Invariant natural killer T cells in lupus patients promote IgG and IgG autoantibody production

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IgG autoantibodies, including antibodies to double-stranded DNA (dsDNA), are pathogenic in systemic lupus erythematosus (SLE), but the mechanisms controlling their production are not understood. To assess the role of invariant natural killer T (iNKT) cells in this process, we studied 44 lupus patients. We took advantage of the propensity of PBMCs from patients with active disease to spontaneously secrete IgG in vitro. Despite the rarity of iNKT cells in lupus blood (0.002–0.05% of CD3-positive T cells), antibody blockade of the conserved iNKT TCR or its ligand, CD1d, or selective depletion of iNKT cells, inhibited spontaneous secretion of total IgG and anti-dsDNA IgG by lupus PBMCs. Addition of anti-iNKT or anti-CD1d antibody to PBMC cultures also reduced the frequency of plasma cells, suggesting that lupus iNKT cells induce B-cell maturation. Like fresh iNKT cells, expanded iNKT-cell lines from lupus patients, but not healthy subjects, induced autologous B cells to secrete antibodies, including IgG anti-dsDNA. This activity was inhibited by anti-CD40L antibody, as well as anti-CD1d antibody, confirming a role for CD40L-CD40 and TCR-CD1d interactions in lupus iNKT-cell-mediated help. These results reveal a critical role for iNKT cells in B-cell maturation and autoantibody production in patients with lupus.

Keywords: Autoantibodies · B cells · IFN-γ · iNKT cells · Lupus

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by inflammation of multiple organs and uncontrolled production of autoantibodies [1]. The serological hallmark of this disease is the presence of high levels of autoantibodies against nuclear antigens. These autoantibodies, especially those directed against double-stranded DNA (dsDNA), are thought to contribute directly to the pathogenesis of SLE [2]. Studies in both humans and animal models have shown that T cells, especially CD4+ T cells, can promote pathogenic autoantibody production by “helping” B cells [3, 4]. The presumptive explanation for this helper activity is that conventional CD4+ T cells, recognizing a particular MHC class II associated autoantigen, interact with B cells

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that have receptors for the same autoantigen, resulting in reciprocal stimulation of T and B cells and the production of autoantibody. NK cells of the innate arm of the immune system have also been reported to help B cells produce IgG [5, 6] and it is possible that such cells may play a role in the development of pathogenic autoantibodies in lupus.

Invariant natural killer T (iNKT) cells in humans are a rare and distinct subset of lymphocytes that share characteristics of both conventional T cells (CD3 and TCR α/β) and NK cells (CD161). They express an invariant TCR α chain (Vα24Jα18) coupled with a variable Vβ11 TCR β chain, and recognize self and exogenous glycolipid antigens presented by CD1d, a nonclassical MHC class I-like antigen-presenting molecule [7]. The most widely studied iNKT-cell agonist is α-galactosylceramide (αGalCer), a marine sponge-derived glycolipid that can be used to selectively activate iNKT cells in vitro and in vivo [8, 9]. Since iNKT cells display a constitutive effector memory phenotype, recognize endogenous glycolipids, and rapidly produce immunoregulatory cytokines, including IL-4 and IFN-γ, upon activation, they are regarded as members of both the innate and adaptive immune system [10]. After glycolipid activation, iNKT cells can also help B-cell proliferation and antibody production in vitro and in vivo in a CD1d-restricted manner [11–14].

The role of iNKT cells in lupus is controversial, as studies in SLE animal models have yielded conflicting results. On the one hand, T cells expressing a transgenic anti-CD1d TCR induced lupus nephritis after transfer into BALB/c nude mice [15]. Treatment of NZBxNZW mice with anti-CD1d mAb or β-galactosylcyeramide to block iNKT-cell function ameliorated lupus and decreased serum levels of IgG2a and anti-dsDNA antibodies [16–18]. Moreover, iNKT cells, but not conventional CD4+ T cells, from NZBxNZW mice with active disease helped B cells to secrete IgG anti-dsDNA antibody via recognition of CD1d on B cells [19]. On the other hand, CD1d−/− NZBxNZW mice developed more severe disease than their wild type littermates [20]. Similarly, in MRL-lpr/lpr mice CD1d deficiency led to exacerbation of skin disease [21], and recent studies in other models revealed that activated iNKT cells can inhibit autoantigen-specific B cells and reduce IgG autoantibody production [22, 23]. Taken together, these findings suggest that iNKT cells may have different effects on lupus in mice, depending on the strain and type or stage of disease.

The relevance of murine lupus models to human SLE is uncertain. Because of their rarity in peripheral blood, human iNKT cells are difficult to study. The situation in SLE is especially challenging, as the frequency of iNKT cells in the blood of lupus patients is decreased relative to that in healthy subjects and the extent of the decrease is related to disease severity [24–27]. Nonetheless, iNKT cells can be extremely potent on a per cell basis and in the current study we took advantage of this property to investigate their role in the regulation of immunoglobulin production in SLE. The results show that iNKT cells from lupus patients, but not conventional CD4+ T cells from the same patients, are potent inducers of IgG and anti-dsDNA IgG autoantibody production. The phenotype and function of these iNKT cells are similar to those of iNKT cells that promote autoantibody production and disease progression in mice [16–19].

**Results**

**PBMCs from lupus patients with active disease spontaneously secrete immunoglobulin**

Previous studies have demonstrated that freshly isolated PBMCs from lupus patients secrete immunoglobulin in the absence of exogenous stimuli [28–31]. In our initial studies, we isolated PBMCs from 23 SLE patients and after culturing these cells for 10 days in the absence of human serum, we measured the level of IgG in the supernatant by ELISA. Significant amounts of IgG were detected in the culture supernatants from 11 of these patients, but not from any of the 10 age- and gender-matched healthy subjects. There was no difference between lupus patients and healthy subjects in the viability of B cells and plasma cells at the beginning or end of the culture period (data not shown), ruling out dead or dying B cells as a significant source of IgG. There was a strong correlation between the amount of IgG secreted and the SLEDAI score ($r_s = 0.6022$, $p = 0.0024$ by Spearman Rank Test) (Fig. 1A). A similar association could also be seen when comparing patients with active (SLEDAI ≥6) versus inactive or minimally active (SLEDAI <6) disease ($p < 0.01$) (Fig. 1B) or when comparing patients receiving ≥10 mg per day of prednisone (who had more severe disease) versus those receiving lower doses or no prednisone ($p < 0.05$) (Fig. 1C).

**Spontaneous immunoglobulin secretion by lupus PBMCs is dependent on iNKT cells**

To assess the possibility that iNKT cells affect spontaneous IgG production in SLE, we selected patients with SLEDAI ≥6 who were positive for spontaneous IgG production and cultured their freshly obtained PBMCs for 10 days in the presence of various blocking mAbs directed at molecules on B cells or iNKT cells, and measured IgG secretion in culture supernatants. Anti-CD1d mAb, but not neutralizing mAbs directed at other molecules on B cells (HLA class I and HLA class II) or the isotype control mAbs, inhibited IgG production by lupus PBMCs (Fig. 2A). In the five lupus patients tested in this manner, anti-CD1d mAb inhibited IgG production by an average of 57%. The PBMCs of these patients produced spontaneous IgM and IgA in addition to IgG, and the secretion of all three classes of antibody was markedly inhibited by anti-CD1d antibody (Fig. 2A and B). A combination of anti-TCRα24 and TCRβ11 mAb, or a single antibody (clone: 6B11) specific for the conserved CDR3 domain of Vα24, or depletion of iNKT cells with anti-TCRα24 antibody coated magnetic beads, all reduced spontaneous IgG secretion by >50% (Fig. 2C-E). By contrast, antibodies of the same subclass (IgG1) directed at CD2 and CD4, molecules that are expressed on both iNKT cells and conventional T cells, had
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Figure 1. Spontaneous immunoglobulin secretion by SLE patient PBMCs correlates with disease activity. PBMCs from SLE patients \( n = 23 \) were cultured for 10 days in medium devoid of human serum. IgG was measured by ELISA in the culture supernatants. (A) Correlation between level of spontaneous IgG production and disease activity (SLEDAI score) was analyzed with the Spearman’s rank correlation test \( r_s = 0.6022, p = 0.0024 \). Each patient was analyzed once. (B) Comparison of spontaneous IgG production between patients with active disease (SLEDAI < 6, \( n = 16 \) samples) and active disease (SLEDAI ≥ 6, \( n = 7 \) samples). (C) Comparison of spontaneous IgG levels between patients treated with no or low dose prednisone treatment (<10 mg/day, \( n = 16 \) samples) and higher dose treatment (>10 mg/day, \( n = 7 \) samples). Each data point represents one individual sample studied in triplicate. (B and C) Data are shown as mean ± SD and (A–C) are pooled from five independent experiments. * \( p < 0.05 \); ** \( p < 0.01 \); Mann–Whitney test.

no effect. FACS analysis confirmed that SLE B cells express CD1d, the main antigen-presenting molecule recognized by iNKT cells, at about the same level as B cells of healthy subjects (Supporting Information Fig. 1). CD1c was also expressed on SLE B cells, but at a slightly lower level than that of healthy control B cells (Supporting Information Fig. 1).

PBMCs of lupus patients with active disease may also spontaneously secrete anti-dsDNA antibody [28–31]. As shown in Figure 2F, PBMCs from patients with active SLE secreted IgG anti-dsDNA antibody. After the cells were cultured for 10 days in the presence of anti-CD1d or anti-iNKT (6B11) mAbs, the frequency of autoantibody secreting cells was reduced by >70% and 50%, respectively, whereas mAbs directed at CD2 and CD4 had no effect. MAb-directed at HLA-class I or HLA-class II, which are expressed on B cells, monocytes and dendritic cells, also did not affect the frequency of anti-dsDNA IgG secreting cells. Finally, when lupus PBMCs were cultured in the presence of anti-iNKT or anti-CD1d antibody, the development of plasma cells, defined as CD19dimCD20−CD27highCD138+ cells, was markedly reduced (Fig. 2G). These results suggest that interaction between the iNKT-cell TCR and its CD1d ligand is required for maturation of B cells into plasma cells and spontaneous secretion of IgG and anti-dsDNA antibody by lupus PBMCs.

Lupus iNKT cells are rare in peripheral blood but secrete large amounts of IFN-γ

Despite the potent B-cell stimulatory activity of iNKT cells in our lupus cohort, the frequency of these cells in peripheral blood was decreased compared with those in healthy age- and gender-matched subjects (0.02% vs. 0.05% of CD3+ T cells), which is in accord with prior reports (Fig. 3A). Nonetheless, CD4+CD8−, CD4+CD8+, and CD4−CD8+ iNKT populations were present in similar proportions in lupus patients and healthy subjects (Fig. 3A). To study cytokine production by iNKT cells, we stimulated PBMCs with αGalCer and 6 days later performed intracellular staining of IFN-γ, IL-4, IL-10, IL-17, and IL-21. The percentage of IFN-γ+ iNKT cells in SLE patients (mean 16%) was higher than that in healthy controls (mean 4%) (Fig. 3B). There was no difference in the frequency of iNKT cells expressing several other cytokines between SLE patients and healthy controls (Fig. 3B). Despite the excess production of IFN-γ by iNKT cells, addition of up to 20 μg/mL anti-IFN-γ blocking antibody to SLE patient PBMC cultures had no effect on IgG production (Fig. 3C).

Expanded iNKT-cell lines from SLE patients help autologous B cells produce IgG and anti-dsDNA IgG

To study pure populations of iNKT cells, we stimulated PBMCs from lupus patients and healthy subjects with αGalCer and expanded the cells for 30 days in the presence of recombinant human (rh) IL-2, rhIL-15 and irradiated autologous PBMCs, and then FACs-purified iNKT cells with antibodies to TCRVα24 and TCRβ11. Using this approach, we obtained expanded iNKT-cell lines from nine female SLE patients who were positive for serum anti-dsDNA IgG and five healthy female control subjects. All of the expanded iNKT cells from lupus patients and healthy controls stained positively with a CD1d-αGalCer tetramer as well as with 6B11 mAb and were comprised mainly of CD4+CD8− cells (Fig. 3D and E). iNKT lines from SLE patients produced more than 12 times the amount of IFN-γ (mean 64.3 ng/mL) produced by iNKT cells from healthy controls (mean 4.9 ng/mL) \( (p < 0.01) \), while there was no significant difference in IL-4 production between...
Figure 2. Spontaneous immunoglobulin production by SLE PBMCs is dependent on iNKT cells. PBMCs from SLE patients were cultured for 10 days in the presence of various neutralizing antibodies or their isotypes. (A and C) IgG (n = 5 samples/group) and (B) IgM and IgA (n = 3 samples/group) concentration was measured by ELISA in culture supernatants. (D) Frequency of iNKT cells, as a percentage of CD3+ T cells, before and after anti-TCRVα24 depletion was determined by flow cytometry. Data are representative of five independent experiments. (E) iNKT cells were depleted from lupus PBMCs with anti-TCRVα24 antibody coated beads (TCRVα24DEP) and spontaneous IgG production by iNKT-depleted PBMCs was measured by ELISA (n = 5 samples/group). (F) B-cell ELISPOT assay was used to detect anti-dsDNA IgG antibody forming cells (AFCs) among SLE PBMCs in the presence or absence of the indicated antibody. The mean number of anti-dsDNA IgG AFC per 10^6 PBMCs is shown (n = 4 samples/group). (G) Frequency of plasma cells in lupus PBMCs cultured for 1 week in the presence or absence of anti-iNKT or anti-CD1d antibody (n = 3 samples/group). (A–C and E–G) Data are shown as mean + SD of three to five samples, and are pooled from (A, C, and E) five, (B and G) three or (F) four independent experiments. *p < 0.05, **p < 0.01; paired t-test.

caption
Figure 3. iNKT cells are rare in lupus patients but expand after αGalCer stimulation. (A) Frequency of iNKT cells (left) and CD4/CD8 distribution (right) on fresh iNKT cells in SLE patients \(n = 13\) and healthy individuals \(n = 18\). (B) Production of IFN-γ, IL-4, IL-10, IL-17, and IL-21 by iNKT cells was analyzed by intracellular staining of PBMCs stimulated with αGalCer for 6 days \(n = 7\) for healthy subjects, \(n = 10\) for SLE patients, except for IL-10 where \(n = 4\) for SLE patients. (C) Total IgG in the supernatant of SLE PBMCs cultured in the presence of blocking antibody against IFN-γ. (n = 5 samples/group) (D) Characterization of expanded iNKT cells \(n = 3\) samples/group) by staining with anti-TCR Vα24/TCR Vβ11, anti-iNKT (clone 6B11), and human CD1d-αGalCer tetramer. (E) Expression of CD4 and CD8 on expanded iNKT cells from healthy subjects \(n = 5\) and SLE patients \(n = 5\). (F) IFN-γ and IL-4 expression in activated iNKT cells. Left: Expanded iNKT cells stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) for 4 h were analyzed by FACS for intracellular IFN-γ. Right: Expanded iNKT cells \(5 \times 10^4\) cells/well) were stimulated with plate-bound anti-CD3 mAb for 24 h. IFN-γ and IL-4 in the supernatants were measured by ELISA. The results represent mean values of triplicate cultures from nine lupus patients and five healthy subjects. Each symbol represents one individual sample. (A, C, E–G) Data are shown as mean ± SD and (D) is representative of three independent experiments, (A, C, and E–G) are pooled from five independent experiments, (B) is pooled from four independent experiments. * \(p < 0.05\), ** \(p < 0.01\); paired t-test.
autologous B cells secrete IgM and IgA through their recognition of CD1d.

Isolated B cells from SLE patients spontaneously secreted slightly higher levels of IgG, IgM, and IgA than healthy controls. When lupus B cells were cocultured with autologous iNKT-cell lines, production of IgM, IgA, IgG was significantly increased ($p < 0.05$), especially in the case of IgG where the increase was more than tenfold (Fig. 4A). Moreover, the antibody production induced by iNKT lines was markedly inhibited by anti-CD1d mAb. More importantly, supernatants from cocultures of lupus iNKT lines and B cells had significantly higher concentrations of anti-dsDNA IgG than the B cells alone, while the coculture of iNKT lines

**Figure 4.** Expanded iNKT cells from SLE patients, but not healthy subjects, can induce IgG production. (A) B cells were cultured for 10 days with expanded autologous iNKT cells from healthy subjects ($n = 5$) or lupus patients ($n = 5$) in the presence or absence of anti-CD1d or isotype control antibody. (B) B cells from healthy subjects were cocultured with autologous iNKT cells or iNKT cells from SLE patients for 10 days in the presence of anti-CD1d or isotype control antibody. Alternatively, B cells from SLE patients were cocultured with autologous iNKT cells or iNKT cells from healthy donors. ($n = 3$ samples/group) (C) B cells from SLE patients were stimulated with PWM and SAC in the presence of anti-CD1d, anti-iNKT (6B11) or anti-iNKT TCR (TCRα24/TCRβ11) antibodies for 10 days. IgG in the supernatant was measured by ELISA. ($n = 3$ samples/group) (D) To assess the ability of iNKT cells from lupus patients to induce Ig class switching, sorted IgM+CD27−CD19+ naive B cells were cultured with autologous iNKT cells for 10 days in the presence or absence of anti-CD1d antibody. Left panel shows total IgG and IgM, and right panel shows anti-dsDNA IgG. ($n = 3$ samples/group). (A–D) Immunoglobulins and antibodies in the supernatant were measured by ELISA. Data are shown as mean ± SD of three to five samples and is pooled from (A) five or (B–D) three independent experiments. *$p < 0.05$, **$p < 0.01$, paired t-test.
and B cells from healthy controls produced no detectable anti-dsDNA IgG (Fig. 4A). We also cultured iNKT lines from patients with freshly isolated B cells from healthy individuals, or iNKT lines from healthy subjects with B cells from lupus patients, but IgG production was not increased in either case compared with the B cells cultured alone (Fig. 4B), suggesting that both iNKT cells and B cells from SLE patients are required for antibody production under these conditions. To rule out nonspecific effects of the anti-CD1d and anti-TCR antibodies used in our studies, we activated purified B cells from SLE patients with pokeweed mitogen (PWM) or Staphylococcus aureus Cowan Strain I (SAC) in the presence of these antibodies. The results showed that both PWM and SAC induced IgG production and anti-CD1d or anti-iNKT TCR antibodies had no effect. (Fig. 4C).

B cells that have been activated by their specific antigens undergo maturation and become either memory IgM, IgA or IgG producing cells. This step is believed to be critical in the pathogenesis of SLE because IgG, but not IgM, anti-dsDNA antibodies can deposit in the kidney and induce destructive inflammation [32]. When purified IgM+CD27−CD19+ naïve B cells from active SLE patients were cocultured with expanded autologous iNKT cells, they produced IgG antibody (Fig. 4D), suggesting that they had been induced to switch from IgM to IgG production by iNKT cells. We also found that expression of activation-induced cytidine deaminase (AID) in SLE B cells cocultured with iNKT cells was much higher than in B cells cultured alone, confirming Ig class switch in SLE B cells cocultured with iNKT cells (Supporting Information Fig. 2). Anti-CD1d antibody inhibited IgG and IgM production, again demonstrating that iNKT helper activity in lupus patients is dependent on recognition of CD1d molecules on B cells (Fig. 4D).

Expanded conventional CD4+ T cells from SLE patients fail to induce B cells to secrete IgG

Conventional CD4+ T helper cells can augment antibody secretion based on specific interactions between their TCRs and peptides associated with MHC class II molecules on B cells. To evaluate the helper activity of conventional CD4+ T cells, we first stimulated iNKT-depleted CD4+ T cells from lupus patients with plate-bound anti-CD3 and anti-CD28 mAbs and then expanded the cells for 10 days with IL-2 prior to their coculture with fresh autologous B cells. Additionally, we obtained TCRβ11−CD4+ T cells from the αGalCer stimulated SLE patient PBMC cultures that were used to generate expanded iNKT cells. As these cultures contained exogenous IL-2 and IL-15, in addition to αGalCer, it is not surprising that conventional non-NKT CD4+ cells were also expanded. The latter cells were purified by sorting for CD4+ TCRβ11−T cells. As shown in Figure 5, both sources of activated TCRβ11−CD4+ T cells failed to increase IgG, IgM or IgA production, as compared with cultures of B cells alone, and IgG anti-dsDNA was not detectable in the supernatants. By contrast, coculture of B cells with iNKT-cell lines from the same patient resulted in markedly increased secretion of all isotypes of Ig, including anti-dsDNA IgG. Moreover, Ig secretion was inhibited by anti-CD1d mAb in the iNKT cocultures.

iNKT-cell mediated B-helper activity is dependent on CD40L-CD40 interaction

CD4+ T helper cells can enhance B-cell antibody secretion through CD40 engagement on B cells, and it has been shown that iNKT cells express CD40 ligand upon activation [33]. To determine whether CD40-CD40L interaction is required for lupus iNKT cells to help B-cell antibody production, we cocultured expanded iNKT cells from SLE patients with freshly sorted autologous CD19+ B cells for 10 days in the presence or absence of anti-CD40L blocking antibody, and IgM, IgA, IgG, and anti-dsDNA IgG were measured in the supernatants. Consistent with our previous data, lupus iNKT-cell lines augmented production of IgM, IgA, IgG, and anti-dsDNA IgG antibodies. Importantly, production of all three classes of antibody, as well as IgG anti-dsDNA, induced by iNKT lines was inhibited by anti-CD40L antibody (Fig. 6).

Discussion

Spontaneous secretion of IgG by freshly isolated PBMCs is a characteristic finding in patients with clinically active SLE but not in patients with most other autoimmune diseases or healthy subjects [28–31]. This phenomenon is known to represent de novo IgG production and depends on T-cell help, but the nature of the T helper cells has not been clearly defined. Our results point to iNKT cells as important inducers of B-cell activation and maturation in lupus, and as the predominant source of T-cell help for spontaneous IgG secretion, which is surprising given the low frequency of these cells. Previous studies have shown that CD4+ iNKT-cell clones obtained from healthy persons can provide help to B cells, but only if they are stimulated with anti-CD3 antibody or αGalCer ex vivo [34–36]. A recent study showed that even in the absence of αGalCer stimulation, certain CD4+ iNKT-cell clones from healthy subjects can help B cells produce IgG antibody [37]. Our study shows that expanded iNKT-cell lines as well as fresh peripheral blood iNKT cells from lupus patients greatly enhance production of total IgG, IgM, and IgA, as well as IgG, anti-dsDNA IgG antibody. It was not clear whether the iNKT-cell enhancement of total IgG and anti-dsDNA IgG production resulted mainly from inducing naïve B cells to class-switch or from helping class switched memory B cells produce more IgG. However, when expanded iNKT cells were added to purified naïve B cells (IgM+CD27−CD19+), they induced IgG isotype class switching. A recent animal study confirmed that iNKT cells help B cells to produce class-switched antibodies in vivo [12].

The helper activity of the expanded iNKT cells in coculture with purified B cells, like that of iNKT cells in fresh PBMCs, did not require exogenous stimulation and was dependent on their recognition of CD1d on B cells. The nature of the CD1d associated antigen(s) on B cells recognized by lupus iNKT cells is unknown.
but presumed to be glycolipid rather than nucleic acid. Such glycolipids might be expressed on lupus B cells as a consequence of their exposure to other stimuli including agonists of toll-like receptors [38]. Although CD1d is expressed on cell types in addition to B cells (e.g., dendritic cells), a role for these cells in the observed B-cell help provided by iNKT-cell lines can be ruled out because dendritic cells were absent from our cultures. This finding is consistent with published data showing that treatment of NZBxNZW mice with anti-CD1d antibody ameliorates lupus disease activity and simultaneously reduces the serum concentration of IgG2a anti-dsDNA antibodies [16, 17]. Moreover, recent studies demonstrated that iNKT cells can replace conventional CD4+ T helper cells with respect to the ability to induce antibody responses in MHC class II deficient mice immunized with a protein antigen and αGalCer [39]. In addition, iNKT cells, but not conventional CD4+ T cells, from NZBxNZW mice help B cells spontaneously secrete IgG and anti-dsDNA IgG antibodies in vitro [19, 40]. However, the results of the current human studies differ from those of recent studies in NZBxNZW mice (40), which show that IL-21 secreted by the CD4+ subset of iNKT cells plays an important role in spontaneous autoantibody secretion. In our studies, lupus iNKT cells produced only small amounts of IL-21 and neutralizing anti-IL-21 antibody had no effect on spontaneous Ig secretion (data not shown).

Our findings indicate that iNKT cells from lupus patients produce large amounts of IFN-γ. Previous studies in mouse models...
have shown that IFN-γ can promote B-cell activation during the initiation of antibody responses and is a key cytokine in murine lupus pathogenesis, promoting autoantibody production and glomerulonephritis [41–44]. In addition, αGalCer activated iNKT cells from lupus prone NZBxNZW mice secrete a high ratio of IFN-γ to IL-4 as compared with iNKT cells from nonautoimmune mice [16, 17]. Harigai et al. reported that increased expression of IFN-γ in SLE patients induces APCs to produce BLYS/BAFF, a well characterized B-cell activating and survival factor that is the target of a drug approved for the treatment of SLE [45]. Given these findings, we were surprised that addition of up to 20 μg/mL of a neutralizing anti-IFN-γ antibody failed to inhibit Ig secretion induced by lupus iNKT cells. This result suggests that IFN-γ does not mediate the helper effect of these cells.

Additional studies of the mechanism by which lupus iNKT cells help B cells secrete antibodies revealed that IgM, IgA, IgG, and IgG anti-dsDNA antibody levels were markedly reduced in coccults of iNKT and B cells to which anti-CD40L had been added. These results suggest that CD40/CD40L interactions are critical for iNKT-cell help, just as these interactions are required for conventional T-cell help. Previous studies have shown that WT mice immunized with a protein antigen and αGalCer had an increased frequency of plasma cells by comparison to mice immunized with antigen alone, indicating that iNKT cells enhance plasma cell differentiation and survival [39]. Our studies showed that lupus iNKT cells can induce B cells to differentiate into plasma cells in the absence of αGalCer, as demonstrated by the dramatic reduction of plasma cell frequency when lupus PBMCs were cultured in the presence of anti-iNKT or anti-CD1d antibody. Taken together, these data indicate that lupus iNKT cells induce B-cell activation and maturation through CD40L-CD40 and TCR-CD1d interactions.

Although conventional CD4+ T-cell subsets can promote antibody and autoantibody production via TCR recognition of endogenous protein antigens associated with MHC class II molecules on B cells, in our experiments in lupus patients, such cells had little or no detectable helper activity for spontaneous Ig secretion. This could not be explained by the absence of a T-cell stimulus, because CD4+ T cells depleted of iNKT cells and activated with anti-CD3 mAb failed to help B-cell antibody secretion. One possibility is that pathogenic conventional helper T cells are sequestered in lymphoid organs or inflamed tissues. At least one subpopulation of CD4+ T cells with potent B-cell helper capability resides mainly in lymph node germinal centers [46, 47] and is likely not present in large numbers in the circulation. Conceivably, that portion of spontaneous antibody production that could not be blocked with antibodies to iNKT cells or CD1d might depend on this or another T-cell population [48, 49]. On the other hand, based on the finding that lupus-like abnormalities can be transferred to immunodeficient mice with PBMCs from SLE patients [50], it seems likely that T and B cells involved in the pathogenesis of SLE are at least partly represented in the circulation.

The reduced frequency of circulating iNKT cells in patients with lupus, combined with the ability of iNKT cells to downregulate immunity in other settings, has led to the suggestion that activation or enhancement of these cells should be a goal of therapy [24–26, 51, 52]. This view was reinforced by a report that lupus iNKT cells, which had been stored frozen prior to being thawed and stimulated with αGalCer, produced large amounts of IL-10 but not IFN-γ [27]. Importantly, the latter study was performed with cells that had been cryopreserved, whereas we studied only freshly isolated cells. In our experience, frozen-thawed peripheral blood iNKT cells from both lupus patients and healthy subjects often differ in their cytokine secretion from fresh iNKT cells obtained from the same individuals (Supporting Information Fig. 3), and this could explain the difference between our data and the prior study. Our findings clearly argue against the view that reduced iNKT-cell activity plays a pathogenic role in lupus and instead suggest that excessive activation of iNKT cells likely contributes to disease development. In this regard, the reduced frequency of iNKT cells in lupus blood could be due to their migration to inflamed tissues. Indeed, IFN-γ-secreting iNKT cells have recently been shown to be enriched at sites of cutaneous inflammation in lupus patients [53]. Regardless of their frequency in blood, our data suggest that iNKT cells play an important role in the induction of autoantibody production in patients with SLE. New therapies directed at neutralizing or antagonizing these cells could prove useful in the treatment of this disease.

Materials and methods

Patients and healthy donors

This study included 44 patients who fulfilled the American College of Rheumatology criteria for the classification of SLE and 32 healthy subjects. Clinical and demographic features of the participating lupus patients are described in Table 1. The disease activity of patients with SLE was quantified by SLE Disease Activity Index (SLEDAI). Patients were recruited from the Immunology and Rheumatology Clinic at Stanford Hospital and the Oklahoma Research Center with approval of the administrative panels on Human Subjects in Medical Research from both institutions. Healthy control PBMCs were obtained from blood donors at the Stanford Blood Center.

Antibodies and cell staining

Antibodies against human CD19, IgM, CD27, NKT (6B11), CD3, CD4, CD8, IFN-γ, IL-4, IL-10, IL-17, IL-21, CD28, and CD1d (blocking) were from BD Biosciences. Antibodies against human CD20, CD38, CD69, CD138, HLA-ABC, HLA-DR, CD1d, CD1c, CD4 (blocking), CD2 (blocking), CD40L (blocking), and isotype control mIgG1 or mIgG2a were purchased from Biolegend. Antibodies against human Vα24 and Vβ11 were from Beckman Coulter. PE-conjugated CD1d–αGalCer tetramer was provided by the US National Institutes of Health Tetramer Core Facility. For surface staining, cells were maintained in the dark at 4°C throughout.
Cells were incubated for 20 min with each antibody in FACS buffer (PBS with 2 mM EDTA and 1% FCS) and were washed thoroughly with FACS buffer. For intracellular cytokine staining, cells were stimulated with PMA (50 ng/mL) and ionomycin (1 μg/mL) for 4 h in the presence of Brefeldin A. Then cells were fixed and permeabilized for 20 min at 4°C with Cytofix/Cytoperm buffer (BD Biosciences) and washed twice with Perm/Wash buffer (BD Biosciences). Anti-cytokine antibodies were added to the cells and incubated in the dark at 4°C for 30 min. LSR II and FACS Diva software (BD) were used for the acquisition of flow cytometry data, and FlowJo software (TreeStar) was used for analysis.

Cell isolation

PBMCs were prepared from human peripheral blood using Ficoll density gradient centrifugation. B cells and CD4+ T cells were purified from PBMCs by MACS with anti-CD19 and anti-CD4 microbeads (Miltenyi Biotec). Naïve B cells (CD19+CD27−IgM+) were sorted by FACS Aria II. Activated conventional CD4+ T cells were obtained from two sources. (1) CD4+ T cells which had been stimulated with plate-bound anti-CD3 (2 μg/mL) and anti-CD28 (0.5 μg/mL) antibodies in the presence of rhlL-2 (10 ng/mL) for 10 days and then depleted of iNKT cells using biotinylated anti-TCRVo24 antibody and anti-biotin microbeads (Miltenyi Biotec). (2) PBMCs which had been stimulated with αGalCer and expanded with rhlL-2/15 for 30 days, after which iNKT cells were depleted as above and conventional CD4+ T cells were then purified by FACS.

In vitro expansion of iNKT cells

PBMCs were cultured in RPMI 1640 medium supplemented with 10% normal human serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. During the first week PBMCs (1 × 106 cells/mL) were maintained in 12-well plates in a 37°C humidified incubator with 5% CO2, αGalCer (100 ng/mL) was added at the start of culture, and rhlL-2 (50 ng/mL) and rhlL-15 (10 ng/mL) (PeproTech) were added 16 h later. Cultures were restimulated every 10 days with 1 × 106 cells/mL αGalCer-pulsed, irradiated (3000 rad) autologous PBMCs in medium containing rhlL-2 in addition to 50% AIM V, 15% human serum, and 20% human serum. After two rounds of restimulation, TCRVo24Vβ11 double positive cells were sorted with mAbs to TCRVo24 and TCRVβ11 (Beckman Coulter) using a FACS Aria II.

Cytokine production by expanded iNKT cells

Sorted iNKT cells were cultured in anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) antibody coated plates for 24 h. The concentrations of IL-4 and IFN-γ in the supernatant were determined by ELISA (BD Bioscience). To measure intracellular cytokines, sorted iNKT cells were stained with APC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4 antibodies (BD Biosciences) and analyzed by flow cytometry.

Assays of antibody production

To assess spontaneous antibody production, fresh PBMCs (2 × 106 cells/mL) were cultured in RPMI medium containing 5% FCS in the presence or absence of the indicated antibodies. The effect of isolated T or iNKT cells on immunoglobulin production was assessed by culturing purified B cells (1 × 106 cells/well) alone or with autologous iNKT-cell lines (1 × 105 cells/well) or iNKT-depleted CD4+ T cells in U-bottom 96-well plates. In some experiments, isolated B cells were cultured with PWM (Life Technologies) at a final dilution of 1:100, or were cultured with S. aureus Cowan I strain (SAC) (Sigma, St. Louis, MO) at a final concentration of 1:10 000 v/v and IL-2 (10 ng/mL) from PeproTech. After 10 days of culture, supernatants were harvested and measured by ELISA for total IgG, IgM, IgA (Bethyl Laboratories) and anti-dsDNA IgG (Signosis). In blocking experiments, anti-human mAbs to CD1d, NKT TCR (clone 6B11), Vα24, Vβ11, HLA-ABC, HLA-DR, CD4, CD2, and isotype control Ig were used at 20 μg/mL. Although plate-bound 6B11 is mitogenic for iNKT cells [54], in its soluble form this mAb does not activate these cells (data not shown).

Detection of antibody-forming cells

IgG anti-dsDNA antibody-forming cells were detected by enzyme-linked immunosorbent spot-forming cell assays (ELISPOT). MultiScreen-IP sterile white plates (Millipore) were prewet with 50 μL of 70% ethanol per well before washing five times with water. Plates were then coated with 100 μL of 100 μg/mL calf thymus dsDNA (Sigma) in PBS (PH 7.4) overnight at 4°C. Coated plates were blocked with culture media for 1 h at room temperature (RT). For detection of IgG anti-dsDNA-producing cells, 5 × 106 fresh PBMCs were added to each well. The plates were incubated for 72 h at 37°C and the cells were washed away with PBS. Biotinylated anti-IgG at 1 μg/mL (MABTECH) was added to the wells and incubated for 2 h at RT, followed by incubation with alkaline phosphatase-labeled streptavidin (MABTECH) diluted at 1:1000 for 1 h at RT. Specific antibody-binding spots were visualized with substrate 5-bromo-4-chloro-3-indolyl phosphate/NBT-plus (MABTECH). Developed plates were evaluated and read by ZellNet Consulting (Fort Lee, NJ). Results are expressed as the number of IgG anti-dsDNA antibody forming cells per 106 PBMCs.

Analysis of AID expression

SLE B cells were cultured alone or cocultured with autologous iNKT cells for 7 days. Total RNA was extracted from B cells and B and iNKT cocultures using RNEasy Micro Plus kit (Qiagen). RNA was converted to cDNA using the High-Capacity cDNA Reverse
Transcription Kit (Applied Biosystems). Primers: AID (Forward, AGACACTCTGGACACCACTAT, Reverse, CTTAGCCCATCGG-GACATT), CD79B (Forward-CCAGGCTGGCTGTGCCTCG, Reverse-AGCCGCTGTGCACTGAGCGT). Quantitative RT-PCR was performed using SYBR green (Applied Biosystems) on a QuantStudio 6 Flex machine (Applied Biosystems). Results were normalized to CD79B gene.

Statistical analysis

Results are expressed as mean ± SD. Statistical significance between groups was determined by an unpaired t test or a Mann–Whitney U test. Correlations between variables were evaluated by Spearman’s rank correlation test. A p value of <0.05 was considered significant. Graphs and statistical analyses were performed using Prism software.

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References


**Abbreviations:** AFC: antibody forming cell • AID: activation-induced cytidine deaminase • GalCer: α-galactosylceramide • dsDNA: double-stranded DNA • iNKT: invariant natural killer T • PWM: pokeweed mitogen • SLE: systemic lupus erythematosus • SLEDAI: SLE disease activity index

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