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The IL-23/IL-17 pathway is important in multiple autoimmune diseases, but its effect on lupus pathology remains unclear, with opposing trials in murine models of the disease. In this study, we show a disease activity–related upregulation of serum IL-23 and IL-23 receptor in patients with systemic lupus erythematosus (SLE) as compared with healthy controls. When added in SLE T cell in vitro cultures, IL-23 induced IL-17 and limited IL-2 production, whereas T follicular helper and double negative (DN) T cells significantly expanded. To further dissect the role of IL-23 in the expression of autoimmunity and related pathology, we generated IL-23 receptor–deficient MRL.*lpr* mice. These IL-23R$^{-/-}$MRL.*lpr* mice displayed attenuated lupus nephritis with a striking decrease in the accumulation of DN T cells in the kidneys and secondary lymphoid organs. Moreover, T cells from IL-23R$^{-/-}$MRL.*lpr* mice produced increased amounts of IL-2 and reduced amounts of IL-17 compared with T cells from wild type animals. In vitro IL-23 treatment promoted IL-17 production and downregulated IL-2 production. The IL-23R$^{-/-}$MRL.*lpr* had fewer T follicular helper cells, B cells, and plasma cells, leading to decreased production of anti-dsDNA Abs. Our results show that IL-23 accounts for the main aspects of human and murine lupus including the expansion of DN T cells, decreased IL-2, and increased IL-17 production. We propose that blockade of IL-23 should have a therapeutic value in patients with SLE. ***The Journal of Immunology*****, 2017, 199: 903–910.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by an imbalance between proinflammatory, such as IFN-γ, and regulatory cytokines, such as IL-2 (reviewed in 1). This imbalance underlies the basic pathophysiologic steps that eventually lead to organ damage: production of autoantibodies, immune complex formation and deposition, inflammatory cell migration, and activation in various tissues such as the skin, joints, and kidneys. Although a multitude of biologic medications have been used to disrupt cytokine signaling in SLE, only belimumab, an anti-B lymphocyte stimulator, has been shown to be modestly effective in the treatment of this disease (2).

Based on some early findings that the proinflammatory cytokine IL-17 is expressed by T lymphocytes in kidney biopsies of patients with lupus nephritis (3), we proposed that Th17 cells may be important in the pathogenesis of the disease. As generation of these cells depends on IL-23, a member of the IL-12 family (4, 5), we examined the effect of this cytokine in the model of autoimmunity B6.*lpr*. These mice bear a mutation (*lpr*) on the Fas gene that results in deficient lymphocyte apoptosis. This leads to massive hyperplasia of their secondary lymphoid organs, expression of a variety of autoantibodies, but only mild kidney and skin inflammation. We found that B6.*lpr* mice that lack the receptor for IL-23 do not develop pathogenic autoantibodies or organ inflammation (6). Moreover, there was a significant reduction of the $CD3^+CD4^-CD8^-$ (double negative [DN]) T cell population that is abnormally expanded in these mice. We then used a neutralizing anti–IL-23 Ab to treat MRL.*lpr* mice that, as opposed to B6.*lpr* mice, develop severe nephritis similar to SLE; the treated mice had less severe nephritis than control-treated mice, suggesting but not conclusively proving that IL-23 plays a role in the development of autoimmunity and ensuing inflammation (7).

Based on well-established models of autoimmunity, it was further hypothesized that the proinflammatory effects of IL-23 depend on the production of IL-17A and/or F by differentiated Th cells (Th17). Targeting through IL-17 directly showed opposing effects in different murine lupus trials (8, 9), suggesting that IL-23 potentially has a broader effect on the development of SLE.

Both MRL.*lpr* lymphocytes and SLE T cells produce reduced amounts of the regulatory cytokine IL-2 in vitro (10). This is particularly true for the DN T cell population that is expanded in MRL.*lpr* mice and SLE patients, and is reduced in the absence of IL-23R. We therefore hypothesized that IL-23 not only induces IL-17 but also decreases IL-2 production, leading to a proinflammatory/anti-inflammatory cytokine imbalance.

To answer the question of whether IL-23 is central in the pathophysiology of SLE, we evaluated its concentration in the serum of SLE patients, its relation to disease flares, and its direct effect on SLE T cells ex vivo. Furthermore, we generated MRL.*lpr* mice that lack the IL-23 receptor and studied in vivo the development of nephritis. To our knowledge, for the first time we present evidence that IL-23 supports the generation of extrafollicular Th cells in SLE T cell cultures, promoting the production of anti-dsDNA Ab. Similarly, IL-23 receptor deficiency in MRL.*lpr* mice prevented the development of nephritis by inhibiting the generation of DN T cells and extrafollicular T helper cells. Besides the expected effect of IL-23 on IL-17 production, we show that IL-23 critically influences the production of IL-2, limiting the activation of the transcription factor NF-κB.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DN, double negative; SLE, systemic lupus erythematosus; Tfh, T follicular helper cell; Treg, regulatory T cell.

Materials and Methods

Patients

Patients who fulfilled the American College of Rheumatology criteria for the diagnosis of SLE (11) were enrolled in the study by donating 50 ml of blood. The Beth Israel Deaconess Medical Center Institutional Review Board approved the study protocol and informed consent was obtained from all study subjects. We included 69 patients in the studies (64 female and 5 male; age: 39.8 ± 8.9 y; racial distribution: 54% white, 35% African-American, 10% Asian, 1% other). SLE disease activity was measured by the Systemic Lupus Erythematosus Disease Activity Index (12). Medications used included hydroxychloroquine (86%), prednisone (65%, dose: 5–50 mg/d), mycophenolate mofetil (32%), cyclophosphamide IV (two patients), belimumab (one patient), and azathioprine (17.4%). We included 27 healthy controls (26 female, 1 male; age: 40 ± 8.5 y; racial distribution: 48% white, 41% African-American, 7% Asian, 4% other).

T cells were isolated by negative selection using the RosetteSep T Cell Isolation Kit (Stemcell Technologies) unless otherwise indicated. PBMCs were isolated using a Ficoll gradient. Serum was isolated from 10 ml serum separator tubes.

Mice

The IL-23R–deficient C57BL/6 mice were generated as previously described (6). All mice were housed at the Beth Israel Deaconess Medical Center pathogen-free animal facility (Boston, MA). Our protocol was approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

We generated IL-23R–deficient MRL.*lpr* mice using a backcross-intercross scheme. After 12 generations of breeding, the mice were PCR screened for the *Fas/lpr* and mutated *IL-23R* gene. Primer for *Fas/lpr* genetic screen: 5′-GTAAATAATTGTGCTTCGTCAG-3′, 5′-TAGAAAGGTGCACGGGTGTG-3′ and 5′-CAAATCTAGGCATTAACAGTG-3′; *IL-23R* genetic screen: 5′-ACCCCTAGGAATGCTCGTCAAG-3′ and 5′-TGGTTGCCTGCACCAATTTAAAAG-3′; and for homozygous versus heterozygous mutated: 5′-GATCATCTTATGGCTGGTCCTC-3′ and 5′-GAGTGAGACAGTGTAGCCACAGAT-3′.

Flow cytometry

Briefly, 2 × 10^6 cells were suspended in 50 μl FACS buffer containing fluorescent Abs. All cells were stained with Zombie aqua (BioLegend). The following Abs were used for staining: CD45, CD3, CD4, CD8, TCRβ, CXCR5, PD-1, CXCR4, PSGL-1, CD138, CD19, CD25, IL-23R, Thy1.2, CD44, and CD62L (BD Pharmingen). For Ki67 (BioLegend) and Foxp3 (eBioscience) measurements, after surface staining cells were fixed, rendered permeable, and intracellularly stained following the manufacturer's instructions. Cells were analyzed by flow cytometry (FACSCalibur; BD Biosystems, San Jose, CA).

Cell culture, cytokine, and protein measurement

Murine lymphocytes were cultured in RPMI 1640 with 10% (v/v) FCS (supplemented with 50 μM 2-ME, 1 mM sodium pyruvate, nonessential amino acids, L- glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere of 10% CO_2 in a culture incubator.

SLE patients or murine lymphocytes were incubated (2 ×10^6/ml) in plates coated with 5 μg/ml anti-CD3/28. IL-23 (25 ng/ml; R&D Systems) was added as described. Before collection, PMA, calcium ionophore (A23187), and 1 μl/ml brefeldin A (Golgi-Plug; BD Pharmingen) were added. The cells were subsequently collected and IL-17, IL-2, and/or IFN-γ production was measured by intracellular staining. For Western blots, cells were lysed using the Nuclear Extraction Kit (Abcam) and immunoblotted using anti-NFκBp65 and β-actin Abs (Santa Cruz).

ELISA was used for the measurement of IL-2, IFN-γ, and IL-17 in supernatants (BioLegend). Anti-dsDNA Ab titers in serum and supernatants were also measured using ELISA (Alpha Diagnostic).

Histopathology and tissue cell isolation

The kidneys were fixed in 10% formalin overnight at 4°C, embedded in paraffin, and cut at 6 μM before being stained with H&E and periodic acid-Schiff reagent. We evaluated in a blinded fashion glomerular pathology (in 100 glomeruli per kidney) using a semiquantitative scale as before (13, 14).

Cells were extracted from murine spleens and lymph nodes by filtering the tissue through a 100 μm BD Biosciences Falcon cell strainer. The extracts were centrifuged at 1200 rpm for 5 min. ACK lysing buffer (Quality Biological) solution was added in the cell pellet to lyse the red cells. The treated cell pellet was subsequently washed once with DMEM cell culture medium and resuspended in medium for further treatment or staining.

Murine kidney tissue was incubated with collagenase type A (10 μg/ml; Sigma-Aldrich) in cold Dulbecco's PBS buffer with EDTA (2 μM) for 15 min at 37°C. The digested kidney tissue suspension was strained through a 100 μm BD Biosciences Falcon cell strainer (Fisher Scientific). The cells were centrifuged at 1200 rpm for 5 min, and the cell pellet washed and resuspended for treatment or staining.

BrdU assay

Mice were injected with BrdU (100 mg/kg i.p.; BD Biosciences, San Jose, CA) and then sacrificed 3 h later. BrdU staining was performed using a BrdU labeling kit (BD).

Statistical analysis

Statistical analyses were performed in GraphPad Prism version 6.0 software. Statistical significance was determined by *t* tests (two-tailed). Statistical significance was defined as $p < 0.05$. Three to five mice were used for each experiment as indicated. Subsequently the results were replicated in two additional independent experiments using three to five mice per experiment.

Results

IL-23 promoted IL-17 production and generation of $CD3^+$ $CD4^-CD8^-$ DN T cells in SLE patients

To address the role of IL-23 in SLE patients, we first measured IL-23 levels in the sera of patients and sex-, age-, and race-matched healthy donors. Patients with Systemic Lupus Erythematosus Disease Activity Index (12) scores of more than four were defined as active. IL-23 was significantly elevated in patients with active disease compared with patients with inactive disease and healthy controls (Fig. 1A). IL-23 levels did not correlate with the type of medications the patients were taking or their dose. Specifically, IL-23 levels did not correlate with prednisone dose (Pearson $r = -0.17$, $p = 0.19$). We noted a nonstatistically significant positive correlation between IL-23 and anti-dsDNA Ab titers (Pearson $r = 0.25$, $p = 0.054$). We found no other statistically significant correlation between serum IL-23 levels and specific disease manifestations, or laboratory values. We then measured IL-23 receptor expression on T cells. Contrary to the serum IL-23 concentration, IL-23R expression on T cells of active and inactive SLE was similar and higher than healthy controls (Fig. 1B). We then asked how IL-23 affects SLE T cells in vitro. Treatment with IL-23 resulted in the doubling of the percentage of T cells that expressed T follicular helper cell (Tfh) markers (Fig. 1C) and an increase in DN T cell numbers (Fig. 1D). DN T cells did not express Tfh markers (Supplemental Fig. 1A). To address whether the IL-23 induced increase in Tfh results in B cell hyperactivity, we incubated T and B cells from patients with active autoantibody-positive SLE, in the presence or absence of IL-23. After stimulation of T cells with anti-CD3/CD28, we measured the production of anti-dsDNA Abs by B cells. There was a 2-fold increase of anti-dsDNA Ab production in the presence of IL-23 (Fig. 2A), suggesting a key role of IL-23 in anti-dsDNA Ab production in this limited number of patients. To dissect the effect of IL-23 on T cell function, we measured the cytokine profile of activated SLE T cells cultured in the presence or absence of IL-23. As expected, IL-23 increased the production of IL-17, but limited the production of IFN-γ (Supplemental Fig. 1B) and IL-2 by SLE T cells (Fig. 2B), a critical cytokine that boosts regulatory T cells (Tregs) and inhibits Tfh. IL-23 did not induce $IL\text{-}17A^+IFN\text{-}\gamma^+$ double positive T cells (Supplemental Fig. 1C) as observed in other disease states. Because IL-2 production by SLE T cells is due to deficient transcription of the *IL-2* gene (15), we asked whether IL-23 influences the recruitment of transcription factors

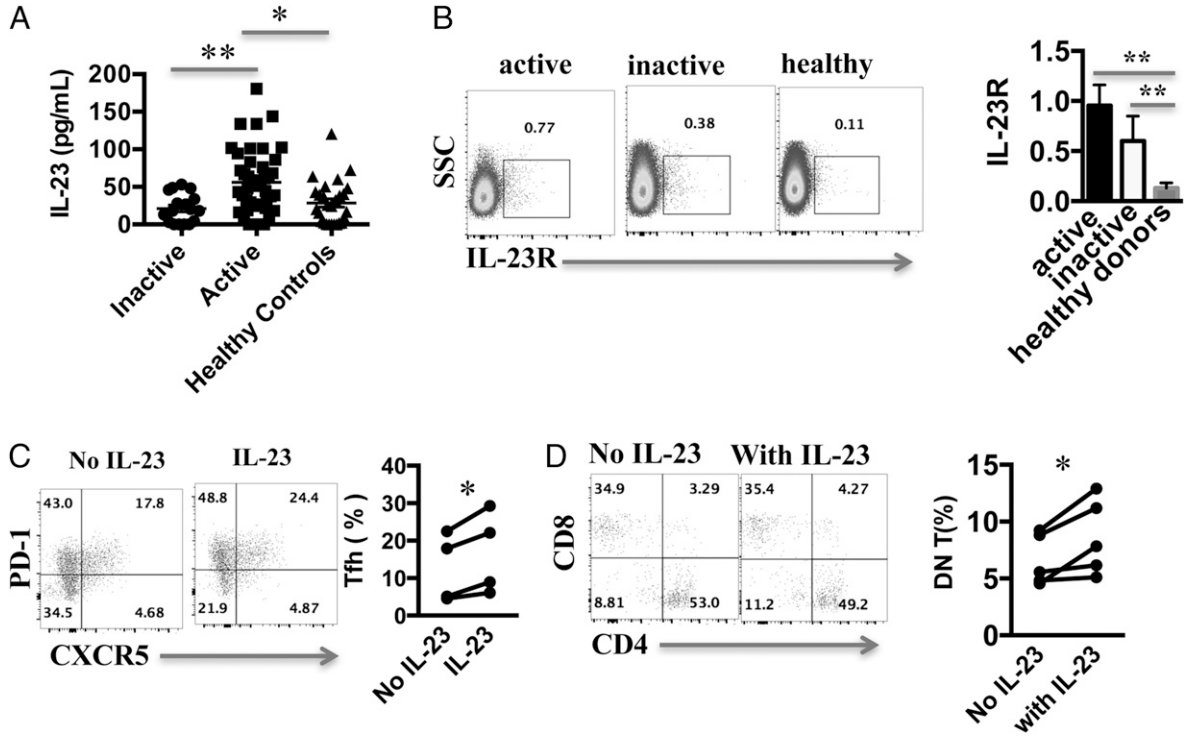

FIGURE 1. IL-23 is elevated in SLE and promotes the generation of Tfh and DN T cells. (**A**) IL-23 level was measured in sera of healthy donors, active, and inactive SLE patients. (**B**) $CD3^+$ T cells from healthy donors, active, and inactive SLE patients were stained with an anti–IL-23R Ab. A representative dot plot and cumulative results are shown. (**C**) SLE T cells were stimulated with anti-CD3/CD28 Abs with or without IL-23 for 5 d and then stained for the Tfh markers ($CD3^+CD4^+PD\text{-}1^+CXCR5^+$) (representative dot plot and cumulative results from five patients). (**D**) SLE T cells were stimulated with anti-CD3/aCD28 Abs with or without IL-23 for 5 d and then stained with CD3, CD4, and CD8 (gated for $CD3^+$, representative experiment and cumulative data from five patients). Error bars represent SD. $*p < 0.05$, $**p < 0.01$.

in the nucleus of activated SLE T cells. We found that the *IL-2* enhancer NFκBp65 (16) recruitment in SLE T cell nuclei is suppressed in the presence of IL-23 (Fig. 2C). NFκBp65 is known to be decreased in SLE T cells and its forced replenishment restores IL-2 production (14). Our data therefore suggest that chronic exposure of SLE T cells to IL-23 may account for the

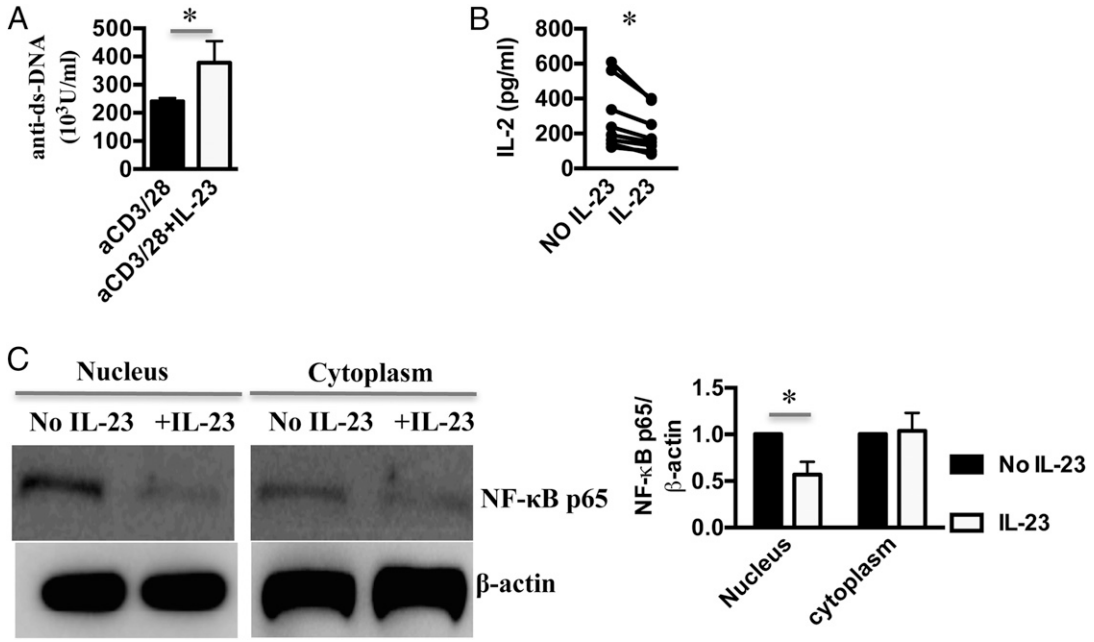

FIGURE 2. IL-23 promoted the production of autoantibodies, although limited IL-2. (**A**) 3×10^4 CD4 T cells and 3×10^4 B cells from SLE patients were stimulated with anti-CD3/28 in the absence or presence of 25 ng/ml IL-23 for 6 d, and anti-dsDNA Abs were measured by ELISA ($n = 6$). (**B**) SLE T cells were stimulated with anti-CD3/CD28 Abs in the presence or absence of IL-23 for 16 h, and IL-2 production in the supernatant was determined by ELISA ($n = 8$). (**C**) 4×10^6 T cells from SLE patients were stimulated with anti-CD3/28 in the absence or presence of 50 ng/ml IL-23 for 6 h; nuclear and cytoplasmic proteins were extracted and relative to β-actin expression of NFκBp65 was measured by Western blot (representative blot and cumulative data, $n = 5$ patients). Error bars represent SD. $*p < 0.05$, $**p < 0.01$.

decreased production of IL-2 by limiting translocation of necessary transcription factors.

*Decreased lupus disease severity and accumulation of DN T cells in kidneys in IL-23R$^{-/-}$ MRL.*lpr *mice*

To address mechanistically the effect of IL-23 on lupus pathogenesis, we generated IL-23R$^{-/-}$ MRL.*lpr* mice after backcrossing the B6IL-23R$^{-/-}$ with MRL.*lpr* for 12 generations. As opposed to B6.*lpr* mice, which develop autoantibodies but not severe autoimmune disease, these mice develop severe nephritis, which is similar to SLE and therefore can be far more useful in addressing the effects of IL-23 on both the development of autoimmunity and the ensuing inflammatory organ damage. By 3 mo of age, IL-23R$^{+/+}$ MRL.*lpr* mice developed enlarged kidneys and severe skin lesions, whereas IL-23R$^{-/-}$ MRL.*lpr* mice did not (Fig. 3A, 3B). On microscopic analysis, we found that the IL-23R$^{-/-}$ MRL.*lpr* mice had significantly attenuated glomerulonephritis as compared with IL-23R$^{+/+}$ MRL.*lpr* mice (Fig. 3C, representative H&E staining and cumulative scores). We then performed flow cytometric analysis of kidney-infiltrating ymphocytes and found that IL-23R deficiency resulted in decreased kidney infiltration of CD45$^+$ cells (Fig. 3D). Among the infiltrating lymphocytes, the majority were DN T cells (Fig. 3E). To further evaluate the phenotype of infiltrating T cells, we stained the kidney-derived lymphocytes for expression of IL-17 and IFN-γ (Fig. 3F, left panel). We found that the IL-23R$^{-/-}$ MRL.*lpr* had a 2-fold decrease in both IL-17– and IFN-γ–expressing cells versus IL-23R$^{+/+}$ MRL.*lpr*. Of note, the majority of IL-17$^+$ cells were DN T cells (Fig. 3G–H). Taken together, these data suggest that IL-23R deficiency fundamentally changes the inflammatory infiltrate in the kidneys of MRL.*lpr* mice and prevents tissue damage.

IL-23R deficiency resulted in decreased accumulation of DN T cells in secondary lymphoid organs

IL-23R$^{+/+}$ MRL.*lpr* mice had significantly larger spleens and peripheral lymph nodes than IL-23R$^{-/-}$ MRL.*lpr* mice (Fig. 4A, 4B), with both spleen and lymph nodes being profoundly more hypercellular (Fig. 4C). As we examined the cellular composition, we found that IL-23R deficiency primarily affected the DN T cells by reducing both their relative frequency and their absolute numbers (Fig. 4D, 4E). The relative proportion of CD4$^+$ and CD8$^+$ T cells increased in the absence of IL-23R, but their total numbers were not statistically significantly different in the presence or absence of the IL-23R (Fig. 4D, 4E). Taken together, these data suggest that genetic deletion of the IL-23 receptor in MRL.*lpr* mice reduced the numbers of DN T cells in both the kidneys and peripheral lymphoid organs.

IL-23R deficiency resulted in impaired T cell–dependent autoimmune humoral responses

Murine and human lupus is characterized by a profound increase in the production of autoantibodies that are thought to be pathogenic. B cell activation and eventual production of class-switched autoantibodies are assisted by their interaction with T cells (17). It has

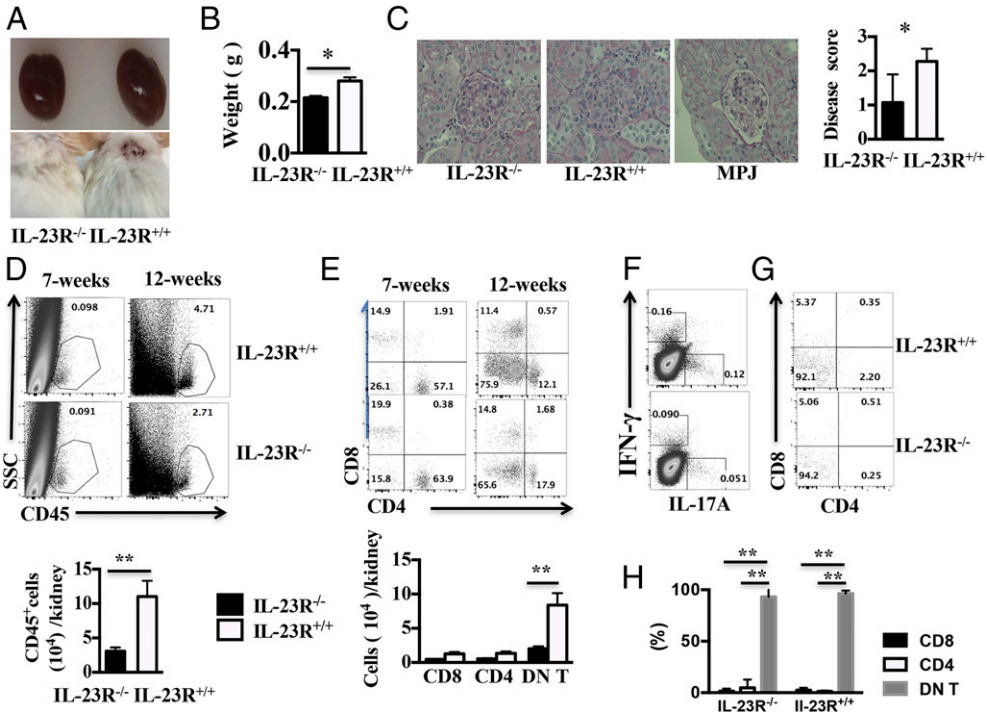

FIGURE 3. IL-23R deficiency prevented the development of nephritis in MRL.*lpr* mice. (**A**) Kidneys (top panel) and skin (lower panel) from a representative pair of 12-wk-old female IL-23R$^{+/+}$ and IL-23R$^{-/-}$ MRL.*lpr* mice is shown. (**B**) Harvested kidneys from IL-23R$^{+/+}$ and IL-23R$^{-/-}$ weight is shown (n = 3). (**C**) Representative H&E staining of kidney sections and cumulative results of kidney scores in IL-23R$^{+/+}$, and IL-23R$^{-/-}$ MRL.*lpr* are shown (n = 4). H&E staining of kidney section from healthy MRL.MPJ mice is shown for comparison. (**D**) Kidneys from 7- and 12-wk-old IL-23R$^{+/+}$ and IL-23R$^{+/+}$ MRL.*lpr* mice were harvested and cells were isolated. Frequency of CD45$^+$ cells among total live kidney cells (upper panel) and absolute number of CD45$^+$ cells (lower panel) are shown. (**E**) Kidneys from 7- and 12-wk-old IL-23R$^{+/+}$ MRL.*lpr* and IL-23R$^{-/-}$ MRL.*lpr* mice were harvested, cells were isolated, and analyzed with flow cytometry for CD4 and CD8 expression after gating for CD45$^+$CD3$^+$T cells. The relative percentage to total T cells (upper panel) and the absolute number of the major T cell subsets (lower panel) are shown (n = 3). (**F** and **G**) Cells were isolated from the kidneys of three mice and were stimulated with PMA and ionomycin in the presence of Golgi plug for 4 h; then the cells were fixed and subjected to surface staining for TCRβ, CD3, CD4, CD8, and intracellular staining for IL-17 and IFN-γ. The expression of IFN-γ and IL-17 in TCRβ$^+$CD3$^+$ cells (F) and the expression of CD4 and CD8 on IL-17$^+$ cells (G and **H**). Cumulative results (lower panel), n = 3) are shown. Error bar represents SD. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$.

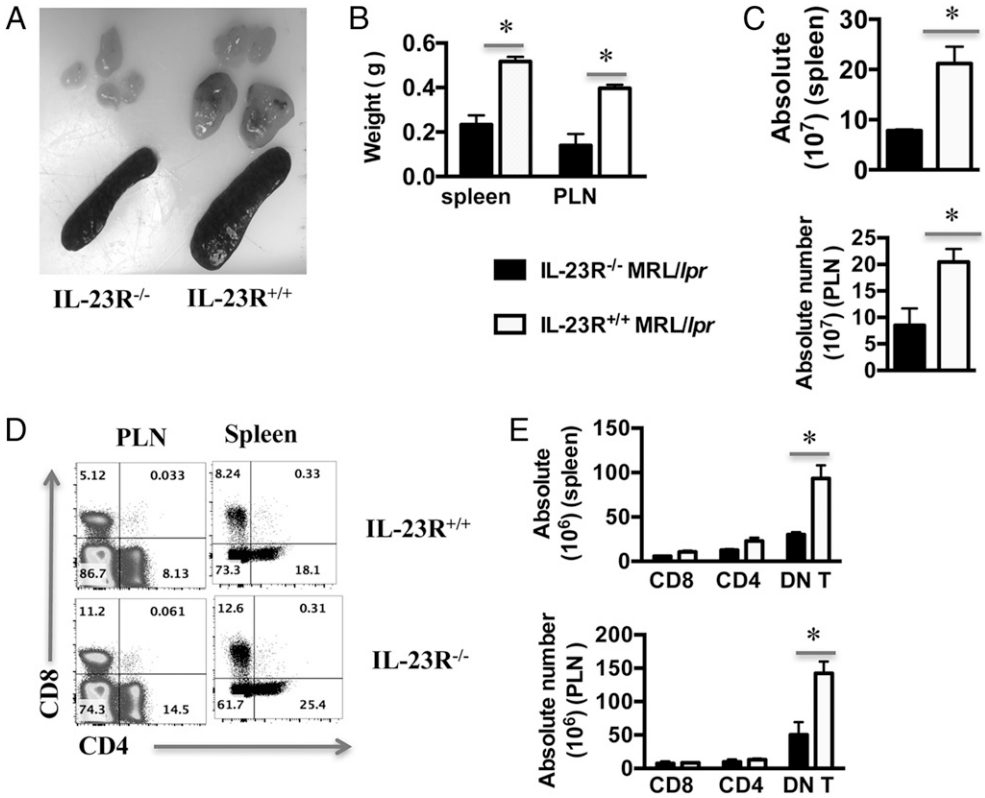

FIGURE 4. IL-23R$^{-/-}$ MRL.*lpr* mice had decreased T cell accumulation in secondary lymphoid organs. (**A**) A representative pair of harvested spleens and peripheral lymph nodes (PLNs) from 12-wk-old IL-23R$^{+/+}$ and IL-23R$^{-/-}$ MRL.*lpr* mice are shown. (**B**) Spleen and PLN weights from the two groups ($n = 3$) are plotted. (**C**) Total number of splenocytes and PLN cells from the two groups ($n = 3$) are shown. (**D** and **E**) Cells from spleens and lymph nodes were isolated from IL-23R$^{+/+}$ and IL-23R$^{-/-}$ MRL.*lpr* mice, stained for Thy1.2, CD3, CD4, and CD8, and analyzed with flow cytometry. Representative plot of CD4 and CD8 staining of Thy1.2$^+$CD3$^+$ splenocytes and PLN cells from both groups (D) and cumulative results (E, $n = 3$). Error bar represents SD. Data are representative of three independent experiments. *$p < 0.05$.

been shown that not only CD4$^+$ but also DN T cells (18, 19) can be effective in providing help to B cells to produce anti-dsDNA Abs. We found that serum anti-dsDNA autoantibody levels were reduced by more than 60% in the IL-23R–deficient mice as compared with wild type mice (Fig. 5A). This finding was in accordance with the observation that extra Tfh, plasma cells, and

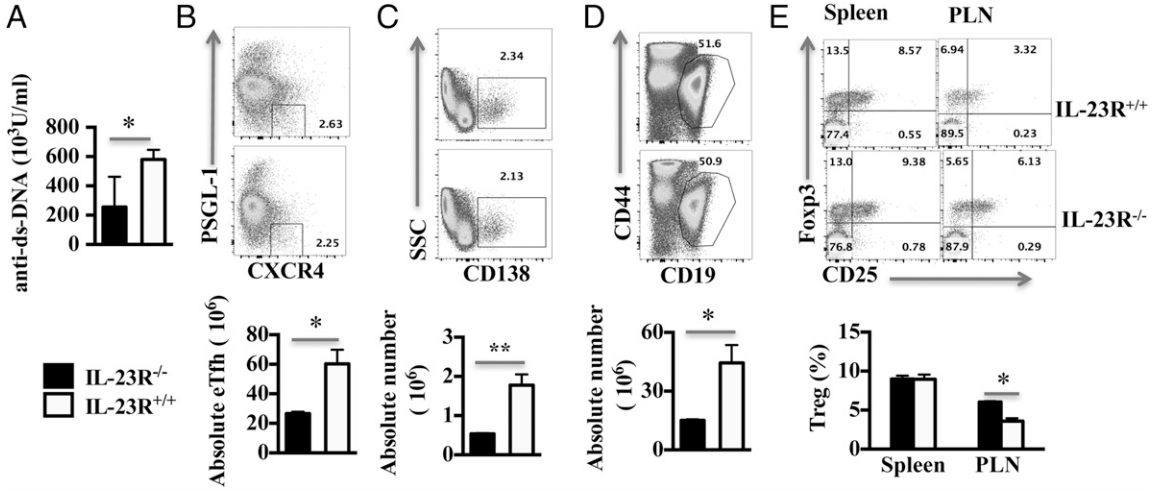

FIGURE 5. IL-23R$^{-/-}$ MRL.*lpr* mice displayed decreased spontaneous humoral responses. (**A**) Anti-dsDNA Abs were measured in the serum of 12-wk-old IL-23R$^{-/-}$ and IL-23R$^{+/+}$MRL.*lpr* mice (representative experiment and cumulative data from five mice per group). (**B**) Splenocytes were isolated from 12-wk-old IL-23R$^{-/-}$ and IL-23R$^{+/+}$ MRL.*lpr* mice and stained for PSGL-1, CXCR4, Thy 1.2, CD8, CD4, CD44, and CD62L. The extra Tfh (Thy1.2$^+$ CD8$^-$CD62L$^-$CD44$^+$CD4$^+$PSGL-1$^-$CXCR4$^+$) were measured (representative experiment, gated for Thy1.2$^+$CD4$^+$CD8$^-$CD44$^+$CD62L$^-$ [upper panel], and cumulative results [lower panel], $n = 3$). (**C**) The same samples as in (B) were stained for CD138, CD44, and CD3 (representative dot plot of CD138$^+$ cells after gating from CD3$^-$CD44$^+$ CD19^{low} [upper panel], and cumulative results [lower panel], $n = 3$). (**D**) The same samples as in (B) were stained for CD19, CD44, and CD3 (representative dot plot of CD44$^-$CD19$^+$ cells after gating from CD3$^-$ [upper panel], and cumulative results [lower panel], $n = 3$). (**E**) Cells from the spleens and peripheral lymph nodes of IL-23R$^{+/+}$ and IL-23R$^{-/-}$ MRL.*lpr* mice were stained for TCRβ, CD4, CD8, and CD25 followed by intracellular staining for Foxp3 (representative experiment of CD25$^+$Foxp3$^+$ cells after gating for CD3$^+$CD4$^+$CD8$^-$ [upper panel], and cumulative results [lower panel], $n = 3$). Error bar represents SD. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$.

B cells were all reduced in absolute numbers (Fig. 5B–D). Tregs were proportionally higher in the lymph nodes but not the spleens of IL-23R$^{-/-}$ MRL.*lpr* versus wild type mice (Fig. 5E). These results clearly demonstrate that IL-23R signaling is important for T cell–dependent humoral responses in lupus-prone mice.

IL-23R signaling influences IL-17 production and DN T cell generation

As expected, we found that Th17 cells were decreased in IL-23R–deficient compared with sufficient mice (Fig. 6A). In both IL-23R–deficient and sufficient animals, DN T cells were the main IL-17 producing cells when tested directly ex vivo (Fig. 6B). To explain the IL-23R influenced preferential production of IL-17 by DN T cells, we stained MRL.*lpr* splenocytes for IL-23 receptor and found that the majority of T cells expressing IL-23R were DN T cells with a minority being CD4$^+$, whereas very few CD8$^+$ were IL-23R$^+$ (Fig. 6C). We then tested whether exogenous IL-23 (25 ng/ml) can boost the production of IL-17 (Fig. 6D) when added to MRL.*lpr* lymphocytes in culture. Indeed, there was an increase in both IL-17$^+$ T cells and IL-17 secretion after the addition of IL-23 (Fig. 6D, 6E).

We observed that the addition of IL-23 in the culture of total splenocytes with anti-CD3/CD28 Abs resulted in increased numbers of DN T cells (Fig. 6F). We then cultured purified splenic CD4$^+$ and CD8$^+$ T cells in vitro from IL-23R$^{-/-}$ and IL-23R$^{+/+}$ MRL.*lpr* mice. We found that >10% CD4$^+$ T cells from wild type MRL.*lpr* mice become DN T cells, significantly higher than both IL-23R$^{-/-}$ MRL.*lpr* CD4$^+$ and control MRL.MPJ CD4$^+$ cells (Fig. 6G). Of note, very few CD8$^+$ T cells became DN T cells either in the presence or absence of IL-23R (Fig. 6H).

Given the effect of IL-23R deficiency on DN T cell numbers, we postulated that not only is there a decrease in the generation of DN T cells, but also that DN T cells may proliferate less and/or die at a higher rate in the absence of IL-23R. To this end, we isolated lymphocytes from IL-23R$^{-/-}$ and IL-23R$^{+/+}$ MRL.*lpr* mice, and analyzed the proportion of Ki67$^+$ cells using flow

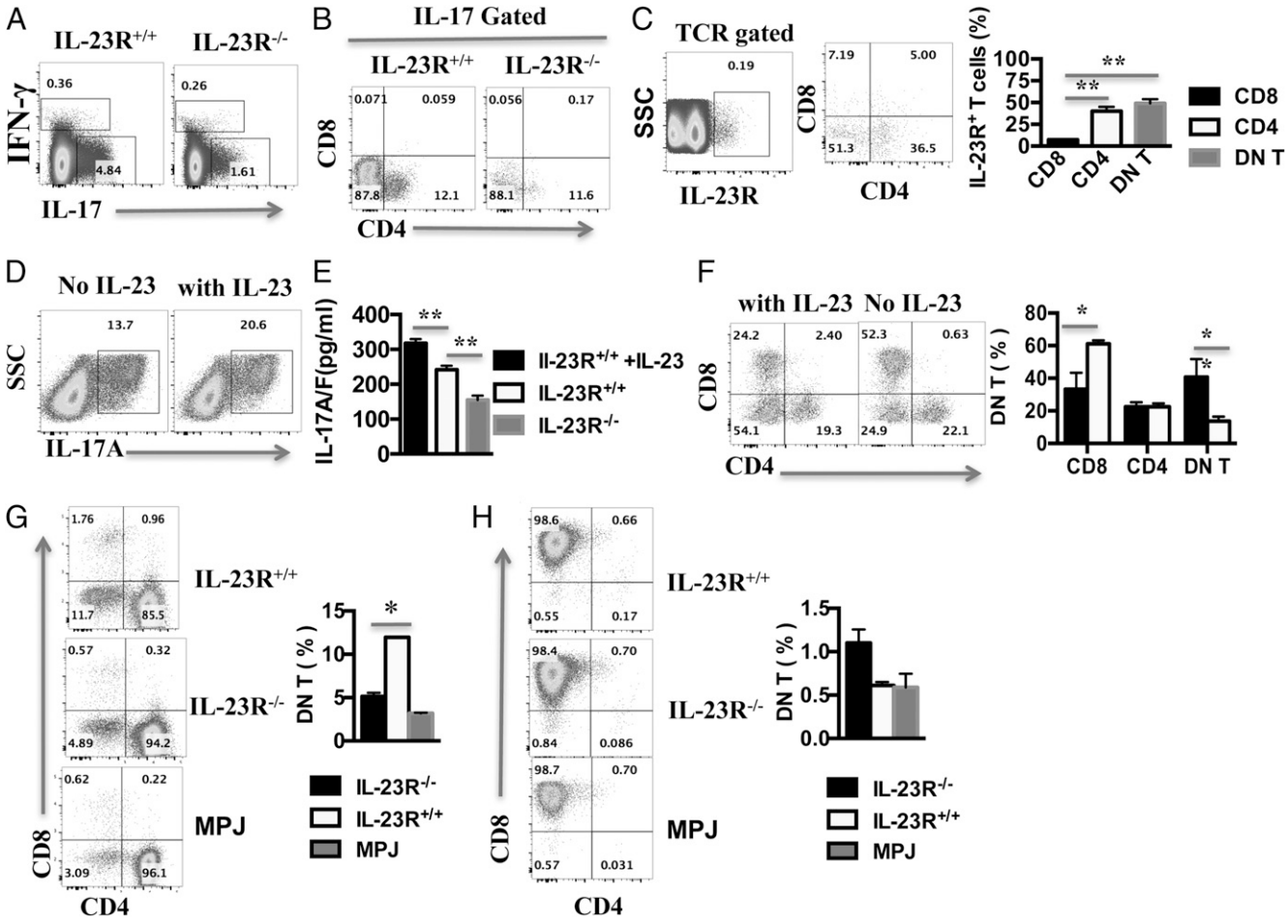

FIGURE 6. IL-23R deficiency limited the production of IL-17 and the generation of DN T cells. Spleens were harvested from IL-23R$^{-/-}$ MRL.*lpr* and IL-23R$^{+/+}$MRL.*lpr* mice as indicated, and single cell suspension was prepared. (**A**) IFN-γ and IL-17 produced by T cells were analyzed by intracellular staining and cytometry after gating on live TCRαβ$^+$ T cells (see *Materials and Methods*). (**B**) IL-17–producing T cells were analyzed for CD4 and CD8 expression by cytometry after gating on TCRβ$^+$IL-17$^+$; (**C**) TCRβ$^+$ splenocytes from IL-23R$^{+/+}$ MRL.*lpr* were stained with anti-IL-23R Ab and evaluated using flow cytometry (cumulative results [right panel], $n = 3$). (**D**) Splenocytes from IL-23R$^{+/+}$ MRL.*lpr* were stimulated with anti-CD3/anti-CD28 Abs for 3 d with or without IL-23; then the cells were harvested to analyzed for IL-17 expression by cytometry via gating on TCRβ$^+$ T; (**E**) The supernatants from the 3 d cell cultures described in (D) including a IL-23R$^{-/-}$ control were used for IL-17A/F level measurement with ELISA ($n = 3$ mice in each plot). (**F**) 4×10^6 splenocytes from 8-wk-old MRL.*lpr* mice were stimulated with anti-CD3/28 in the presence or absence of 25 ng/ml IL-23 for 2 d; the cells were then harvested and analyzed for CD4 and CD8 expression by cytometry after gating on CD3$^+$ T cells (representative plot of three mice, left panel; cumulative results, right panel). (**G** and **H**) CD4$^+$ (G) and CD8$^+$ (H) T cells were sort-purified from the spleens of 12-wk-old IL-23R$^{+/+}$ MRL.*lpr*, IL-23R$^{-/-}$MRL.*lpr*, and MRL.MPJ mice. They were then stimulated with anti-CD3/CD28 for 3 d before being (re)stained for CD3, CD4, and CD8 expression (representative experiment, left panel; cumulative data from three mice per group, right panel). Error bar represents SD. Data are representative of three independent experiments. *$p < 0.05$.

cytometry. As shown in Supplemental Fig. 2A and 2B, in both spleen and peripheral lymph nodes, DN T cells from IL-23R$^{+/+}$ MRL.*lpr* mice exhibited increased proliferation as compared with those from IL-23R$^{-/-}$ MRL.*lpr*. CD8$^+$ T cells exhibited the opposite, whereas there was no difference in CD4$^+$ T cell proliferation between the two strains. To assess DN T cell proliferation dynamically, we injected Brdu i.p. into the two strains of mice, and found that the proliferation of T cells from spleens was not significantly different between the two groups (Supplemental Fig. 2C). However, CD4$^+$ T cells from peripheral lymph nodes of IL-23R$^{+/+}$ MRL.*lpr* mice exhibited decreased proliferation compared with those from IL-23R$^{-/-}$ controls. In contrast, DN T cells from IL-23R$^{+/+}$ MRL.*lpr* mice displayed more proliferation than those from IL-23R$^{-/-}$ mice (Supplemental Fig. 2D). These results demonstrated that IL-23R deficiency resulted in decreased DN T cell proliferation. The effect on CD4$^+$ and CD8$^+$ T cells was opposite, although not consistent between the spleens and lymph nodes.

Next, we asked whether deficiency of IL-23R influences T cell death in MRL.*lpr* mice. To this end we isolated lymphocytes from spleen and lymph nodes of IL-23R$^{-/-}$ MRL.*lpr* and IL-23R$^{+/+}$ controls. IL-23R deficiency resulted in increased death of DN T cells in the spleen as compared with Il-23R$^{+/+}$ animals (Supplemental Fig. 3A), whereas in peripheral lymph nodes there were no significant differences among the groups (Supplemental Fig. 3B). There was no apparent effect of IL-23R on single positive T cell death. Taken together, these data suggest that IL-23R signaling enhances DN T cell accumulation in MRL.*lpr* mice by increasing their generation from CD4 cells, promoting their ability to proliferate, and decreasing their demise.

IL-23R deficiency resulted in increased IL-2 production

As discussed above, lupus T cells are characterized by poor production of IL-2 upon stimulation, whereas exogenous IL-2 ameliorates the disease (13). We therefore asked whether IL-23R deficiency will affect IL-2 production. In Fig. 7A, we show that IL-23R$^{-/-}$ lymphocytes produced higher levels of IL-2 when activated in vitro than wild type MRL.*lpr* mice. Using intracellular staining, we found that the IL-23 deficiency promoted IL-2 production by CD4 T cells with a minimal effect on CD8 (Fig. 7B) and no effect on the very low IL-2–producing DN T cells (Supplemental Fig. 4). Then, we cultured IL-23–sufficient MRL.*lpr* lymphocytes in the presence of IL-23, and measured IL-2 production (Fig. 7C). IL-23 resulted in a 2- and 3-fold decrease in the production of IL-2 by spleen and lymph node cells respectively. To further analyze the effect of the altered cytokine milieu in MRL.*lpr* mice, we cultured wild type MRL.*lpr* lymphocytes with or without IL-23 and/or IL-2. We observed that the positive effect of IL-23 on IL-17 production can be limited in the presence of IL-2 (Fig. 7D). These data taken together show that IL-23 not only affects IL-17 production but fundamentally alters the cytokine milieu leading to a low IL-2/high IL-17 proinflammatory state.

Discussion

In this study, to our knowledge we showed for the first time that IL-23 and its receptor are upregulated in SLE and may account at least in part for the expansion of extrafollicular Tfh, the decreased IL-2, and aberrant anti-dsDNA Ab production. In murine lupus, deficiency of IL-23R attenuated the severity of nephritis by inducing the counter-inflammatory cytokine IL-2, limiting the expansion of DN T cells, and inhibiting critical steps in the production of pathogenic autoantibodies.

DN T cells are present in the thymus, and are generated at an early stage in the development of mature T cells. DN T cells are also generated in the periphery as the result of downregulation and internalization of CD4 or CD8. It has been shown that activated CD8 cells from healthy individuals convert to DN T cells (20). In MRL.*lpr* mice DN T cells are expanded due to deficient apoptosis and have proinflammatory features, producing IL-17 among other cytokines (3). DN T cells from SLE patients share certain features with the MRL.*lpr* DN T cells: they produce IL-17, infiltrate the kidneys (3), and provide help to B cells (19). Our data show that CD4$^+$ T cells

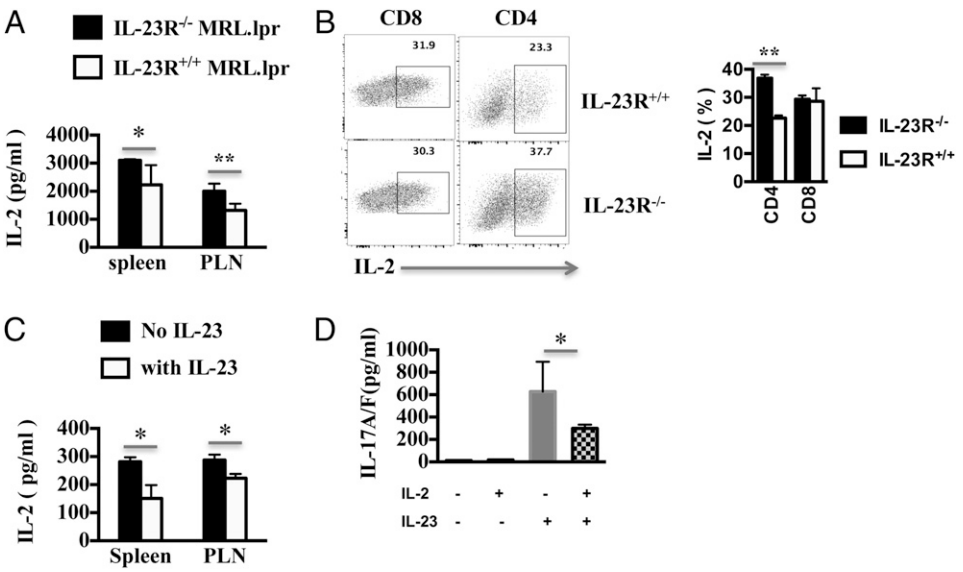

FIGURE 7. IL-23 limited the production of IL-2 in MRL.*lpr* mice. (**A**) 4×10^6 cell suspensions were prepared from spleens or peripheral lymph nodes (PLNs) of IL-23R$^{+/+}$ or IL-23R$^{-/-}$ MRL.*lpr* mice and were stimulated with anti-CD3/CD28 Abs for 16 h. IL-2 levels in the supernatant were measured by ELISA ($n = 3$). (**B**) 4×10^6 cell suspensions were prepared from spleens of IL-23R$^{+/+}$ or IL-23R$^{-/-}$ MRL.*lpr* mice and were stimulated with anti-CD3/CD28 Abs for 16 h. PMA, ionomycin, and brefeldin A were added 4 h prior to harvesting and then IL-2 expression was analyzed by flow cytometry after gating on live TCR$\alpha\beta^+$ CD4$^+$ or CD8$^+$ T cells. (**C**) 4×10^6 cells from spleens or PLN of IL-23R$^{+/+}$ MRL.*lpr* mice were stimulated with anti-CD3/CD28 with or without IL-23 (25 ng/ml) for 3 d and the level of IL-2 in the supernatant was measured by ELISA ($n = 3$). (**D**) 4×10^6 cells from spleens of IL-23R$^{+/+}$MRL.*lpr* mice were stimulated with anti-CD3/CD28 Abs with or without IL-23 (25 ng/ml) and/or IL-2 for 3 d. Subsequently IL-17A/F level in the supernatant was measured by ELISA (cumulative results, $n = 3$ mice). Error bar represents SD. Data are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$.

from lupus-prone mice and patients with SLE can become DN T cells upon exposure to IL-23. Moreover, we found that IL-23R deficiency results in decreased proliferation and increased death of DN T cells, suggesting an effect of IL-23 on DN T cells at various stages of their development. The underlying mechanism may reflect a direct effect of STAT3-mediated prosurvival signal (21) to DN T cells or an indirect one by influencing IL-2 production, which can alter both cell activation and post activation cell death (22).

IL-23R deficiency resulted in decreased production of anti-dsDNA Abs in MRL.*lpr* mice, whereas in vitro exposure of T and B cells from SLE patients to IL-23 resulted in higher T dependent anti-dsDNA Abs production. The production of these potentially pathogenic autoantibodies depends on the activity of Tfh (23). Circulating Tfh-like cells have been well described in SLE and are associated with disease activity (24). Our data show that IL-23 significantly increases Tfh and Tfh-like numbers in both mice and in in vitro SLE lymphocyte cultures, potentially due to the observed decrease in IL-2 production. Future studies are needed to further dissect the molecular mechanisms whereby IL-23R leads to the expansion of Tfh in lupus-prone mice and SLE patients.

Besides the aforementioned effects of IL-23, we showed that IL-23 affects the production of key cytokines. IL-23 promoted the production of IL-17 as expected, but also limited the production of IL-2 by impairing the *il-2* gene enhancer NFκBp65. NFκBp65 is known to be deficiently recruited in SLE T cell nuclei and to contribute to the limited production of IL-2 by activated SLE T cells (16). Low-dose IL-2 has been claimed to suppress autoimmunity and pathology in mice and in uncontrolled trials in humans (25, 26). It has been suggested that the effect of low-dose IL-2 in abrogating lupus disease activity depends on empowering regulatory T cells. Our data suggest that IL-23 deficiency in lupus-prone mice induces IL-2 production and Treg generation. The reciprocal relation between IL-23/IL-17 on one hand and IL-2/Treg on the other may be key in the ongoing inflammatory response in SLE. This observation furthermore explains why IL-23 targeting is efficacious in mitigating lupus nephritis in MRL.*lpr* mice as opposed to IL-17A targeting, which does not increase IL-2/Treg or improve lupus nephritis.

IL-23, a cytokine that is elevated in the serum of patients with active SLE, therefore emerges as a key molecule that dictates the balance between pro- and anti-inflammatory cytokines in this disease. We propose that targeting IL-23 may restore this balance in SLE, limiting both autoantibody production and cell infiltration in organ targets. Furthermore, as biologic therapies move to dual cytokine targeting, our studies point to the possibility of combining low-dose IL-2 along with blockade of IL-23 as a higher clinical value therapeutic intervention.

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Disclosures

The authors have no financial conflicts of interest.

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