Guinamard et al., 2000). In addition to DNA, several lipid autoantigens are described in systemic autoimmune disease (Valesini and Alessandri, 2005), and a significant increase in IgG reactivity against both cardiolipin and phosphorylcholine was seen in the injected $NKT^{-/-}$ mice (Fig. 1 C). In agreement with the autoantibody production, a significantly increased glomeruli deposition of IgG immune complexes (ICs) was found in kidneys of the $NKT^{-/-}$ mice (Fig. 1 D). The transient nature of the used model does not allow for changes related to the chronic phase of IC-mediated disease. Still, in the absence of NKT cells, lymphocyte infiltration was observed in glomeruli of the experimental group by hematoxylin and eosin (H&E) staining (Fig. 1 D).

Years of studies on autoimmune diseases have identified the importance for both central and peripheral tolerance. It is known that SLE patients have defects in checkpoints that normally limit autoreactive B cell development (Yurasov et al., 2005). Because CD1d is highly expressed on developing and mature B cells, with the highest expression found on the mature MZB population (Fig. S1), we examined if the increased autoreactive activation seen in the $NKT^{-/-}$ mice could be caused by an altered B cell development and thus be attributed to a general effect on B cell responses. However, when analyzing the splenic MZB cell population with FACS (fluorescence activated cell sorter) in WT and the two different $NKT^{-/-}$ mice used, no difference was seen (Fig. 1 E). The same was also true for conventional/follicular B cells (FoB; not depicted). Furthermore, no difference was seen in the response to the T cell-independent type II antigen TNP-Ficoll (a response where the MZB cells are involved; (Guinamard et al., 2000), in the response to the T cell-dependent antigen NP-OVA or in natural phosphorylcholine (PC) antibody levels (Fig. 1 F-H), suggesting functionally normal B cell compartments. Moreover, ex vivo stimulation of splenocytes with the polyclonal activator LPS did not show any difference in anti-DNA response, demonstrating that the $NKT^{-/-}$ mice do not have an obvious increase in autoreactive B cell clones (Fig. 1 I). It has been found that iNKT cells can provide help for B cell activation if the strong iNKT cell activating ligand αGalCer is presented by CD1d on the B cell (Barral et al., 2008; Leadbetter et al., 2008). This establishes that activated B cells do interact with iNKT cells via CD1d and, taken together with the data presented here, that the outcome of B – iNKT cell interaction is context and/or ligand dependent. Recently, an IL-10 producing $CD1d^{hi}$ B cell has been described, which could be involved in

regulating autoimmune responses (Yanaba et al., 2008). The question was raised whether the CD1d^{hi} B cell could be involved in the used model and be affected in the CD1d$^{-/-}$ mice. However, no expansion of IL-10 producing B cells was observed in mice injected with apoptotic cells (Fig. S2). As a similar phenotype was seen in the two different NKT$^{-/-}$ strains, one lacking iNKT cells and the other both iNKT and type II NKT cells, we conclude that (a) the absence of iNKT (type I) cells increases the autoreactive B cell response to apoptotic cells and (b) that this is not caused by an altered B cell development or a general B cell deregulation.

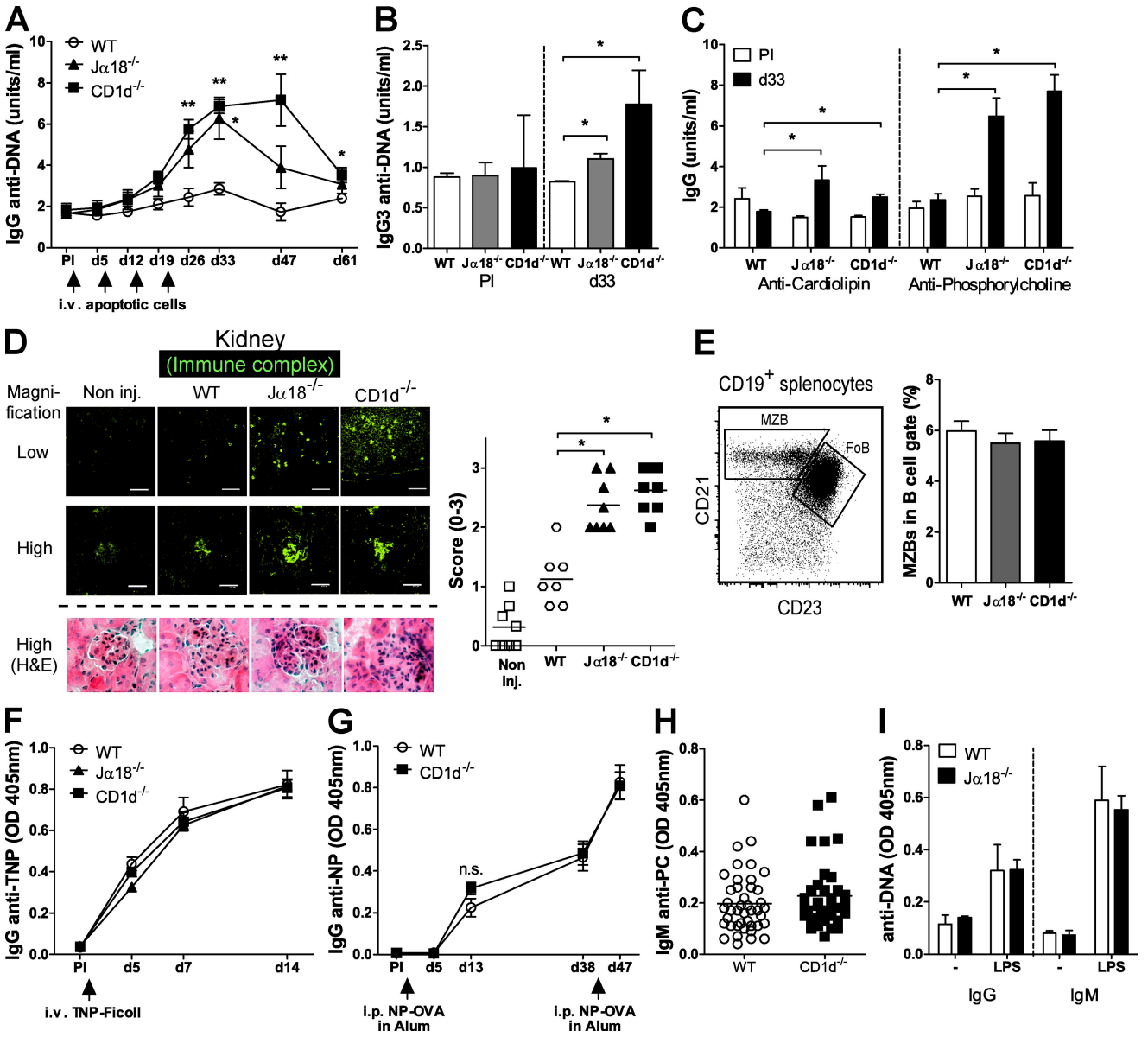

Figure 1. Increased activation of autoreactive B cells in iNKT cell–deficient mice. (A) WT and NKT-deficient mice (Jα18$^{-/-}$ and CD1d$^{-/-}$) injected four times i.v. with syngenic apoptotic cells. Serum IgG anti-DNA response is followed preimmune (PI) to day 61. (B) IgG3 anti-DNA PI and day 33. (C) IgG reactivity against lipid antigens (cardiolipin and PC) PI and day 33. (D) Representative kidney histology from day 61. 10× and 100× magnification of IgG immune complex (IC) deposition, H&E staining, and quantification of IC deposition (score 0–3). Bars: 300 µm (low); 47 µm (high). (E) FACS plot showing MZB cell gating of viable CD19^{+} splenocytes and quantification of the MZB cells in WT and NKT$^{-/-}$ mice. (F) IgG response to the T cell–independent type II antigen TNP-Ficoll in WT and NKT$^{-/-}$ mice. (G) IgG response to the T cell–dependent antigen NP-OVA in WT and CD1d$^{-/-}$. (H) IgM anti-PC in serum of untreated WT and CD1d$^{-/-}$ mice. (I) IgG anti-DNA response after ex vivo stimulation of splenocytes with LPS for 72 h. Antibody levels (A–C and F–I) were measured by ELISA. Results expressed as mean ± SEM in A–C (n = 6–8), E (n = 5), F (n = 10–11), G (n = 6), I (n = 3), and individual mice and mean in D (n = 7–8) and H (n = 35–42). n.s., not significant. *, P < 0.05; **, P < 0.01. In A, the significance is related to the WT response. Data are representative of at least two independent experiments or (H) pooled from three independent experiments.

iNKT cells sense apoptotic cell death and alter their cytokine producing profile

We next turned our focus to the iNKT cell response to injected apoptotic cells, to examine if the phenotype in the iNKT$^{-/-}$ mice could be supported. Because we have previously defined involvement by splenic B cells in this model (Wermeling et al., 2007), and established that these B cells express high levels of CD1d, we focused on the splenic iNKT cell population in WT mice. Interestingly, splenic iNKT cells up-regulated the activation marker CD69 after a single injection of apoptotic cells (Fig. 2 A). Thus, the iNKT cells rapidly recognize the apoptotic cell death event.

iNKT cells are potent cytokine producers, which in many systems has been defined as their main function. In autoimmune models, a protective role for iNKT cells has often been attributed to a shift in their cytokine signature toward a less proinflammatory one (Miyamoto et al., 2001). To investigate if apoptotic cell death could alter cytokine production from iNKT cells, two different experiments were performed comparing splenocytes from WT mice that were injected or not

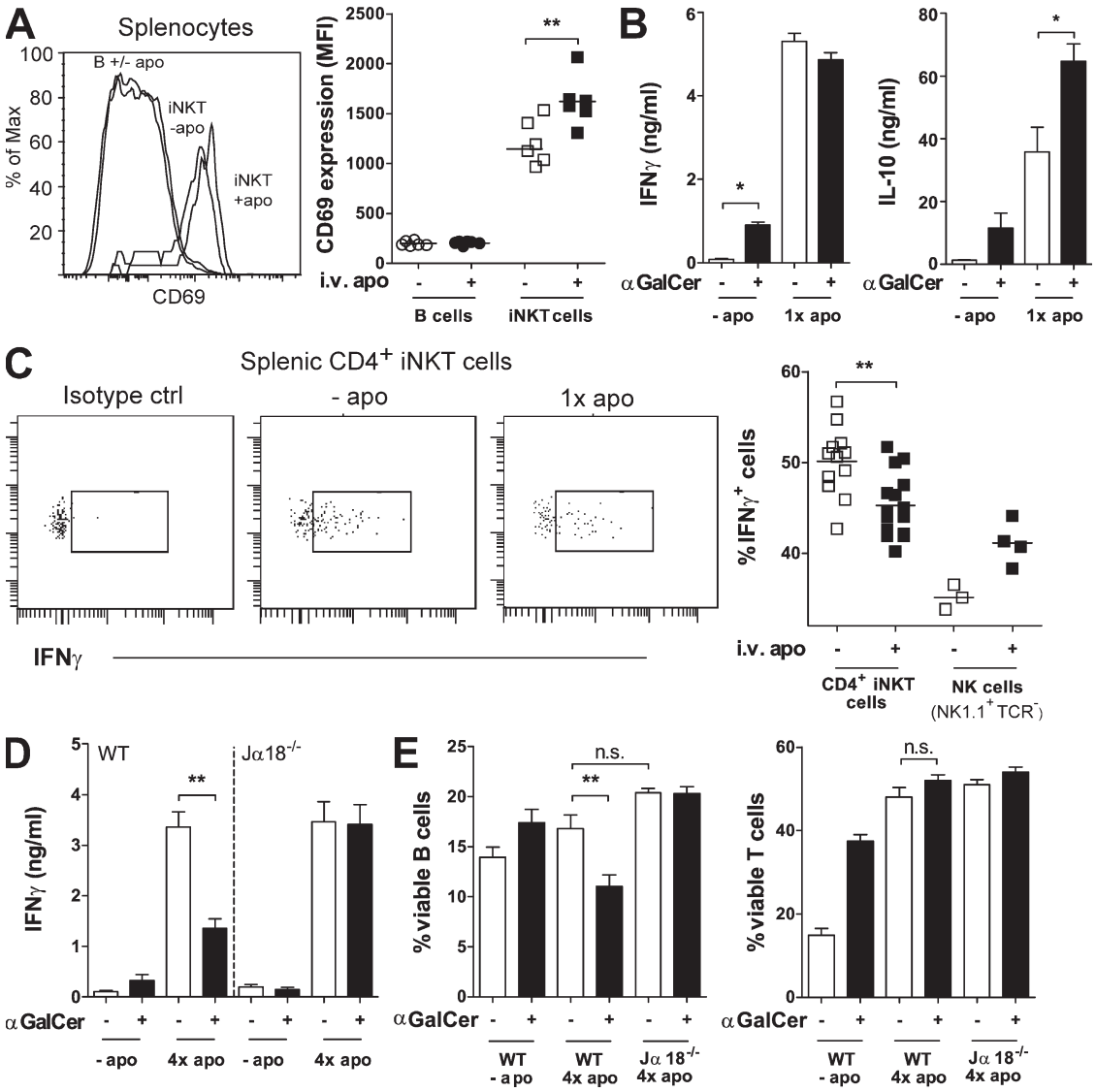

Figure 2. iNKT cells rapidly sense apoptotic cell death and alter their cytokine profile. (A) Plot showing CD69 expression on viable splenic B cells and iNKT cells 5 d after i.v. injection of apoptotic cells or control. (B) Data from supernatants of splenocyte cultures prepared from mice 5 d after injection of apoptotic cells and stimulated with αGalCer for 72 h. IFN-γ and IL-10 measured by ELISA. (C) Intracellular IFN-γ FACS data of splenic CD4$^+$ iNKT and NK cells 5 d after injection of apoptotic cells and ex vivo stimulation with PMA/ionomycin/Brefeldin A. (D) IFN-γ measurement of WT and Jα18$^{-/-}$ splenocytes stimulated with αGalCer as in B, but comparing cells from mice injected with apoptotic cells four times, instead of once. (E) Viable B and T cells in cultures from D measured using propidium iodine exclusion with FACS. Results are expressed as individual mice and mean in A (n = 6) and C (n = 3–16) and mean ± SEM in B (n = 6), D (n = 7), and E (n = 4–7). n.s. = not significant. *, P < 0.05; **, P < 0.01. Data are representative of at least two independent experiments.

with apoptotic cells. These experiments were initially performed 5 d after a single injection of apoptotic cells to match when the B cell compartment was evaluated by measuring antibody levels. First, we used αGalCer to selectively activate iNKT cells in splenocyte cultures and measured secreted cytokines. Interestingly, αGalCer was not able to induce IFN-γ production in splenocyte cultures from injected mice, in contrast to noninjected, instead a significant IL-10 production was seen (Fig. 2 B). Second, using intracellular FACS we found a significant reduction in IFN-γ producing iNKT cells after ex vivo stimulation with PMA and Ionomycin. In contrast to NK cells and $CD4^+$ T cells that increased their IFN-γ producing ability after the injection (Fig. 2 C and Fig. S3). Furthermore, after four injections of apoptotic cells the addition of αGalCer strongly decreased the spontaneous IFN-γ production seen in splenocyte cultures (Fig. 2 D). Interestingly, the activation of iNKT cells in these cultures also led to a decreased B cell (but not T cell) survival (Fig. 2 E). We conclude that iNKT cells rapidly detect apoptotic cell death, which alters their cytokine producing ability toward a seemingly less pro-inflammatory signature and that their activation could affect B cell survival. Together this supports the notion of a regulatory role for iNKT cells in the model.

Adoptive transfer reveals a direct role for CD1d on B cells limiting autoreactive activation

CD1d is expressed on several cell populations, including DCs, which could have a role in regulating the autoreactive response to an increased load of apoptotic cells. To determine whether iNKT cells directly interact with autoreactive CD1d-expressing B cells, adoptive transfer experiments were performed. Purified B cells from WT or $CD1d^{-/-}$ mice were transferred to $CD19^{-/-}$ recipient mice and the autoreactive response to injected apoptotic cells was followed (Fig. 3 A). $CD19^{-/-}$ mice have a severe B cell defect with nonresponsiveness in the model used, as well as other models; the mice also display the selective absence of MZB and B1 B cells, but normal iNKT cell levels (Rickert et al., 1995; unpublished data). The expression of CD19 can also easily be used to track the transferred B cells. The injections of apoptotic cells in recipients of WT or $CD1d^{-/-}$ B cells led to a significantly higher IgM and IgG3 anti-DNA response from the $CD1d^{-/-}$ B cells (Fig. 3 B). Interestingly, a higher proportion of the remaining transferred cells in the spleen had a MZB phenotype in the recipients of $CD1d^{-/-}$ B cells after four injections of apoptotic cells, suggesting an increased survival in the absence of CD1d expression (Fig. S4).

Additionally, immunofluorescence examination of spleen sections, after four injections with apoptotic cells, showed transferred cells ($CD19^+$), in the recipient of $CD1d^{-/-}$ B cells in GCs (Fig. 3 C). This was also seen when quantifying splenic GCs with histology from WT and $CD1d^{-/-}$ mice injected four times with apoptotic cells (Fig. 3 D). Thus, we conclude that CD1d on autoreactive B cells can directly regulate their activation to apoptotic cell–derived antigens; the data suggests that this occurs before GC entry.

A CD1d-dependent peripheral tolerance checkpoint for GC B cells

The results suggested that iNKT cell interaction with CD1d on autoreactive B cells could directly limit their autoantibody

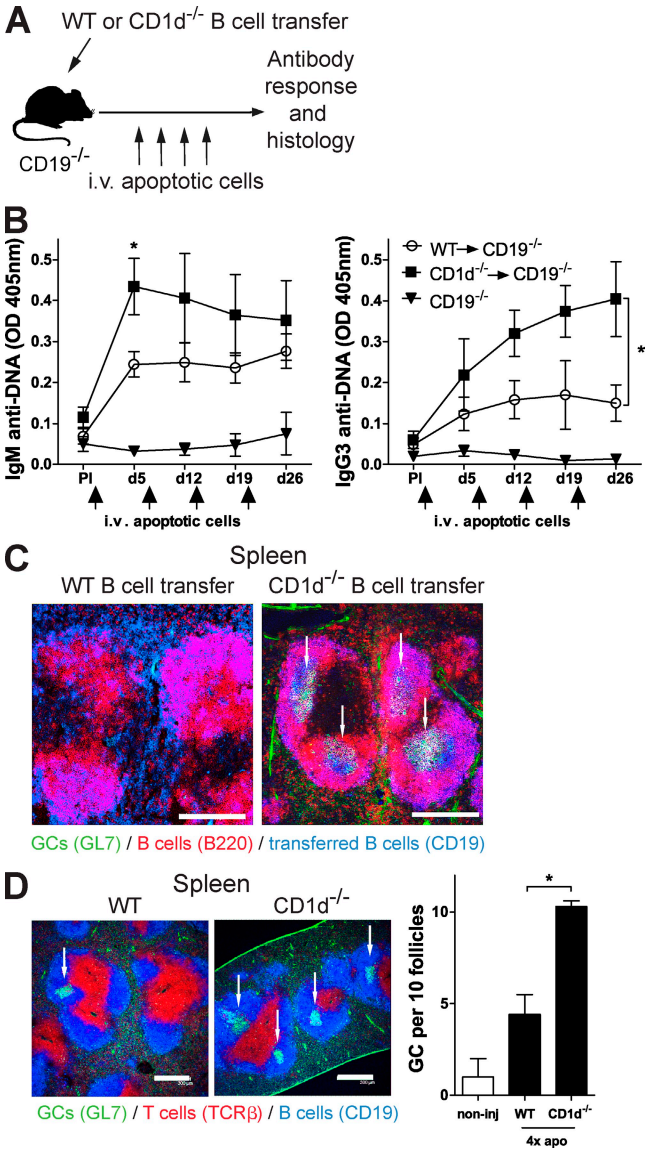

Figure 3. Increased autoantibody production from adoptively transferred splenic $CD1d^{-/-}$ B cells. (A) Schematic illustration of the transfer of purified B cells from WT or $CD1d^{-/-}$ mice to a $CD19^{-/-}$ recipient, followed by four injections of apoptotic cells. (B) IgM and IgG3 anti-DNA measured by ELISA in apoptotic cell–injected recipients and control mice. (C) Representative splenic immunofluorescence image from transferred WT B cells and $CD1d^{-/-}$ B cells at day 26. Transferred cells are visualized by their CD19 and B220 expression in contrast to the recipient B cells ($CD19^{-/-}$) only expressing B220. GCs are visualized by GL7 staining and indicated by arrows. (D) Representative splenic GC histology of WT and $CD1d^{-/-}$ mice injected four times with apoptotic cells. Bars: (C) 150 µm; (D) 300 µm. Results are shown as mean ± SEM in B (n = 6–12 for recipient groups; n = 4 for $CD19^{-/-}$ without transfer) and D (n = 6). *, $P < 0.05$. Data are representative of at least two independent experiments.

production. To more carefully investigate the GC phenotype, mixed BM chimeric mice were created using WT and CD1d$^{-/-}$ BM and subsequently injected with apoptotic cells (Fig. 4 A). The B cells in the chimeric mice do, side by side, experience the same conditions during the injections, allowing for direct examination of the role of CD1d on them. After only two injections of apoptotic cells, a significantly higher proportion of the CD1d$^{-/-}$ B cells had a GC phenotype (Fig. 4, B–D), which was even more pronounced after four injections (Fig. 4 E). To control whether the increased GC phenotype was a general phenomenon in the absence of iNKT cells, two experiments were performed. First, we analyzed GC B cells in Peyer's patches (a location with high levels of spontaneous GC B cells, because of the influence of the gut microbiota) in mixed BM chimeras created as described above (Fig. 4 F). Second, we examined the levels of splenic GC B cells after two injections of NP-OVA (as described in Fig. 1 G) in WT and CD1d$^{-/-}$ mice (Fig. 4 G). No difference was seen between WT and CD1d$^{-/-}$ mice in these two experiments, showing that CD1d does not have a general effect on the levels of GC B cells. Furthermore, examining CD1d expression on B cells in WT mice injected with apoptotic cells showed an impressive down-regulation of CD1d on the triggered GC B cells and an up-regulation on MZB cells (Fig. 4 H). We conclude that a CD1d-dependent peripheral tolerance checkpoint exists, which regulates the activation of autoreactive B cells against apoptotic material, and that this checkpoint most probably is located before the GC entry. Interestingly, a

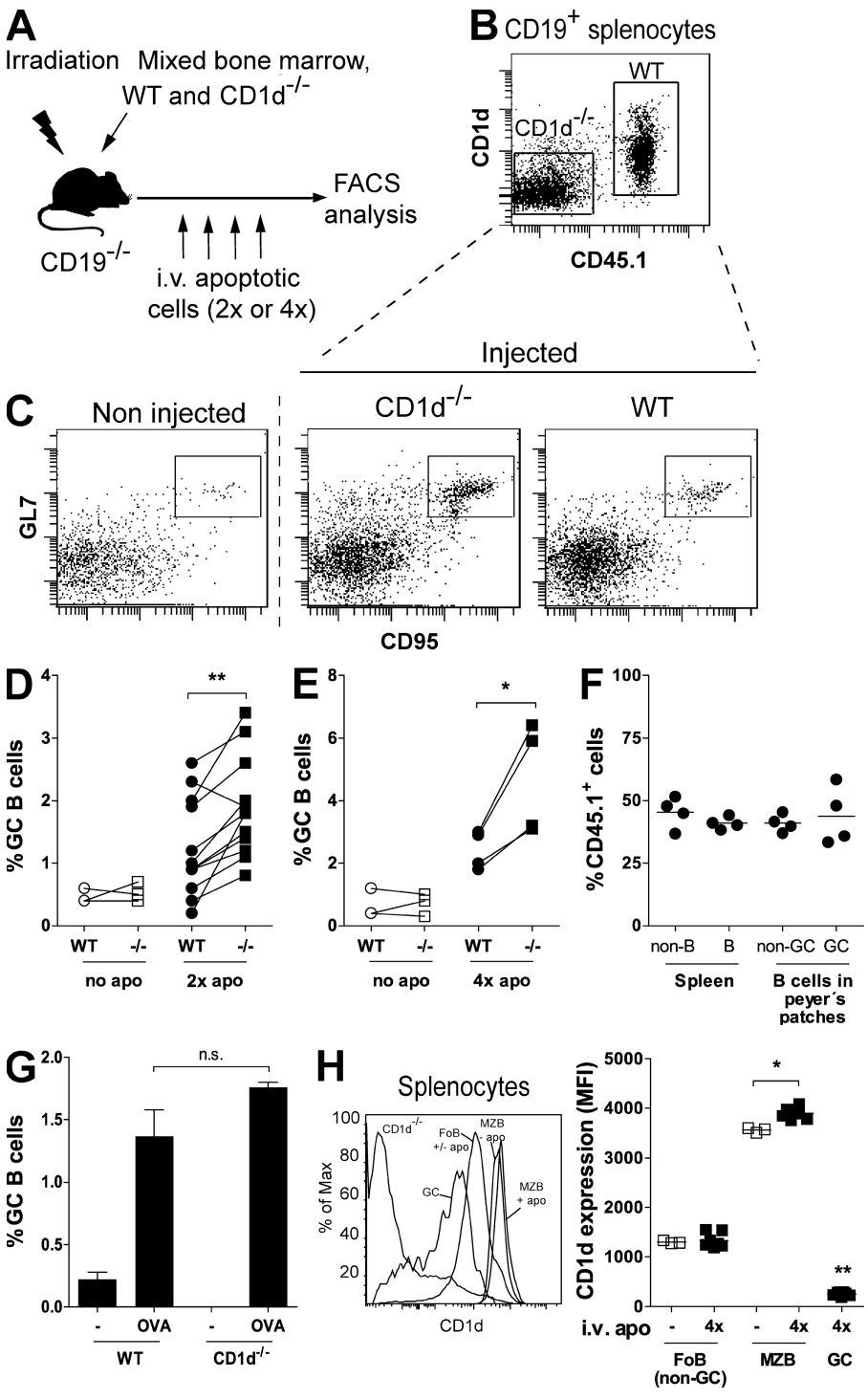

Figure 4. A CD1d-dependent peripheral tolerance checkpoint limits GC B cell levels. (A) Schematic illustration of the experimental setup comparing GC entry after injections of apoptotic cells in mixed WT and CD1d$^{-/-}$ BM chimeric mice. (B and C) Representative FACS gating of splenic GC B cells (GL7^{hi} and CD95^{hi}) from noninjected and injected mice of the indicated genetic background (WT = CD45.1^{+} and CD1d$^{-/-}$ = CD45.1^{-}). Data shown as percentage of B cells with GC phenotype of total B cells with the indicated genotype after two (D) or four (E) injections. Lines connect the CD1d$^{-/-}$ and the WT populations in individual mice. n = 3–10. (F) Examination of the role of CD1d expression on GC B cell levels in Peyer's patches in a mixed BM chimera, as in B and C. (G) Splenic GC B cells in WT and CD1d$^{-/-}$ after the second injection of NP-OVA (see Fig. 1 G for protocol) measured by FACS. (H) FACS staining of CD1d expression on WT splenocytes injected four times with apoptotic cells. FoB, MZB, and GC cells gated as in Fig. 1 E and Fig. 4 C. Results are shown as individual mice and mean in F (n = 4) and H (n = 3–7) and mean ± SEM in G (n = 4–6). *, P < 0.05; **, P < 0.01. Data are representative of two independent experiments.

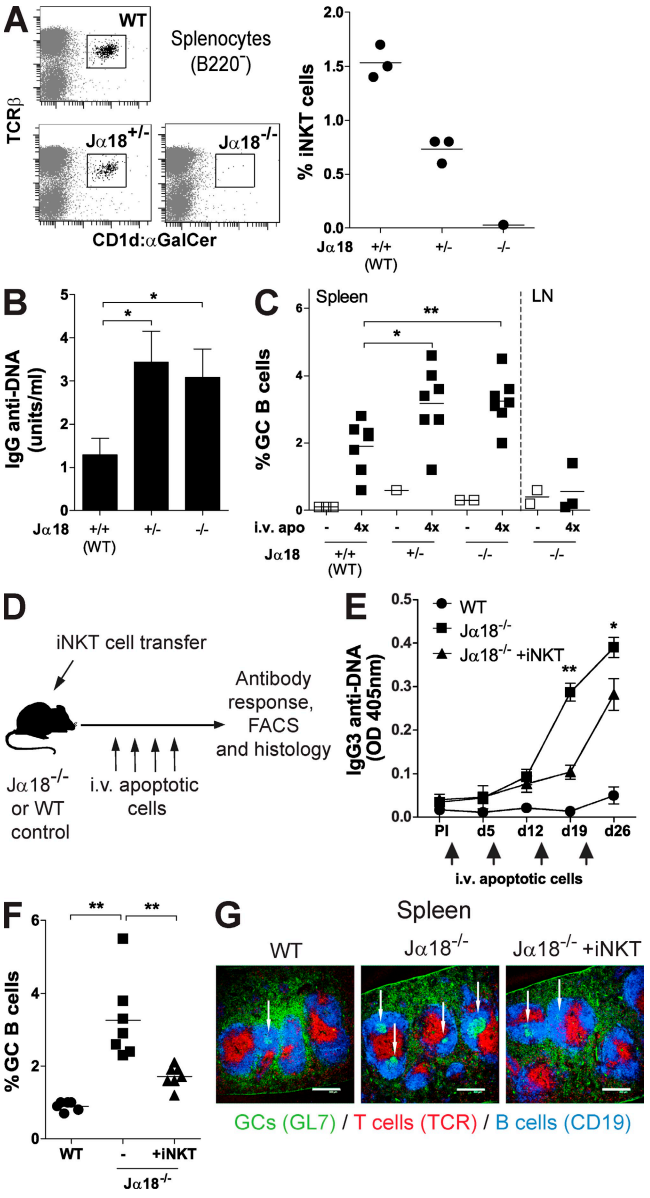

Figure 5. Reduced levels of iNKT cells are sufficient to see increased autoreactive B cell activation to injected apoptotic cells, and this can be rescued by transferring iNKT cells. (A) FACS analysis of the splenic iNKT cell population (TCRβ$^+$ and αGalCer loaded CD1d-dimer$^+$) in WT, Jα18$^{+/-}$ and Jα18$^{-/-}$ mice. (B) IgG anti-DNA response and (C) analysis of splenic and lymph node GCs after four injections of apoptotic cells in mice described in (A). (D) Schematic illustration of the iNKT cell transfer to a Jα18$^{-/-}$ recipient followed by four injections of apoptotic cells. (E) IgG3 anti-DNA response in mice described in (D) measured by ELISA. (F) Quantification of splenic GC B cells with FACS day 26. (G) Representative histology of splenic GCs (GL7$^+$, indicated by arrows) day 26. Bars, 300 µm. Results are shown as individual mice and mean in A (n = 3), C (n = 1–7), and F (n = 7) and mean ± SEM in B (n = 7) and E (n = 7). *, $P < 0.05$; **, $P < 0.01$. Data are representative of two independent experiments (A–C) or pooled from two independent experiments (D–G).

similar B cell activation checkpoint has been described to be defective in SLE patients (Cappione et al., 2005).

Reduced numbers of iNKT cells is sufficient to see increased autoreactive B cell activation, and this can be rescued by transfer of iNKT cells to deficient mice

Examination of mice with total absence of iNKT cells in disease models can give insights in the role of this cell population. One can, however, argue that the translation to human disease can be problematic, as the link to disease in e.g., SLE patients is to a reduced level of iNKT cells, not to complete absence. We therefore made mice heterozygous for the Jα18 deletion (i.e., Jα18$^{+/-}$) and examined their response to injections of apoptotic cells. Deletion of one Jα18 allele resulted in a reduction of almost exactly 50% of the peripheral iNKT population (Fig. 5 A). This reduction was somewhat unexpectedly sufficient to change the phenotype to that of the Jα18$^{-/-}$ mice, with significantly increased IgG anti-DNA levels and splenic germinal center B cell levels (Fig. 5, B and C). This finding supports that a reduction in iNKT cells is enough to affect disease parameters in lupus patients. As we propose that the B cells and the iNKT cells interact as the activated B cells migrate toward the GC, the reduced iNKT cell population might not be sufficient to interact with the B cells during the limited period before the B cell down-regulate CD1d.

To further establish the potential role for iNKT cells as a therapeutic target in SLE patients, we performed a transfer of iNKT cells to Jα18$^{-/-}$ mice and observed whether this could ameliorate the autoreactive response to injections of apoptotic cells (Fig. 5 D). The transferred population was enriched for untouched iNKT by purification from Vα14-Cα$^{-/-}$ mice (Lehuen et al., 1998). These mice express a transgenic TCRα chain used by iNKT cells (Vα14-Jα18) on a TCRα chain–deficient background. Repopulation of the iNKT cell population showed significant effects on all the autoreactive B cell activation parameters previously studied, including anti-DNA response (Fig. 5 E) and both the size of the GC

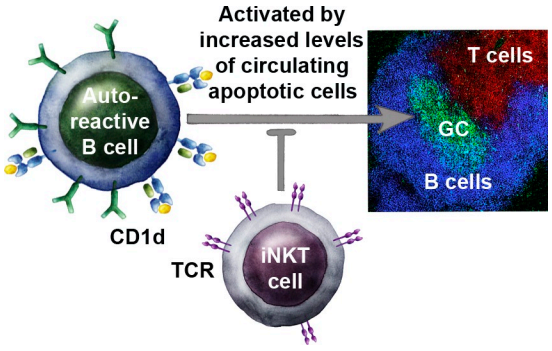

Figure 6. Schematic illustration describing that iNKT cells limit the levels of autoreactive GC B cells, and thereby autoantibody production in response to an increased load of circulating apoptotic cells.

B cell population (Fig. 5 F) and the amount of GCs (Fig. 5 G and Fig. S5).

These data suggest the presence of an inhibitory iNKT–CD1d$^+$ B cell axis limiting autoreactive response to increased levels of apoptotic cells. The need for CD1d on B cells suggests that the iNKT cell recognizes ligands presented in CD1d that, at the time, is interpreted as a signal to limit B cell activation. Explanations for this include presentation of inhibitory ligands, or that the iNKT cell is affected by other factors, like TLR engagement or cytokines, to perceive the ligand as inhibitory. Several candidate lipid derivatives, relevant for this study, have been shown to be presented in CD1d context, e.g., the phospholipid phosphatidylcholine (PC) and its derivative lyso-PC, which is released during apoptosis (Giabbai et al., 2005; Chang et al., 2008). Furthermore, lyso-PC–specific type II NKT cells have been identified and are expanded in myeloma patients (Chang et al., 2008). However, it is not known if this population could be antiinflammatory, although the tumor-associated lipid derivative gangliotriaosylceramide has been confirmed to mediate immunosuppressive activity on iNKT cell activation (Sriram et al., 2002).

In conclusion, we suggest a model where iNKT cells are linked to the anti-inflammatory milieu created by apoptotic cell death. In response to dying cells they acquire an antiinflammatory phenotype and are able to limit activation of autoreactive B cells (Fig. 6). The exact mechanism involved is not completely clear, hampered by the lack of knowledge about the precise nature of the B cell activation, but seems to involve both effects on cytokines and direct B cell survival. In the absence of iNKT cells, or CD1d expression on B cells, the balance in the response to an increased load of apoptotic cells is shifted. This is manifested as increased autoreactive B cell activation with increased GC B cell levels and autoantibody production. SLE is a multigenetic disease and subgroups displaying different symptoms can readily be found in the patient group (Kotzin, 1996). Our data indicate that iNKT cells could have an important protective role in patients where autoreactivity is driven by defects in apoptotic cell homeostasis, and that this could be modulated by increasing the iNKT cell numbers.

MATERIALS AND METHODS

Mice. CD1d$^{-/-}$ (Chen et al., 1997) and Jα18$^{-/-}$ (Cui et al., 1997) mice on C57BL/6 background were provided by M. Johansson and P. Höglund (Karolinska Institutet, Stockholm, Sweden). iNKT transgenic Vα14-Cα$^{-/-}$ mice (Lehuen et al., 1998) were provided by A. Lehuen (INSERM U561, Paris, France). CD19$^{-/-}$ mice (Rickert et al., 1995) were kindly provided by N. Lycke (Göteborg University, Göteborg, Sweden). C57BL/6 mice congenic for CD45.1 were obtained from Charles River Laboratories. Age- and sex- matched mice were used in all experiments, aged 6–12 wk. Animals were kept and bred under pathogen-free conditions and experiments were approved by the local ethical committee (the North Stockholm district court).

Injections, antigens, and ELISA. Apoptosis was induced in syngenic thymocytes with dexamethasone (Sigma-Aldrich) as previously described (Wermeling et al., 2007), with the modification that thymi were collected from CD1d-deficient mice. This was to eliminate the transfer of apoptotic iNKT cells and CD1d-expressing cells. Mice were injected i.v. with 10^7 apoptotic cells weekly and bled for 4 wk. TNP-Ficoll and NP-OVA (Biosearch Technologies, Inc.) were injected i.v. (20 μg/mouse) in PBS and i.p. (50 μg/mouse) in alum (Thermo Fisher Scientific), respectively.

Antibody levels against DNA and TNP-Ficoll were measured with ELISA as previously described (Wermeling et al., 2007) and reactivity against the injected NP-OVA was measured using NP-BSA (Biosearch Technologies, Inc.) coated ELISA plates (Thermo Fisher Scientific). Reactivity against PC was measured with PC-BSA (a gift from Athera Biotechnologies AB) and cardiolipin reactivity was measured with cardiolipin purchased from Sigma-Aldrich as described in (Delgado Alves et al., 2003) using standard ELISA techniques and secondary reagents anti-IgG/IgG3/IgM-AP (SouthernBiotech). IL-10 and IFN-γ were measured in supernatants following the suggested protocol (MabTech).

Adoptive transfer and BM chimeras. Naive splenic B cells were isolated from WT and CD1d$^{-/-}$ mice using CD43, NK1.1, and CD11c microbeads (Miltenyi Biotec). Naive B cells were collected as the negative population and purity was confirmed with FACS. 3×10^7 purified B cells were injected i.v. in recipient CD19$^{-/-}$ mice, and the next day apoptotic cell injections started. Untouched iNKT cells were enriched from the liver and spleen of Vα14-Cα$^{-/-}$ mice (Lehuen et al., 1998) using established methods (Bezbradica et al., 2006), i.e., depletion of B220$^+$, CD8$^+$, CD11c$^+$, and CD11b$^+$ cells using microbeads (Miltenyi Biotec). This isolation technique provides mainly iNKT cells and some CD4$^+$ T cells (Fig. S6). Approximately 0.8×10^6 untouched viable iNKT cells were transferred to each recipient and apoptotic cell injections started the following day.

CD19$^{-/-}$ mice were lethally irradiated (900 rad) and reconstituted i.v. with 10^6 mixed BM cells from CD1d$^{-/-}$ (CD45.2$^+$) and C57BL/6-CD45.1 mice. 7 wk after BM transfer, the mice were sacrificed and analyzed for chimerism with FACS or injected with apoptotic cells as described above.

FACS. Single-cell suspensions of organs were prepared and erythrocytes were lysed. Nonspecific labeling was blocked with anti-CD16/32 (Fc Block) before specific labeling. Samples were analyzed using a FACSCalibur or a FACSAria (BD). iNKT cells were labeled using αGalCer (KRN7000; Larodan Fine Chemicals) loaded CD1d-dimerX as suggested by the manufacturer (BD), and defined as viable (DAPI$^-$), B220$^-$, TCRβ$^+$ CD1d:αGalCer$^+$ lymphocytes. For intracellular cytokine staining, single-cell suspensions of splenocytes were cultured in complete medium for 5 h in the presence of PMA (50 ng/ml), ionomycin (500 ng/ml; both from Sigma-Aldrich), and Brefeldin A (BioLegend). In Fig. S2, the stimulation protocol was adopted from a previous study (Yanaba et al., 2008), also including LPS (10 μg/ml; Sigma-Aldrich). Extracellular proteins were then labeled and after several washing steps, fixation and permeabilization (buffers from BioLegend) followed. Intracellular IFN-γ was labeled (antibody clone XMG1.2) and samples were analyzed on a FACSAria (BD). LIVE/DEAD was used to remove dead cells when intracellular staining was performed before the fixation step, as suggested by the manufacturer (Invitrogen). All FACS plots show log10 fluorescence.

Ex vivo stimulation and histology. Cytokine and antibody release was measured from splenocyte cultures after 72 h in complete media with αGalCer (100 ng/ml) or 10 μg/ml LPS (Sigma-Aldrich) stimulation. Immunohistology was performed as previously described (Wermeling et al., 2007). H&E staining was performed using standard protocol. Immune complex deposition in kidneys was quantified by three blinded observers and arbitrarily scored from 0–3 dependent on the intensity and amount of the anti-IgG staining. Quantification of GC was done by one blinded observer counting GC (GL7) staining and total amount of follicles (CD19$^+$ cells surrounding TCRβ$^+$ cells). More than 20 follicles were counted on each sample, and a number corresponding to the amount of GCs per 10 follicles was calculated. Antibodies for FACS and histology were obtained from BD or BioLegend.

Statistical analysis. Data were compared using nonparametric tests; Kruskal-Wallis test followed by Dunns post-test (Fig.1), Mann-Whitney test

(Figs. 2, 3, 4 H, and 5), and Wilcoxon matched pairs test (Fig. 4, D and E). $P < 0.05$ was considered significant.

Online supplemental material. Fig. S1 shows CD1d expression on splenic B cell and DC subsets. Fig. S2 shows IL-10$^+$ B and T cells, and Fig. S3 shows IFN-γ producing CD4$^+$ T cells by intracellular FACS after four injections of apoptotic cells and ex vivo stimulation. Fig. S4 shows the remaining splenic MZB in B cell transferred mice injected four times with apoptotic cells. Fig. S5 shows histological quantification of GCs in spleens of Jα18$^{-/-}$ mice that received an iNKT cell transfer. Fig. S6 shows the transferred population enriched for iNKT cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091314/DC1.

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