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Interleukin-23 deficiency alters thymic selection in lupus prone mice

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Abstract

We have previously reported that IL-23 receptor deficiency in MRL.*lpr* mice ameliorates lupus by altering the balance of pro- and anti-inflammatory cytokines in secondary lymphoid organs. As IL-23 may also impact thymic selection, we evaluated the effect of IL-23 on thymic T cell development in lupus prone mice. We generated IL-23p19 deficient MRL.*lpr* mice and harvested their thymus at 8-week of age. We found that the late stage double negative DN4 population was increased in IL-23p19^{-/-}MRL.*lpr* mice when compared to IL-23p19^{+/+}MRL.*lpr* mice. Despite this, mature thymocytes (CD24⁻TCRβ⁺) were decreased by more than 50% in the IL23p19 deficient mice vs. wild type controls. This was associated with a decrease in the generation of CD8⁺ T cells, possibly through downregulation of the IL-7 receptor. CD8⁺ T cells were not only fewer in numbers but also had decreased expression of the migration related receptors CD44 and CD62L in the thymus and spleens of IL-23p19 deficient vs. wild type mice. We propose that IL-23 promotes the development of lupus-like autoimmunity not only through T cell polarization and cytokine production in the peripheral lymphoid organs but also by influencing T cell thymic development.

Keywords

MRL.*lpr*, SLE; interleukin-23; thymus

Introduction

Interleukin 23 (IL-23), a member of the IL-2 family of cytokines, plays a significant role in the pathophysiology of several autoimmune diseases such as psoriasis, psoriatic arthritis and inflammatory bowel disease. We have previously shown that IL-23 influences the (auto)-immune response in both patients with systemic lupus erythematosus (SLE) and lupus prone mice by a variety of mechanisms including increase in IL-17 production and inhibition of IL-2^{1, 2}. Moreover, IL-23 induces follicular T helper cells (Tfh) while limiting Treg activity, a hallmark of T cell profile in SLE. In turn Tfh provide excessive help to antibody producing

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Author Contributions

Hong Dai designed and performed most of the experiments (criterion 1a,1b,1c,2,3). Vasileios C Kytaris designed the studies, and together with Hong Dai prepared the manuscript (criterion 1a,1c,2,3).

cells, increasing the production of dsDNA antibodies. Although its pro-inflammatory role is undisputed, the effect of IL-23 on T cell development in SLE is unclear.

T cell development largely takes place in the thymus, where T cell precursors migrate from the bone marrow³. At the very early stages of thymic development, T cell precursors do not express CD4 or CD8 (double negative cells, DN). These DN cells undergo maturation from DN1 to DN4 stage, eventually expressing both CD4 and CD8 receptors and becoming double positive (DP) thymocytes. DP cells in turn undergo positive selection based on the recognition of major histocompatibility complex (MHC) molecules. During this process DP thymocytes commit to one of the two lineages, CD4 or CD8, thus become mature single positive (SP) cells. SP undergo negative selection and eventually emigrate to secondary lymphoid organs.

Intra-thymic IL-23 production has been shown to play a regulatory role in T cell development in naïve mice⁴. IL-23 induces apoptosis of late stage DP thymocytes, affecting the process of T cell selection, which by limiting thymic Treg output in mice, may promote a pro-inflammatory state. In this manuscript we examined whether IL-23 influences thymic selection in lupus prone mice and how this may impact T cell phenotype in the periphery.

Materials and Methods

Mice

We generated IL-23p19-deficient MRL.*Fas*^{lpr/lpr} (MRL.*lpr*) mice using a backcross-intercross scheme. MRL.*lpr* mice purchased from The Jackson Laboratory (Bar Harbor, ME) were crossed with IL-23p19^{-/-}C57/BL6 mice, a kind gift from Dr. Nico Ghiraldi⁵. After 12 generations of breeding, the mice were PCR screened for the *Fas/lpr* and mutated *IL-23p19* gene. Primer for *Fas/lpr* genetic screen: 5' GTAAATAATTGTGCTTCGTCAG-3', 5'-TAGAAAGGTGCACGGGTGTG-3', and 5'-CAAATCTAGGCATTAACAGTG-3'; *IL-23p19* genetic screen was performed as before⁵. All mice were housed at the Beth Israel Deaconess Medical Center pathogen-free animal facility (Boston, MA). Our protocol was approved by the BIDMC IACUC.

Flow cytometry

2×10⁶ cells were suspended in 50 µl FACS buffer containing fluorescent antibodies. All cells were stained with Zombie aqua (Biolegend). The following antibodies were used for staining: CD3, CD4, CD5, CD8, CD24, TCRβ, TCRγδ, CD44, CD25, CD69, CD62L, CD127 (BD Pharmingen). RORγt was analyzed by intracellular staining (BD Pharmingen). Cells were analyzed by flow cytometry (FACS Caliber; BD Biosystems, San Jose, USA).

Cell culture, cytokine and protein measurement

Murine thymocytes and splenocytes were cultured in RPMI1640 with 10% (v/v) FCS (supplemented with 50 µM 2-ME, 1 mM sodium pyruvate, non-essential amino acids, Lglutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin) at 37°C in a humidified atmosphere of 10% CO₂ in culture incubator.

Histopathology and tissue cell isolation

Cells were extracted from murine thymus and spleens by filtering the tissue through a 100- μ m 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.

Statistical analysis

Statistical analyses were performed in GraphPad Prism version 5.0 software. Statistical significance was determined by t-tests (two-tailed). Statistical significance was defined as $p < 0.05$. 3–5 mice were used for each experiment as indicated. Subsequently the results were replicated in an additional independent experiment using 3–5 mice/experiment.

Results

Thymocyte development in the absence IL-23p19 in MRL.*lpr* mice.

We harvested thymus from 8 weeks-old MRL.*lpr* mice that were deficient or sufficient (wild type) for the p19 subunit of IL-23. The thymus was not significantly different in size between the two mouse strains. We then stained the thymocytes for expression of CD4 and CD8 and observed a modest but significance increase in the proportion of cells that were in the double positive (DP, CD4⁺CD8⁺) stage vs. single positive (SP, CD4⁺CD8⁻ or CD4⁻CD8⁺) cell stage ($p < 0.05$, Figure 1A and B).

Contrary to DP and SP thymocytes, double negative (DN, CD4⁻CD8⁻) cells, which represent the earlier stages of thymocyte development, were not significantly different in their overall number between the two mouse strains. The expression though of the DN maturation markers CD44 and CD25 were altered; IL-23p19^{-/-} derived DN thymocytes were less likely to express those two molecules. This CD44⁻CD25⁻ DN thymocyte population (DN4), that was modestly expanded in the IL-23p19^{-/-}MRL.*lpr* mice, represents the later stage of DN development just before the DP stage and the ensuing positive selection.

We then asked whether the observed increase in DN4 population results in more cells entering the DP stage, undergoing positive selection and eventually becoming mature T cells. To this end we stained the thymocytes of both strains with CD24, a marker of early stage development and TCR β . We found that in the absence of IL-23p19, MRL.*lpr* thymocytes were more likely to be immature (CD24⁺TCR β ⁻) (Figure 1C, $p < 0.05$) than mature (CD24⁻TCR β ⁺). When the different populations were analyzed for the expression of CD4 and CD8, we found that IL-23p19 deficient mice had a decrease in mature CD8⁺ cells and a proportional increase in CD4⁺ (Figure 1D, $p < 0.05$).

These data suggest that IL-23p19 deficiency, although associated with increase in late stage double negative thymocytes (DN4), results in relative decrease in mature thymocytes in MRL.*lpr* mice.

IL-23p19 deficient MRL.*lpr* mice show a decrease in thymocyte positive selection.

To account for the discrepancy between apparent accelerated early stage thymocyte development and late stage decrease in mature thymocytes, we asked whether positive selection is affected in the thymus of MRL.*lpr* mice that do not express IL-23p19. First, we examined the percentage of thymus cells that co-express TCR β and CD69 in IL-23p19 deficient and wild type MRL.*lpr* mice. We found that the deficient mice had a relative increase in prepositive selection TCR β ⁺CD69⁺ thymocytes (population P1, Figure 2A, $p<0.05$) with reciprocal decrease in thymocytes that are at the post positive selection (populations P3 and P4, $p<0.05$, Figure 2A). We found similar results by comparing CD5, another marker of thymocyte maturation, and TCR β expression in the two groups (data not shown).

Then we examined whether IL-23p19 affects not only positive selection but also the relative generation of CD4 and CD8 single positive cells. We found that IL-23p19 deficient MRL.*lpr* mice single positive thymocytes were more likely to express CD4 than CD8 while the opposite was true for MRL.*lpr* wild type mice (Figure 2B, $p<0.01$ for populations P3 and P4). This is in keeping with the finding that mature CD24⁺ thymocytes were more likely to be CD4⁺ rather than CD8⁺ (Figure 1D). To account for this change in lineage commitment, we examined the expression of IL-7R, a receptor that provides a survival signal that is critical for CD8⁺ single positive thymocytes but not CD4⁺. We found that the expression of CD127 (the α subunit of the IL-7R) was decreased in both CD4 and CD8 single positive T cells ($p<0.01$, Figure 2C). This explained in part the decrease in relative proportion of CD8 SP T cells among maturing thymocytes in the IL-23p19 deficient mice. Another molecule that is associated with thymocyte survival is the transcription factor ROR γ t, that is regulated by IL-23. We found that although ROR γ t overall was not decreased in the thymus of the IL-23p19^{-/-} mice vs controls (data not shown), it was modestly decreased in the double positive cells ($p<0.05$, Figure 2D).

These data suggest that IL-23 deficiency in lupus prone MRL.*lpr* mice decreased thymocyte selection possibly through its effect on ROR γ t expression in DP thymocytes. Moreover, potentially through its effect on IL-7R expression, IL-23 deficiency changed lineage commitment in the thymus, decreasing the proportion of CD8⁺ cells among mature thymocytes.

IL-23p19 deficiency delays CD8⁺ thymocyte maturation.

We then asked whether IL-23p19 not only affects CD8⁺ SP cell proportion but also their phenotype. We found that IL-23p19 deficient MRL.*lpr* CD8⁺ thymocytes had decreased expression of the surface molecules CD44 and CD62L, both of which are important for lymphocyte trafficking and homing to peripheral lymphoid organs (Fig 3A). IL-23p19 deficiency did not alter the expression of CCR7, an important molecule for intra-thymic migration (data not shown), suggesting that thymic emigration rather than intra-thymic migration is affected by IL-23.

To answer whether the changes in the thymocyte development may directly impact T cell population in the secondary lymphoid organs, we determined the expression levels of CD44

and CD62L in CD4⁺ and CD8⁺ splenocytes of mice that had not yet developed clinical disease (8-weeks old). We found that indeed, CD8⁺ but not CD4⁺ T cells had significantly decreased expression of both CD62L and CD44 in the absence of IL-23p19.

These data suggest that IL-23 impacts T cell emigration from the thymus, allowing for population of the secondary lymph organs with T cells that are more likely to migrate to tissues. We observed this effect only on CD8⁺ T cells.

Discussion

Herein we show that IL-23 deficiency in lupus prone mice results in altered thymocyte maturation, which is more pronounced at the DP stage. This is possibly related to decreased levels of the IL-23 dependent transcription factor ROR γ t. Besides being a survival factor for DP thymocytes, ROR γ t is important for the TCR α repertoire generation and natural Treg development. It is therefore not surprising that its inhibition ameliorated experimental autoimmune encephalomyelitis in mice⁶.

Another striking finding in this study was the effect of IL-23 on the generation of CD8⁺ cells. IL-23 deficiency led to a relative decrease in the CD8 SP cells among mature thymocytes. Normal maturing DP cells downregulate CD8 expression, a phenomenon that can be reversed by IL-7⁷. We observed that IL-23p19^{-/-}MRL.*lpr* mice had significantly reduced expression of IL-7R when compared to wild type mice. This explains why DP preferentially develop into CD4 SP cells in these mice. IL-7:IL7R signaling is also responsible for lymphoid cell homeostasis in both central and peripheral lymphoid organs. In murine lupus, enhanced IL-7 signaling has been linked to lymphoproliferation. Blockade of the IL-7R in MRL.*lpr* mice resulted in amelioration of the disease⁸, suggesting that IL-7 is important for the initiation and the propagation of the autoimmune response in murine lupus. Whether IL-23 influences IL-7 signaling in the periphery as well as in the thymus remains an open question.

Finally, we observed that IL-23 deficiency resulted in the percent decrease of mature CD44⁺CD62L⁺ T cells in both the thymus and the periphery, disproportionally affecting the CD8⁺ cells. We have previously shown that SLE T cells overexpress CD44^{9, 10}, which determines their homing to target-tissues. We have also reported that lymphocytes from B6.*lpr* mice treated with IL-23 *in vitro* were more likely to infiltrate the kidneys of recipient Rag 1^{-/-} lymphopenic mice¹¹. This effect on lymphocyte migration may be mediated by the signal transducer and activator of transcription (STAT) 3 which becomes activated following IL-23:IL-23R engagement; STAT3 enhances T cell adhesion and migration¹² while its inhibition alleviates nephritis in MRL.*lpr* mice¹³. IL-23 therefore may be important in lymphocyte trafficking between primary, secondary lymphoid organs, and target-tissues.

In conclusion, the results presented in this report suggest that IL-23 plays a significant role in the development of autoimmunity by affecting not only peripheral T cell function but also T cell thymic development.

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Figure 1. IL-23p19^{-/-} deficiency increased early but not late stage maturation of thymocytes in MRL.lpr mice.

(A) Thymocytes were isolated from 8-week old IL-23p19^{-/-} and IL-23p19^{+/+} MRL.lpr mice. The cells were stained with CD4 and CD8. Using flowcytometric analysis, the populations of CD4⁺, CD8⁺, CD4⁺CD8⁺ (double positive, DP) and CD4⁻CD8⁻ (double negative, DN) were calculated. A representative experiment (left panel) and cumulative data (right panel) are shown here (n=5). (B) The double negative thymocytes were stained for CD44 and CD25 and the different DN population percentages (DN1->4) were calculated using flowcytometry. A representative experiment (left panel) and cumulative data (right panel) are shown here (n=3). (C) Thymocytes were isolated from 8-week old IL-23p19^{-/-} and IL-23p19^{+/+} MRL.lpr mice. The cells were stained with CD4, CD8, CD24 and TCRβ and underwent flowcytometric analysis. Three populations of maturing thymocytes using the CD24 and TCRβ markers were analyzed (P1:CD24⁺TCRβ⁻, P2:CD24⁺TCRβ⁺, P3:CD24⁻TCRβ⁺). A representative experiment (left panel) and cumulative results (right panel) are shown here (n=5). (D) Thymocytes were gated as in 1C and the expression of CD4 and CD8 was evaluated using flowcytometry. A representative experiment (left panel) and cumulative data (right panel, n=5). * = p<0.05, ** = p<0.01. Error bar represents SEM.

Figure 2. IL-23p19^{-/-} deficiency decreased positive selection in the thymus of MRL.*lpr* mice. (A) Total thymocytes were isolated from 8-week old IL-23p19^{-/-} and IL-23p19^{+/+} MRL.*lpr* mice. The cells were stained with CD69, TCRβ. A representative experiment (upper panel) and cumulative data (lower panel) are shown here (n=5). (B) The cells from Fig 2A, were gated according to the expression of CD69 and TCRβ; the expression of CD4 and CD8 surface molecules was evaluated in the depicted 4 populations of thymocytes undergoing positive selection. A representative experiment (upper panel) and cumulative data (lower panel) are shown here (n=5). (C) Thymocytes were isolated from 8-week old IL-23p19^{-/-} and IL-23p19^{+/+} MRL.*lpr* mice. The cells were stained with CD4, CD8 and CD127 (IL-7Rα). The expression of the IL7Rα in CD4 and CD8 positive cells is shown here. A representative experiment (left panel) and cumulative data (right panel) are shown here (n=5) (D) CD4⁺CD8⁺ thymocytes were intracellularly stained for the expression of RORγt. A representative experiment (left panel) and cumulative data (right panel) are shown here (n=3). * = p < 0.05, ** = p < 0.01. Error bar represents SEM.

Figure 3. IL-23p19^{-/-} deficiency is associated with altered expression of maturation molecules among CD8 T cells.

(A) Total thymocytes were isolated from 8-week old IL-23p19^{-/-} and IL-23p19^{+/+} MRL.*lpr* mice, stained with CD4, CD8, CD44 and CD62L and used for flowcytometric analysis. The expression of CD62L and CD44 on cells gated for CD4 or CD8 is shown here. A representative experiment (upper panel) and cumulative data (lower panel) are shown here (n=5). (B) Total splenocytes were isolated from 8-week old IL-23p19^{-/-} and IL-23p19^{+/+} MRL.*lpr* mice, stained with CD4, CD8, CD44 and CD62L and used for flowcytometric analysis. The expression of CD62L and CD44 on cells gated for CD4 or CD8 is shown here. A representative experiment (upper panel) and cumulative data (lower panel) are shown here (n=5). * = p<0.05, ** = p<0.01. Error bar represents SEM.