Increased cathepsin S in Prdm1−/− dendritic cells alters the T_{FH} cell repertoire and contributes to lupus

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Aberrant population expansion of follicular helper T cells (T_{FH} cells) occurs in patients with lupus. An unanswered question is whether an altered repertoire of T cell antigen receptors (TCRs) is associated with such expansion. Here we found that the transcription factor Blimp-1 (encoded by Prdm1) repressed expression of the gene encoding cathepsin S (Cts), a cysteine protease that cleaves invariant chains and produces antigenic peptides for loading onto major histocompatibility complex (MHC) class II molecules. The increased CTS expression in dendritic cells (DCs) from female mice with dendritic cell–specific conditional knockout of Prdm1 (CKO mice) altered the presentation of antigen to CD4+ T cells. Analysis of complementarity-determining region 3 (CDR3) regions containing the β-chain variable region (V_{β}) demonstrated a more diverse repertoire of T_{FH} cells from female CKO mice than of those from wild-type mice. In vivo treatment of CKO mice with a CTS inhibitor abolished the lupus-related phenotype and reduced the diversity of the T_{FH} cell TCR repertoire. Thus, Blimp-1 deficiency in DCs led to loss of appropriate regulation of Cts expression in female mice and thereby modulated antigen presentation and the T_{FH} cell repertoire to contribute to autoimmunity.

The TCR repertoire is determined through positive and negative selection of T cells on the basis of recognition by the TCR of complexes of peptide and major histocompatibility complex (MHC) molecules presented by antigen-presenting cells (APCs). In the periphery, CD11c classical dendritic cells (cDCs) are the main APCs, with a critical role in both innate immune responses and adaptive immune responses. DCs activate natural killer cells, natural killer T cells and innate lymphocytes at the site of infection or sterile inflammation. They also process antigens and migrate to local lymphoid organs, where they activate naive T cells. T cells require signals from a peptide–MHC II complex, co-stimulatory molecules and cytokines provided by DCs for differentiation into various subsets of CD4+ effector T cells or CD4+ regulatory T cells (T_{reg} cells), with each CD4+ effector T cell subset executing unique functions and secreting different cytokines.

The cytokine milieu is critical for the differentiation of CD4+ T cells. Dendritic cytokines help to establish the initial polarization of CD4+ helper T cell subsets: interleukin 12 (IL-12) establishes the T_{TH1} subset; IL-4 establishes the T_{TH2} subset; IL-6 and TGF-β establish the T_{TH17} subset; IL-6 establishes the T_{TH1} cell subset; and TGF-β and IL-10 establish the T_{reg} cell subset. The differentiation of CD4+ T cells can be modulated by several other factors, such as the type of antigen and dose of exposure, affinity of the TCR for the MHC class II complex, and duration of stimulation.

The antigen-processing pathways in mouse DCs have been investigated extensively. After being taken up, antigens are transported into the endolysosomal compartment, where they are cleaved, and some of the fragments that are generated enter the groove of the MHC class II molecule for presentation to CD4+ T cells. This process is dependent on the action of endocytic proteases in endosomal–lysosomal compartments that fall into three main classes: cysteine proteases (cathepsins B, E, H, L, S and Z), aspartate proteases (cathepsins D and E) and serine proteases (cathepsins A and G). While all cathepsins can function in antigen processing and many show an overlapping expression pattern, CTS has been shown to be expressed mainly in professional APCs, including B cells and DCs, in which it has a critical role in cleavage of the invariant chain to permit loading of peptides into MHC class II molecules. CTSS also contributes to antigen processing through degradation of antigen in the endolysosome and helps to establish the pool of peptides that is available for presentation by MHC class II. Appropriate expression of CTSS is critical for establishment of the repertoire of immunocompetent cells. Modulation of the expression of cathepsins CTS and CTSL can change the pool of peptides that are presented in vitro to CD4+ T cells. Overexpression of CTSS in DCs and medullary epithelial cells in the thymus has been shown to permit autoreactive T cells to escape negative selection, presumably through too-exuberant degradation of autoantigens. Whether negative regulation in the periphery is also affected by CTSS has not been addressed.

**PRDM1**, which encodes Blimp-1, was identified as a risk allele for systemic lupus erythematosus (SLE) in genome-wide association studies of Chinese Han and Northern European populations. CD14+ monocyte-derived DCs (MO-DCs) derived from carriers of the...
risk single-nucleotide polymorphism (SNP) (C/C) have lower expression of PRDM1 transcripts than do MO-DCs from carriers of the control (non-risk) SNP (T/T). To investigate the pathological function of Blimp-1 in SLE, we generated mice with DC-specific conditional knockout of Prdm1 (CKO mice) by mating mice expressing Cre recombinase from the gene encoding the integrin CD11c to mice withloxP-flanked Prdm1 alleles. Mice withloxP-flanked Prdm1 alleles but no expression of Cre recombinase served as the control (called ‘wild-type’ here). In female CKO mice, DCs that lacked Blimp-1 exhibited an activated phenotype with enhanced expression of MHC class II and increased production of pro-inflammatory cytokines following stimulation via Toll-like receptors. Those DCs resembled DCs from people with the SLE-associated PRDM1 risk allele, characterized by increased expression of MHC class II and hyper-responsiveness to stimulation via Toll-like receptors. The frequency of T\textsubscript{FH} cells is greater in the blood of patients with lupus, which correlates with disease activity. Female CKO mice have population expansion of T\textsubscript{FH} cells that is associated with a greater number of germinal center B cells that produce autoantibodies and cause antibody-mediated glomerulonephritis. This phenotype is also critically dependent on greater production of IL-6 by Blimp-1-deficient DCs, as all autoimmune features that is associated with a greater number of germinal center B cells.

RESULTS

Increased CTSS expression in Blimp-1-deficient DCs

Blimp-1 has been suggested to participate in antigen presentation through downregulation of the gene encoding the MHC class II transactivator (Ciita) in B cells and CD11c+ DCs. Consistent with those studies, in Blimp-1-deficient DCs have increased MHC class II expression. To investigate whether Blimp-1 deficiency in DCs also leads to altered antigen processing, we assayed Blimp-1-deficient DCs from female C57BL/6 mice for expression of H2-Aa, Ciita, H2dmb, Ctss and other genes encoding products involved in antigen presentation. The expression of H2-Aa, Ciita, H2dmb and Ctss was significantly higher in Blimp-1-deficient DCs than wild-type DCs, whereas other genes showed no difference in expression (Fig. 1a).

To investigate whether Blimp-1 can directly regulate the expression of mouse cathepsin-encoding genes, we searched for the Blimp-1

Figure 1. Increased expression of Ctss in DCs from female CKO mice. (a) qPCR analysis of mRNA from various genes (horizontal axis) in splenic CD11c+ cDCs purified from 6- to 8-week-old female wild-type (WT) or CKO mice (key); results (obtained with specific primers) were normalized to those of the control gene Pol2a. (b) qPCR analysis of Ctss and Ctsf mRNA in splenic cDCs or BM-DCs from age-matched female wild-type and CKO mice (key); results were normalized to those of the control gene Hprt. (c) Flow cytometry (top) and qPCR analysis of Ctss mRNA (below) of blood cDCs from a female CKO mouse (top) or from age-matched female wild-type and CKO mice (horizontal axis, below); qPCR results were normalized as in b. (d) Enzymatic activity in vitro of CTSS in cDCs from wild-type and CKO mice (key); results (relative fluorescence units (RFU)) are presented relative to the fluorescence of cultures with no substrate. (e) qPCR analysis of Ctss mRNA in splenic DCs from male wild-type and CKO mice (horizontal axis); results were normalized as in b. (f) qPCR analysis of PRDM1 and CTSS mRNA in MO-DCs from carriers of the control SNP (Control) or the risk SNP (Risk); results were normalized to those of the control gene POLR2A. Each symbol (b,c,e) represents an individual mouse (n = 9 (splenic DCs), b, n = 6 (BM-DCs), b, n = 4 (c) or n = 3 (e) mice per genotype); small horizontal lines indicate the mean (± s.e.m.). P values, nonparametric, Mann-Whitney test. Data are from three independent experiments (a,b, n = 6 mice per genotype in a: horizontal bars, median; boxes, 25th percentile to 75th percentile; whiskers, 10th percentile and 90th percentile), two independent experiments (c), two independent experiments with n = 6 mice per group (d); plotted as in a), two independent experiments (e) or seven experiments with n = 19 female control carriers, n = 15 female risk carriers, n = 7 male control carriers and n = 11 male risk carriers (f; plotted as in a).
Figure 2 Blimp-1 regulates Ctss expression in DCs. (a) ChIP of DCs purified from the spleen of female wild-type or CKO mice, with antibody to Blimp-1 (anti-Blimp-1) or goat polyclonal IgG (Control IgG), followed by qPCR with primers specific for the Ctss; presented as agarose-gel electrophoresis (left) and qPCR results (right). (b) Flow cytometry (left) of wild-type BM-DCs 24 h after transfection with a luciferase plasmid driven by the Ctss promoter alone or also transfected with (2:1 ratio) control vector encoding green fluorescent protein (GFP) alone (Control) or vector encoding Prdm1 and GFP (Prdm1), analyzing transfection efficiency via GFP expression (quantitation (as GFP+ cells), middle), followed immediately by analysis of luciferase activity of the Ctss promoter, normalized to the activity of renilla luciferase (right). (c) qPCR analysis of Prdm1 and Ctss mRNA in BM-DCs 48 h after transfection of control siRNA with a scrambled sequence (Control) or Prdm1-specific siRNA (Prdm1) (below plot); results are presented relative to those of Polr2a. (d) qPCR analysis of Prdm1 mRNA, Ctss mRNA and Ctsk mRNA (irrelevant control) (bottom row) and immunoblot analysis of Blimp-1, CTSS and actin (loading control) (top right) in Blimp-1-deficient BM-DCs left untransfected (−) or transfected with a plasmid encoding Prdm1 and GFP or control plasmid encoding GFP only (2 µg), analyzed at day 5 after transfection in GFP+ (successfully transfected) populations (red asterisks) purified 2 d after transfection (top left); qPCR results are presented relative to those of Polr2a. Each symbol (c,d) represents an individual experiment; small horizontal lines indicate the mean (± s.e.m.). P values, nonparametric, Mann-Whitney test. Data are from one experiment representative of four independent experiments (a (bottom plotted as in Fig. 1a), b, top), are from four independent experiments with n = 4 mice per group (b, bottom left; plotted as in Fig. 1a), are representative of three experiments with n = 4 mice per group (b, right; plotted as in Fig. 1a), are from one experiment representative of three independent experiments (c, d, bottom).

consensus core binding sequence (5′-GAAAGT-3′) in a region 1,500 base pairs upstream of the transcriptional start site of the first exon of each gene. Ctss, Ctsf, Ctsd, Ctsr, Ctsk, Ctsl1, Cts, Ctss and Ctsw contained one or more of such sequences (Supplementary Table 1). We therefore measured the expression of each cathepsin-encoding gene in DCs from female wild-type and CKO mice. The expression of Ctss and Ctsl, but not that of other cathepsin-encoding genes, was higher in splenic DCs and BM-DCs of female CKO mice than in those of female wild-type mice (Fig. 1b and Supplementary Fig. 1). Expression of Ctss was also higher in blood DCs of female CKO mice than in those of female wild-type mice (Fig. 1c). To confirm that the increased expression of Cts in DCs correlated with increased functional activity of CTSS, we assayed the enzymatic activity of CTSS in DCs purified from the spleen of female CKO and wild-type mice. We measured in vitro enzymatic activity by assaying fluorescence generated after cleavage of a CTSS substrate. Blimp-1-deficient DCs exhibited more catalytic activity than that of wild-type DCs (Fig. 1d). Male CKO mice do not exhibit altered DC function and do not develop lupus-like disease. Both the abundance of Ctss transcripts and the catalytic activity of CTSS were similar in DCs derived from male wild-type mice and those from male CKO mice (Fig. 1d,e), which suggested that Blimp-1 deficiency in combination with sex-specific alterations in DCs altered Ctss expression. To address whether CTSS expression is associated with Blimp-1 expression in human MO-DCs, we measured CTSS expression in MO-DCs from the carriers of risk or control SNPs. MO-DCs from female carriers of the risk SNP exhibited lower PRDM1 expression, as reported, and higher CTSS expression than that of MO-DCs from carriers of the non-risk SNP (Fig. 1f). Given the presence of a Blimp-1 consensus binding site within the Ctss promoter and the enhanced expression of CTSS in DCs of female CKO mice, we performed chromatin-immunoprecipitation (ChIP) assays to determine whether Blimp-1 bound to the Ctss promoter region in splenic CD11c+ DCs from wild-type mice. As expected, the Ctss promoter region was immunoprecipitated with antibody to Blimp-1 (Fig. 2a), which indicated that Blimp-1 bound in vivo to the Ctss promoter in wild-type mice but not in CKO mice. To investigate whether binding by Blimp-1 negatively regulates the transcription of Ctss, we performed assays with a luciferase expression reporter driven by the Ctss promoter (positions −1300 to +10 relative to the transcription start site). There was significant suppression of luciferase expression when Blimp-1 was expressed (Fig. 2b), which demonstrated that Blimp-1 downregulated activity of the Ctss promoter. Moreover, transfection of small interfering RNA (siRNA) specific for Prdm1 in wild-type DCs led to increased Ctss expression, but transfection of nonspecific (control) siRNA did not (Fig. 2c), and restoration of Blimp-1 expression suppressed the abundance of Ctss transcripts and CTSS protein in Blimp-1–deficient DCs (Fig. 2d). These data demonstrated that Ctss was a target of Blimp-1 in DCs.
and that the binding of Blimp-1 to the promoter region of CtsS suppressed its transcription.

Sex specificity of altered CtsS expression

The differential expression of CtsS in Blimp-1-deficient DCs from male mice relative to that in such cells from female mice suggested the presence of additional regulators in the cells of female mice that contributed to Blimp-1-mediated CtsS regulation. DCs from female CKO mice exhibit higher expression of IL-6 following activation of Toll-like receptors than do their counterparts from female wild-type mice, while DCs from male CKO and wild-type mice exhibit similar IL-6 expression. We also investigated whether STAT3 bound to the CtsS promoter (Fig. 3a). We employed two approaches. First, we analyzed CtsS expression in DCs of female CKO mice that were haplosufficient for IL-6 (Ile6<sup>−/−</sup>). Equivalent CtsS expression was observed in DCs from female CKO Ile6<sup>−/−</sup> mice and those from wild-type Ile6<sup>−/−</sup> mice (Fig. 3c). We also administered neutralizing antibody to IL-6 (MP5-20F3) to female CKO mice. There was significantly lower CtsS expression in DCs from mice treated with neutralizing antibody than in those from mice treated with the control antibody immunoglobulin G (IgG) (Fig. 3c, right). Thus, IL-6 participated in the positive regulation of CtsS in DCs and probably contributed to the increased abundance of CtsS transcripts seen in Blimp-1-deficient DCs from female mice.

**Blimp-1-deficient DCs ‘preferentially’ induce IL-21* T cells**

The greater number of T<sub>FH</sub> cells in female CKO mice might have resulted from skewed differentiation into T<sub>FH</sub> cells induced by Blimp-1-deficient DCs, as previously reported<sup>19</sup>, or from DC-induced changes in gene expression in T<sub>FH</sub> cells that led to enhanced survival of T<sub>FH</sub> cells in CKO mice. Ifb4, Maf, Batf and Bcl6 encode transcription factors that positively influence the number of T<sub>FH</sub> cells<sup>27</sup>. We isolated T<sub>FH</sub> cells from female wild-type and CKO mice and assessed the expression of these genes. Only Bcl6 expression was significantly higher in T<sub>FH</sub> cells from female CKO mice than in those of female wild-type mice (Supplementary Fig. 4), which suggested the possibility of enhanced survival of T<sub>FH</sub> cells.

We reasoned that the higher expression of CtsS in Blimp-1-deficient DCs might affect antigen presentation by MHC class II molecules, which might influence the TCR repertoire in a manner favorable to the development of autoreactive T<sub>FH</sub> cells. We therefore assayed activation of OT-II CD4<sup>+</sup> cells, which recognize a complex of ovalbumin (OVA) peptide (amino acids 323–339) and MHC class II. CD4<sup>+</sup> T cells purified from OT-II mice were co-cultured for 3 d with CD11c<sup>+</sup> DCs isolated from the spleen of female CKO or wild-type mice and either intact OVA protein or OVA peptide. T cell proliferation, measured as dilution of the division-tracking dye CFSE, was...
Figure 4 ‘Preferential’ differentiation of IL-21-producing T cells by co-culture with Blimp-1-deficient DCs in vitro. (a) Proliferation of OT-II T cells labeled with CFSE (10 μM) and then cultured alone (T alone) or with wild-type or Blimp-1-deficient DCs (key) and OVA (DC + T + OVA), assessed by flow cytometry (left), and division of OT-II CD4+ T cells cultured for 3 d with DCs from the spleen of female wild-type or CKO mice (key) at various ratios (below plot), with (+) or without (−) OVA protein (10 μg/ml) (right), presented as the division index (total divisions / starting cells). (b) qPCR analysis of Il2 and Il21 mRNA among total RNA purified from OT-II T cells cultured with no DCs (−) or with unfixed DCs (+) or fixed DCs (far right) from wild-type or CKO mice (key), in the presence (+) or absence (−) of OVA protein, and a Transwell insert or an inhibitor (inh) of CTSS (below plot); results were normalized to those of Polr2a. (c) Intracellular staining of IL-2 and IL-21 in T cells cultured with DCs from wild-type or CKO mice (left margin) and OVA protein alone or OVA with a Transwell insert or an inhibitor of CTSS (above plots), then stimulated for 6 h with PMA (100 ng/ml) plus ionomycin (1 μg/ml), with brefeldin A (20 μg/ml) added during the final 4 h of stimulation (left), and frequency of IL-2+ T cells (middle) or IL-21+ T cells (right) among CD4+ T cells cultured as in b (below plot) and with PMA plus ionomycin and brefeldin A as at left. (d) Uptake of OVA into DCs from wild-type or CKO mice (left margin), incubated overnight with DQ-labeled OVA protein at various concentrations (above plots), then washed extensively and assessed by flow cytometry (top), and abundance of OVA in those cells, presented as geometric mean fluorescence intensity (geoMFI). Each symbol (d) represents an individual experiment; small horizontal lines indicate the mean (± s.e.m.). P values, nonparametric, Mann-Whitney test. Data are representative of four experiments (a, plotted as in Fig. 1a (right)), twelve experiments (unfixed DCs) or ten experiments (fixed DCs) (b, plotted as in Fig. 1a), or eight experiments (c, middle and right; plotted as in Fig. 1a), or from one experiment representative of four (c, left) or three (d, top) independent experiments, or are from three experiments (d, bottom).

vigorous in the presence of each antigen preparation, regardless of the source of DCs (Fig. 4a).

The activation of CD4+ T cells is characterized also by cytokine production. Splenic DCs pre-incubated with OVA protein were cultured with OT-II T cells, and the expression of cytokine-encoding mRNA by T cells and the frequency of T cells staining positive for cytokine protein were measured by qPCR and flow cytometry, respectively. Culture with Blimp-1-deficient DCs led to modestly but significantly greater IL-2 mRNA in the T cells than that of T cells cultured with wild-type DCs; however, the intracellular abundance of IL-2 in T cells cultured with Blimp-1-deficient DCs did not differ from that in T cells cultured with wild-type DCs (Fig. 4b,c). The concentration of secreted IL-2 was also not different in these two culture conditions (Supplementary Fig. 5), which explained the lack of difference in the proliferation index of T cells stimulated with wild-type DCs relative to that of T cells stimulated with Blimp-1-deficient DCs.

Many cytokines that are ‘preferentially’ expressed by T_{H1}, T_{H2}, T_{H7} or T_{reg} cells were not significantly different in cultures of T cells with Blimp-1-deficient DCs versus cultures of T cells wild-type DCs (Supplementary Fig. 6). While the expression of IL-21, produced by T_{H1} cells, was relatively low compared with that of other cytokines, Il21 transcription and IL-21 production were greater in cultures with Blimp-1-deficient DCs and intact OVA than in cultures with wild-type DCs and intact OVA (Fig. 4b,c and Supplementary Fig. 5).
The increased IL-21 was dependent on antigen processing, since it was abolished in cultures in which OVA peptide (amino acids 323–339) was used as the antigen (Supplementary Fig. 7), and it was dependent on T cell–DC contact, as it was also abolished in Transwell cultures (Fig. 4b,c). Fixation of DCs after incubation with OVA protein also revealed higher expression of IL-21 in cultures with Blimp-1-deficient DCs than in those with wild-type DCs, although IL-21 production by fixed DCs of each strain was less than that of unfixed cells (Fig. 4b), presumably due to both the absence of cytokine secretion and the absence of membrane fluidity.

The activation of OT-II T cells by OVA protein represented a cognate interaction between T cells and DCs, since the OT-II T cells were not activated with an irrelevant antigen (hen egg lysozyme) (Supplementary Fig. 8). To confirm that the enhanced activation of T cells mediated by Blimp-1-deficient DCs resulted from increased CTSS, we treated cultures with the CTSS inhibitor 219393 (with selectivity for CTSS ~400-fold greater than its selectivity for CTSB)28 or with a CTSB inhibitor, as a control. When the CTSS inhibitor was added during OVA processing, there was no increased IL-21 production seen in cultures with Blimp-1-deficient DCs (Fig. 4b,c), but when the CTSB inhibitor was added, that increase did occur (Supplementary Fig. 8). To confirm that CTSS functions to alter antigen processing rather than antigen uptake, we incubated fluorescence (DQ)-conjugated OVA protein overnight with purified DCs from each strain and assessed OVA uptake by flow cytometry. There was no significant difference in the amount of OVA internalized by wild-type DCs and that internalized by Blimp-1-deficient DCs (Fig. 4d). These data suggested that the Blimp-1-deficient DCs had altered antigen processing. Thus, the increased CTSS altered antigen presentation, which induced activated CD4+ T cells to differentiate into TFH cells.

Diversity of the T<sub>FH</sub> cell TCR repertoire

To investigate whether the population expansion of T<sub>FH</sub> cells in CKO mice was accompanied by a change in their antigen specificity, we compared the TCR repertoires of T<sub>FH</sub> cells in wild-type mice with those of CKO mice. T<sub>FH</sub> cells were purified from the spleens of young (6–10 weeks of age) female and male mice. T<sub>FH</sub> cells were identified by their expression of the chemokine receptor CXCR5 and costimulatory molecule PD-1; they also expressed the transcriptional repressor Blimp-1 (~95%) and costimulatory receptor ICOS-1 (~70%) (Fig. 5a). Although no autoantibodies are detected at this age, there was already a greater number of T<sub>FH</sub> cells in female CKO mice than in wild-type mice (Fig. 5a). We focused on V<sub>β</sub> sequences, as these are more diverse than V<sub>γ</sub> sequences. We analyzed over 100,000 V<sub>β</sub> sequences in T<sub>FH</sub> cells from each of five female mice and five male mice of each strain. T<sub>FH</sub> cells from female CKO mice exhibited a more diverse repertoire than that of T<sub>FH</sub> cells from female wild-type mice (Fig. 5b). Notably, T<sub>FH</sub> cells from male CKO mice and those from wild-type mice exhibited similar diversity (Fig. 5b).

To confirm that the diverse TCR V<sub>β</sub> repertoire of T<sub>FH</sub> cells was associated with autoimmunity, we assessed the V<sub>γ</sub> repertoire of wild-type and female CKO Il6<sup>−/−</sup> mice. It has been shown that neither of these strains develops disease19. We found no difference in repertoire diversity (Fig. 5b), consistent with their similar number of T<sub>FH</sub> cells. Thus, the more diverse repertoire was seen only in association with a lupus phenotype.

CTSS inhibitor suppresses lupus development

Given the enhanced activity of CTSS in Blimp-1-deficient DCs and the promising effects of CTSS inhibition on T<sub>FH</sub> cell differentiation in vitro, we investigated whether inhibition of CTSS would suppress development of the lupus-like phenotype in CKO mice, as has been shown in the MRL/lpr mouse model of lupus28. First, we assessed lymphocyte development following long-term treatment with the CTSS inhibitor ROS461111 in chow. Accumulation of the p10 fragment of the invariant chain was seen in splenocytes of wild-type and CKO mice fed ROS461111-treated chow but not in those of mice fed control chow (Fig. 6a), which demonstrated the efficacy of the treatment. Treatment with ROS461111 did not alter the overall frequency of either lymphocytes or DC subsets in wild-type mice (Fig. 6b). DC activation,
as measured by expression of MHC class II, was also not affected by such treatment (Fig. 6b). RO5461111 was given to female CKO mice to investigate whether inhibition of CTSS prevented development of the lupus-like phenotype. Splenomegaly was suppressed by inhibition of CTSS, with a reduction in the number of splenocytes in RO5461111-treated CKO mice relative to that in untreated CKO mice (Fig. 6c). *Cita* expression was not altered in splenic DCs in RO5461111-treated CKO mice relative to that in untreated CKO mice (Fig. 6d). RO5461111-treated CKO mice showed significantly reduced titers of antibodies to double-stranded DNA at both 4 months and 6 months, relative to that in untreated CKO mice (Fig. 6e). Proteinuria and deposition of IgG in glomeruli were also significantly lower in RO5461111-treated CKO mice than in untreated CKO mice (Fig. 6f,g).

The frequency of T<sub>FH</sub> cells and effector memory T cells was significantly lower in RO5461111-treated CKO mice than in untreated CKO mice (~3.5-fold and 2-fold, respectively), while the frequency of naive T cells (CD4<sup>+</sup>CD62L<sup>hi</sup>) was slightly higher (~2-fold) in RO5461111-treated CKO mice than in untreated CKO mice (Fig. 7a). Consistent with the lower frequency of T<sub>FH</sub> cells, the frequency of germinal-center B cells and plasma cells was also much lower (~10-fold) in RO5461111-treated CKO mice than in untreated CKO mice (Fig. 7b,c). These data demonstrated that increased CTSS expression promoted the development of a lupus-like phenotype in CKO mice and that inhibition of CTSS activity was able to suppress the development of disease. Finally, the V<sub>β</sub> repertoire was significantly less diverse in T<sub>FH</sub> cells from RO5461111-treated CKO mice than in
DISCUSSION

Alterations in antigen presentation can lead to autoimmune or inflammatory diseases\textsuperscript{30}. Human genetic studies have shown that genes encoding human leukocyte antigen class II represent prominent risk alleles for autoimmune diseases, including SLE\textsuperscript{31,32}. As human leukocyte antigens are critical in establishing thresholds for the selection and activation of T cells, the TCR repertoire has been presumed to be a key contributor to many autoimmune diseases\textsuperscript{33}.

A role for Blimp-1 in antigen presentation was initially suggested in a study showing that Blimp-1 regulates expression of Ciita, which encodes a positive regulator of MHC class II expression in B cells\textsuperscript{20}. Subsequently, Blimp-1 was shown to regulate MHC class II in DCs and thereby affect the threshold for activation of CD4\textsuperscript{+} helper T cells in a mouse model of experimental autoimmune encephalomyelitis\textsuperscript{34}.

The importance of antigen presentation prompted us to investigate whether Blimp-1 alters antigen presentation to T cells and, thus, the TCR repertoire. Blimp-1-deficient DCs showed altered expression of several genes encoding molecules involved in antigen presentation, including Ctsx. Deletion of Blimp-1 led to increased Ctsx transcripts in DCs because Blimp-1 is a transcriptional repressor of Ctsx and of Il6. IL-6 had high expression in Blimp-1-deficient DCs from female mice, which led to increased Ctsx expression. Thus, Blimp-1 directly and indirectly regulated Ctsx expression. As described in a published study, increased activation of IL-6 and STAT3 also decreases expression of the gene encoding cystatin C, which subsequently enhances CTSS activity\textsuperscript{24}. The increased CTSS activity in DCs of female KO mice led to increased diversity in the TCR repertoire of T\textsubscript{FH} cells, which was abolished by a CTSS-specific inhibitor. These findings suggested that an increase in CTSS expression associated with absent or low expression of Blimp-1 generated a repertoire more skewed toward self-reactivity. While it is likely that the PRDM1 risk allele for SLE also leads to a more-diverse repertoire of T\textsubscript{FH} cells, this will require confirmation in studies of human subjects with the risk allele or the non-risk allele.

The importance of appropriate expression of CTSS in antigen presentation is now widely accepted, and its molecular mechanism has been investigated in other studies. Modulation of the expression of CTSS and CTLA has been shown to lead to alterations in the pool of peptides presented by MHC class II molecules in an in vitro study\textsuperscript{10,35}. Thymic DCs express CTSS and efficiently cleave many known autoantigens; when CTSS concentrations are increased, it is presumed those T cell epitopes that mediate negative selection are destroyed, which allows autoreactive T cells to escape to the periphery\textsuperscript{12}. Conversely, CTSS-deficient mice are very resistant to the development of experimental autoimmune myasthenia gravis\textsuperscript{36}. Our study here has shown that enhanced CTSS in DCs can lead to an alteration of the T cell repertoire in the T\textsubscript{FH} cell compartment. Notably, increased CTSS has been observed in serum from patients with SLE and lupus nephritis\textsuperscript{37}.

An increased frequency of T\textsubscript{FH} cells in peripheral lymphoid organs is a phenotype commonly observed in animal models of SLE\textsuperscript{38,39}. However, it is not clearly understood whether an increased number of T\textsubscript{FH} cells results in a predisposition to autoimmunity or whether the antigenic specificity of the T\textsubscript{FH} cells in these models is also altered. The antigenic specificity of activated CD4\textsuperscript{+} T cells is determined largely by encounter with peptide--MHC class II complexes on DCs\textsuperscript{40,41}. While TCR selection occurs in the thymus, the differentiation of effector T cells occurs in the periphery when CD4\textsuperscript{+} T cells encounter APCs presenting specific peptide--MHC class II complexes\textsuperscript{42}. We do not yet know whether the more-diverse TCR repertoire of T\textsubscript{FH} cells observed in female KO mice than in female wild-type mice was determined by selection in the thymus or activation in the periphery. Nonetheless, this study has demonstrated the importance of the precise regulation of CTSS in APCs in determining the T cell repertoire and the fate of autoreactive T cells.
An inhibitor of CTSS has been shown to prevent disease onset in the MRL/lpr mouse model of lupus. Our study also demonstrated that inhibition of CTSS was able to prevent disease onset and the lupus-related phenotype in CKO mice. More notably, treatment with this inhibitor made the TCR repertoire of T cell populations less diverse. Because long-term treatment with the inhibitor did not reduce the expression of MHC class II on DCs, we speculate that the main function of the inhibitor is to change the pool of peptides presented on MHC class II in vivo. Our data help explain how the SLE-associated PRDM1 risk allele, with low expression of Blimp-1 and high expression of IL-6 in DCs, contributes to the risk of developing SLE.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.J.K. designed and performed experiments, analyzed data and interpreted results; S.S. performed the sequencing experiments and analyzed data; S.S.A. and W.H. provided RO5461111, and critically reviewed the manuscript; S.H.J. performed the promoter assay; P.K.G. provided the samples for the human study; G.G. designed the sequencing experiments and interpreted data; B.D. designed the study and interpreted results; S.I.K., S.S., G.G. and B.D. participated in the interpretation of the study and wrote the manuscript, and also provided critical review of the paper; and all contributing authors agreed to the submission of this manuscript for publication.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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Online Methods

Mice. CKO mice (Prdm1<sup>fl/fl</sup>Cd11c-Cre<sup>+</sup>) and their control littermates (Prdm1<sup>fl/fl</sup>Cd11c-Cre<sup>-</sup>) mice on a C57BL/6 background were bred and maintained in a specific pathogen-free facility at the Feinstein Institute for Medical Research. OT-II mice (B6.Cg-Tg (TcraTcrb) 425Cbn/J) were purchased from Jackson Laboratory.

All the experiments conducted in this study strictly followed the guidance in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the committee on the Ethics of Animal Welfare of The Feinstein Institute for Medical Research (protocol number 2009-048). All the animals were euthanized at the end time point of experiments by COegasulation.

Sample size to achieve adequate power was chosen on the basis of our previous studies with similar methods. We randomized the female or male mice from different cages and different time points to exclude cage or batch variation. Experiments and data analysis was performed without aforesaid genotype or treatment information.

Preparation of PBMCs and blood DCs and in vitro differentiation of MO-DCs. The protocol for study of human samples was approved by the IRB of the Feinstein Institute for Medical Research (FIMR) (approval number 09-081A). Healthy carriers of the PRDM1<sup>−/−; Ciita<sup>−/−</sup> genotype or treatment information.

Purification of T<sub>H1</sub> cells for TCR V<sub>β</sub> sequencing. Age- and sex-matched control and CKO mice or female CKO I<sup>−/−</sup> mice were killed at 6–8 weeks of age, and spleens were collected for T<sub>H1</sub> cell purification. CD4<sup>+</sup> T<sub>H1</sub> cells were labeled with 10<sup>5</sup> cells per ml with 1 ml of TRI reagent (Life Technologies) and total RNA was isolated according to the manufacturer’s protocol of the RNeasy Micro Kit (Qiagen). First-strand cDNA was generated from total RNA using a Superscript RT II kit (Invitrogen) and oligo(dT) primer. The resulting cDNA was used as template for FastStart High Fidelity PCR amplification (Roche) using mouse-specific barcoded constant-region primer (5′-GCACCATGGTGTCGTAGGAGGA-3′) and 23 V<sub>β</sub>-specific primers<sup>34</sup>. The PCR products were gel-purified to isolate the amplified TCR V<sub>β</sub> DNA, and total quantities were determined with a 2100 Bioanalyzer (Agilent). 200 ng of DNA per mouse was processed for Illumina MiSeq DNA sequencing according to the manufacturer’s protocol and sequenced, yielding 2 × 10<sup>6</sup> to 5 × 10<sup>6</sup> raw sequences per mouse.

All sequencing data were first processed using the sequence quality and signal filters of the Illumina MiSeq pipeline and then were subjected to bioinformatics analysis that relied on homologies to conserved framework regions using the MiXCR software package. V<sub>β</sub> CDR3 sequences were then clustered using the MiXCR software package. The PCR products were gel-purified to isolate the amplified TCR V<sub>β</sub> DNA, and total quantities were determined with a 2100 Bioanalyzer (Agilent). 200 ng of DNA per mouse was processed for Illumina MiSeq DNA sequencing according to the manufacturer’s protocol and sequenced, yielding 2 × 10<sup>6</sup> to 5 × 10<sup>6</sup> raw sequences per mouse.

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Transwell experiments were performed in 0.4-µm plates (Corning). cDCs purified from wild-type or CKO mice were plated on upper wells and CD4+ OT-II T cells were cultured with OVA-presenting wild-type DCs on the bottom wells. Purified DCs from wild-type or CKO mice were cultured with OVA protein for 6 h and were fixed with 0.001% glutaraldehyde for 30 s. Fixation was terminated by 0.2 M glycine treatment, followed by washing three times with PBS.

**Measurement of CTSS activity.** *In vitro* CTSS enzymatic activity was measured by a fluorometric analysis kit (Abcam). DCs were purified by cell sorter, and an equal number of DCs (1 × 10⁶) from wild-type or CKO mice were lysed in cell lysis buffer. An equal volume of reaction buffer was added into the cell lysis, and amino-4-trifluoromethyl coumarin (AFC)-labeled CTSS substrate peptide (Ac-VVR-AFC) (200 µM) was added, followed by incubated for 2 h at 37 °C. For a negative control, CTSS inhibitor was added to the substrate mixture, or a reaction mixture that does not contain cell lysate was prepared. After incubation, samples were read in a fluorometer with a 400-nm excitation and 505-nm emission. Change in CTSS activity was determined by comparison of the relative fluorescence units (RFU) to the level in the negative control sample.

**ChIP assay.** 5 µg of polyclonal antibody to Blimp-1 (sc-13206, Santa Cruz Biotech) or naïve goat IgG (AB-108-C, R&D System) were conjugated with 50 µl of protein G magnetic beads (Invitrogen) by overnight incubation. CD11c+MHCII+ cDCs were purified from the spleen of wild-type or CKO mice. Purified DCs (5 × 10⁵ to 10 × 10⁵ cells per experiment) were cross-linked in 1% formaldehyde (final concentration) for 10 min at 20 °C. To quench cross-linking, 1.375 M glycine (100 µl/ml) was added, and cells were washed three times with ice-cold PBS. Cells were lysed in cell lysis buffer and subjected to sonication. Antibody-conjugated magnetic beads were incubated with sonicated DNA at 4 °C with rotation. The next day, unbound DNA was washed away and the antibody-bound complex was eluted in elution buffer. The protein and DNA complex was de-cross-linked at 65 °C overnight. DNA was further purified by RNase treatment and DNA cleanup using a PCR cleanup kit (QIAGEN). Purified DNA was used for PCR.

**Kidney histology.** Kidneys collected from (6-month-old) mice at the end of experiments were cut into two pieces longitudinally. One half was frozen with tissue freezing in Tissue-Tek O.C.T. compound medium (Fisher) and the other half was immediately fixed with 4% formaldehyde (Sigma-Aldrich) and kept in 70% alcohol at 4 °C. Frozen or fixed sections were sliced to 7 mm thickness and were stored until staining. To detect immunoglobulin deposition, frozen kidney sections were fixed with acetone (−20 °C for 5 min) and were blocked with blocking solution (2% BSA, 0.5% TritonX-100, 2% normal goat serum (Invitrogen) in PBS) for 1 h at 20 °C. Alexa Fluor 488-conjugated anti-mouse IgG (1010-30, SouthernBiotech) (1:200 in blocking solution) was incubated with tissue for 2 h at 20 °C. Tissues were washed with PBS three times and analyzed by microscope.

**ELISA of antibody to double-stranded DNA and albumin.** To measure the titers of antibodies t double-stranded DNA, CKO mice or CKO mice fed RO5461111-treated chow were bled at 2 months, 4 months, and 6 months of age. Mouse serum was prepared from blood and was frozen at −20 °C until assay. 96-well plates (Costar) were coated with 100 µg/ml of sonicated and filtered calf thymus DNA (Sigma-Aldrich). The plate was blocked with 3% FCS for 1 h at 20 °C, and diluted samples from experimental mice or positive control mice (6-month-old NZB/W F1 mice) were incubated for 2 h. The plate was washed and alkaline-phosphatase-conjugated anti-mouse IgG (1031-04, SouthernBiotech) was applied. The reaction was developed with p-nitrophenyl phosphate (Sigma-Aldrich). Absorbance was monitored at 405 nm.

**Urinary albumin.** Urine samples were collected from 6-month-old mice maintained in metabolic cages. Albumin concentrations in urine samples were measured by mouse albumin ELISA quantitation set (Bethyl Laboratories) following the manufacturer’s protocol.

**Luciferase assay.** BM-DCs (2 × 10⁶ cells) were transfected with a wild-type or mutated *Ctss* promoter construct by Nucleofector kit (Lonza). Luciferase plasmid and Tk-Renilla luciferase plasmid was added to the cells at a 10:1 ratio. Relative light units were measured 6–12 h after transfection using Dual-Luciferase reporter assay system (Promega).

**Transfection of siRNA and Blimp-1 expression vector.** siRNA (SR420429) targeting *Prdm1* was purchased from Origene, and 200 nM of each siRNA or control scrambled siRNA was used for each transfection. *Prdm1* plasmid and control plasmid were purchased from Genecopoeia. siRNA or plasmid was mixed with DCs in Nucleofector solution and transfected by Amaxa electroporation (program Y-01). Transfected DCs were immediately transferred to culture medium (supplemented with GM-CSF) and were further cultured for 2 d.

**Bio-Plex.** Multiple cytokines were measured by Bio-Plex pro mouse cytokine standard 23-plex, group I and group III (Bio-Rad) according to the manufacturer’s protocol. The assay was measured by the Bio-Plex suspension array system and the data were analyzed by Bio-Plex manager software.

**Statistics.** Statistical significance was calculated and determined by a non-parametric, Mann-Whitney test, and *P* values less than 0.05 were considered significant. No exclusion of sample was done.

**Data availability.** All TCR Vβ sequences of T196 cells sequences raw files that support the findings of this study have been deposited at BioSample database (NCBI) under accession codes SAMN06927868, SAMN06927869, SAMN06927870, SAMN06927871, SAMN06927872, SAMN06927873, SAMN06927874, SAMN06927875, SAMN06927876 and SAMN06927877. Other data are available from the corresponding author upon request. A *Life Sciences Reporting Summary* for this paper is available.

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Experimental design

1. Sample size
   Describe how sample size was determined.
   Sample size to achieve adequate power was chosen based on our previous studies with similar methods.
   Described in material and method section (mice, page 17).

2. Data exclusions
   Describe any data exclusions.
   No exclusion of sample was done.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All the experiment was repeated minimum twice and all attempts at replication were successful.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   We randomized the mice from different cages and different time points to exclude cage or batch variation.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Experiments and data analysis was performed without aforementioned genotype or treatment information.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

   n/a | Confirmed
   □   The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   □   A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
   □   A statement indicating how many times each experiment was replicated.
   □   The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   □   A description of any assumptions or corrections, such as an adjustment for multiple comparisons.
   □   The test results (e.g. $p$ values) given as exact values whenever possible and with confidence intervals noted.
   □   A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range).
   □   Clearly defined error bars.

   See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

GraphPad Prism 5 was used for statistical analysis. FlowJo v.10.0.8 was used for flow cytometry analysis. MiXCR and CD-HIT software packages were used for sequencing. Bio-Plex manager software was used for cytokine analysis.

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The Nature Methods guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

NGS data was deposited at BioProject under the accession numbers SAMN06927868-77. All other materials are available commercially as described. Cathepsin s inhibitor, RO5461111. ROS461111 is only available from Hoffman La-Roche.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

We used antibodies which were validated from published database (multiple publications from independent groups).

- anti-STAT3: 124H6, Cell Signaling
- anti-BLIMP-1: sc-13206, Santa Cruz
- anti-p10: cat# HAF016, R&D Systems
- anti-Actin: mAbcam8226, Abcam
- anti-mouse IgG AF488: Cat#1010-30, Sourthen Biotech
- anti-CXCR5: J252D4, Biolegend
- anti-PD1: EF12.2H7, Biolegend
- anti-CD4: GK1.5, BDPharmingen
- anti-Actin: mAbcam8226, Abcam
- anti-mouse IgG AF488: Cat#1010-30, Sourthen Biotech
- anti-CXCR5: J252D4, Biolegend
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- anti-CD4: GK1.5, BDPharmingen

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

b. Describe the method of cell line authentication used.

c. Report whether the cell lines were tested for mycoplasma contamination.

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

HEK293 purchased from ATCC

Authenticated by ATCC.

not tested since we used newly purchased cell line.

No commonly misidentified cell line was used.
Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Blimp-1 flox/flox; Cre-Tg and IL-6 +/-; Blimp-1 flox/flox; Cre-Tg mice are on C57BL/6 genetic background, both female and male, from 6 weeks old through 8 month old. (page 17)

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

IRB approval number is provided in Material and Method (page 17). Both female and male (hormonally active, age 55 and younger) individuals were included. Participants were genotyped and registered in the Genotype and Phenotype Registry at The Feinstein Institute for Medical Research. Any genotyped individuals with chronic inflammatory disorders, cancers or under hormonal therapy were excluded.
Flow Cytometry Reporting Summary

Corresponding Author: Betty Diamond
Date: June 14 2017

Data presentation

For all flow cytometry data, confirm that:

☑ 1. The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☑ 2. The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
☑ 3. All plots are contour plots with outliers or pseudocolor plots.
☑ 4. A numerical value for number of cells or percentage (with statistics) is provided.

☑ 5. Describe the sample preparation.

For blood samples (Fig. 1C), mouse blood was obtained and PBMCs were prepared by Ficoll gradient centrifugation.
For samples from spleen (Fig. 5A, Fig. 6B, Fig. 7A-B, and Supplementary Fig. 4), splenocytes were prepared by through nylon mash and RBCs were removed (RBC lysing buffer). In vitro stimulated cells (Fig. 2D and Fig. 4A) were collected after stimulation. All the staining was performed in immediately after collection in ice-cold staining buffer. Dead cells were excluded by live-dead staining.

Methodological details

☑ 6. Identify the instrument used for data collection.

BD LSRII new (7-3-2-2)

☑ 7. Describe the software used to collect and analyze the flow cytometry data.

FACSDiva software was used for data collection and FlowJo software v.10.0.8 was used for analyses.

☑ 8. Describe the abundance of the relevant cell populations within post-sort fractions.

FACSaria instrument was used for cell sorting. Purity of post-sorted samples was 90-95% for all samples. Abundance of population is variable depending on samples; 50,000 for Fig 1C, 1-2 millions for Fig. 2D, 0.1-0.2 millions of cells for Fig. 5B and Fig. 7D.

☑ 9. Describe the gating strategy used.

All the samples was analyzed from live cell population except the case of intracellular staining (Fig. 4c). Positive and negative population was defined by isotype control staining. Specific gating strategies which were used: Fig.1C (from the live cells, lineage markers negative, and CD11chiMHCIIhi cells, Fig. 2B,D (from live cells, GFP-positive population was defined by null transfected GFP negative cells), Fig 4A (T alone without stimulation was defined as a positive population and CFSE-unlabelled T cells was used as a negative population), Fig. 4C (unstimulated T cells stained with IL-2 and IL-21 was one negative control and stimulated T cells stained with isotype controls was another negative control), Fig. 4D (DC cultured with unlabeled OVA was a negative control), Fig 5A (for BCL-6 intracellular staining, isotype control staining was a negative control, otherwise all other gating strategy is described in the figure), Fig. 6B (for B and T cell, lymphocyte population was
defined from FSC/SSC gating, otherwise specific gating strategies are described in the figure), Fig. 7A (cells were analyzed from lymphocyte gating defined by FSC/SSC gating), Fig. 7B-C (broad lymphocyte gating was applied to include plasmablast).

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information. ☑