Tolerogenic function of Blimp-1 in dendritic cells

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Blimp-1 has been identified as a key regulator of plasma cell differentiation in B cells and effector/memory function in T cells. We demonstrate that Blimp-1 in dendritic cells (DCs) is required to maintain immune tolerance in female but not male mice. Female mice lacking Blimp-1 expression in DCs (DCBlimp-1ko) or haploid for Blimp-1 expression exhibit normal DC development but an altered DC function and develop lupus-like autoantibodies. Although DCs have been implicated in the pathogenesis of lupus, a defect in DC function has not previously been shown to initiate the disease process. Blimp-1ko DCs display increased production of IL-6 and preferentially induce differentiation of follicular T helper cells (Tfh cells) in vitro. In vivo, the expansion of Tfh cells is associated with an enhanced germinal center (GC) response and the development of autoreactivity. These studies demonstrate a critical role for Blimp-1 in the tolerogenic function of DCs and show that a diminished expression of Blimp-1 in DCs can result in aberrant activation of the adaptive immune system with the development of a lupus-like serology in a gender-specific manner. This study is of particular interest because a polymorphism of Blimp-1 associates with SLE.

Tolerance to self-antigens is a key feature of the immune system. Systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), result from dysregulation of B and T cell activation and altered function of macrophages and DCs (Shlomchik, 2009), leading to pathogenic autoantibodies, which are IgG isotype switched, high affinity to self-antigens, and somatically hypermutated. (Rothfield and Stollar, 1967; Diamond and Scharff, 1984). Although B cells are the proximal cells in the phenotypic manifestations of SLE, interactions with other immune cell types are dynamically involved (MacLennan, 1994; Shlomchik et al., 2001; Craft, 2011).

Recently, a polymorphism of Blimp-1 has been identified as a risk factor in SLE by genome-wide association studies, suggesting a critical function of Blimp-1 in SLE (Gateva et al., 2009; Han et al., 2009). Blimp-1 negatively regulates expression of IFN-β in both humans and mice (Keller and Maniatis, 1991; Turner et al., 1994). In B cells, Blimp-1 is a key regulator of plasma cell development (Shapiro-Shelef et al., 2003). In T cells, Blimp-1 regulates the differentiation of Th1 and the function of regulatory T cells (Martins et al., 2006). Blimp-1 was suggested to be a survival factor in monocytes (Chang et al., 2000); however, a more recent study using a Tie2–Cre system has suggested that Blimp-1 may regulate the differentiation and activation of DCs (Chan et al., 2009). In that study, IL-6 and MCP-1 were shown to be direct targets of Blimp-1 and the deletion of Blimp-1 resulted in increased expression of proinflammatory cytokines. The physiological importance of Blimp-1 specifically in DCs, however, could not be addressed in this mouse model in which Blimp-1 was deleted in all hematopoietic cell lineages.

DCs are important in lupus pathogenesis, although a primary defect in DC function has not been reported. Because DCs were discovered by Steinman and Cohn (1973), they have been recognized as the key immune-regulating cells. DCs can mediate both immune tolerance and immune activation (Cools et al., 2007). DCs can acquire tolerogenic phenotype after phagocytosis of apoptotic cells (Qu et al., 2009). They can also generate regulatory T (Treg) cells or cause immune suppression by secretion of cytokines (Yamazaki...
mice produced increased IL-6 and preferentially induced differentiation of T<sub>FH</sub> cells. All aspects of the phenotype were abolished in DCBlimp-1<sup>ko</sup> mice haploid for IL-6. Together, these observations suggest that a defect restricted to DCs can alter T cell differentiation resulting in the production of high titers of lupus-like autoantibodies in a gender-specific fashion.

RESULTS AND DISCUSSION

Gender-dependent development of autoantibodies in DCBlimp-1<sup>ko</sup> mice

Blimp-1 expression was measured by Western blotting (Fig. 1 A). CD11c<sup>hi</sup> DCs were purified from spleens of age-matched DCBlimp-1<sup>ko</sup> (Blimp-1<sup>-flox/flox</sup>; CD11c-CRE<sup>+</sup>) and control
matory infiltrates were observed in 10-mo-old DCBlmp-1 deposition of IgG and mesangial cell proliferation and inflammation for antibodies (Fig. 1 C). Proteinuria was developed, and kidney demonstrated that IgG2b is the major isotype of anti-dsDNA reactivity to dsDNA antibodies. An isotype-specific ELISA (Fig. 1 E). Mice haploid for Blimp-1 also displayed IgG reactivity to both dsDNA and ENA5 stranded (ds) DNA, and anti-ENA5 by ELISA. All the ANA-positive immunoglobulin was IgG; IgM ANA was negligible.

To understand the alterations in Blimp-1+ DCs responsible for the generation of autoantibodies, we analyzed the characteristics of the DCs. Because the expression of IL-6 is regulated by Blimp-1 (Chan et al., 2009) and enhanced expression of IL-6 is associated with SLE, and possibly related to DC activation (Colonna et al., 2006; Jeon et al., 2010), we examined IL-6 production. We observed an increased production of IL-6 by splenic Blimp-1+ DCs compared with control DCs (Fig. 2 A) and by BM-DCs after LPS stimulation (Fig. 2 B). In male mice, however, there was no significant difference in the level of IL-6 produced by either splenic DCs or BM-DCs from control or DCBlmp-1+ mice (Fig. 2, A and B). In fact, production of IL-6 was higher in female than in male control DCs, implicating a sex difference in cytokine production even in wild type DCs. This observation is consistent with data in patients showing an association of an estrogen-sensitive polymorphism of the IL-6 promoter with susceptibility to type 1 diabetes in women (Kristiansen et al., 2003). The level of expression of several genes also increased in Blimp-1+ DCs as measured by quantitative (q) PCR (Table S2). Bcl-6, a molecule negatively regulated by Blimp-1, was up-regulated in Blimp-1+ DCs. Expression of XBP, which has been demonstrated to be a survival factor for DCs (Iwakoshi et al., 2007), was equivalent in control and Blimp-1+ DCs, implicating a sex difference in cytokine production even in wild type DCs.
haploid for IL-6 (IL-6<sup>+/−</sup> DCBlmp-1<sup>−ko</sup>). DCs from IL-6<sup>+/−</sup> DCBlmp-1<sup>−ko</sup> mice express the same level of IL-6 as DCs from control mice after LPS stimulation (Fig. 2 C). Immunization of control and IL-6<sup>+/−</sup> DCBlmp-1<sup>−ko</sup> mice with NP-CGG showed that IL-6<sup>+/−</sup> DCBlmp-1<sup>−ko</sup> mice mount an antibody response that is indistinguishable from that of control mice (Fig. S3 A), demonstrating that B cells from IL-6<sup>+/−</sup> DCBlmp-1<sup>−ko</sup> mice are not defective in antibody production or affinity maturation. Although IL-6<sup>+/−</sup> DCBlmp-1<sup>−ko</sup> mice had a normal antibody response to immunization, they did not develop autoantibodies (Fig. 2 D). These data suggest that the increased expression of IL-6 by DCs contributes to the generation of autoantibodies in female DCBlmp-1<sup>−ko</sup> mice.

Enhanced GC formation in DCBlmp-1<sup>−ko</sup> mice

Because we observed only IgG, and not IgM, autoantibodies, we asked whether the autoantibodies were derived from GC-experienced B cells. We generated hybridomas of splenocytes from 4-mo-old DCBlmp-1<sup>−ko</sup> mice. There were 27 ANA-positive clones from 304 IgG-secreting clones (~10%). From the 27 clones, 13 and 16 clones were successfully sequenced for heavy and light chain, respectively. Sequence analysis revealed a high incidence of mutation in most clones (Fig. 3 A). Interestingly, 3 out of 13 clones contained an arginine residue acquired by point mutation in the complementary determining region 3 in the heavy chain, which is often seen in high-affinity anti-DNA antibodies. These data suggest that autoantibodies are produced by GC-matured plasma cells.

Flow cytometry and immunohistochemistry (IHC) demonstrated an enhanced GC response in young mice (8–12 wk old); many more spontaneous GC B cells, as well as GCs in spleens, were present in DCBlmp-1<sup>−ko</sup> mice (Fig. 3 B). In contrast to the response in IL-6<sup>+/−</sup> DCBlmp-1<sup>−ko</sup> mice, there was an enhanced immune response in DCBlmp-1<sup>−ko</sup> mice after NP-CGG immunization with an increased high-affinity anti-NP IgG response (Fig. S3 B). There was an increased number of GC B cells (Fig. S3 C) and an increased number of total GCs (7/12 ± GCs in DCBlmp-1<sup>−ko</sup> mice and 1/3 ± GCs in control mice).

**Figure 3. Characterization of ANA IgG and GC response.** (A) Sequence analysis of ANA IgG from hybridomas. Hybridomas were generated by splenocytes of 4-mo-old DCBlmp-1<sup>−ko</sup> mice. Total heavy and light chain of ANA-positive IgG was amplified and sequenced. Mutations were determined by comparison with the mouse genomic sequence database. Numbers in each pie graph represent the number of clones categorized by the number of mutation (n = 4). (B) Spontaneous GC formation in the spleen of 6–10-wk-old DCBlmp-1<sup>−ko</sup> mice. GC (PNA<sup>+</sup> B220<sup>+</sup> asterisks) was analyzed by IHC. Pictures are representative images (bars, 100 µm). On the right, GL-7<sup>+</sup>B220<sup>+</sup> GC B cells were quantified by flow cytometry as depicted in representative pictures. Each dot represents an individual mouse and horizontal bars indicate means of three independent experiments.
The importance of IL-6 was further demonstrated in vitro as α–IL-6 neutralizing antibody inhibited the differentiation of T cells co-cultured with control DCs and Blimp-1 ko DCs into T FH cells (Fig. S5). These observations suggest that the increased generation of T FH cells results from increased IL-6 production by DCs in DCBlimp-1 ko mice. Interestingly, there was no significant increase in T H17 cells, another subset for which IL-6 is critical (unpublished data), suggesting that additional requirements for the generation of T H17 must exist and are not provided by Blimp-1 ko DCs.

IL-6–dependent generation of GC B cells and T FH cells

Because increased T FH cells and GC response are important mechanisms for autoantibody production in DCBlimp-1 ko mice and the phenotype was impaired in IL-6−/− DCBlimp-1 ko mice, we compared GC and T FH cells in IL-6−/− DCBlimp-1 ko mice. IL-6−/− DCBlimp-1 flox/− mice showed a reduced number of T FH cells as well as a reduced number of GC cells in the spleen (Fig. 5). These data suggest that the increased expression of IL-6 in Blimp-1 ko DCs is a
major molecular mechanism responsible for the expansion of T\textsubscript{FH} cells and enhanced GC formation, leading to the generation of autoantibodies in DC-Blimp-1\textsuperscript{ko} mice.

In summary, we propose a new mechanism for the development of a lupus-like phenotype mediated by Blimp-1, which is required to maintain tolerogenic function in DCs in a gender-dependent manner. The loss of function of Blimp-1 in female DCs results in increased secretion of the critical proinflammatory cytokine IL-6, and in increased differentiation of T\textsubscript{FH} cells and increased GC responses. It is of considerable interest that a polymorphism of the Blimp-1 gene has now been implicated in both rheumatoid arthritis and SLE and that the phenotype of the DC-Blimp-1\textsuperscript{ko} mouse is analogous to human SLE with a female bias, enhanced T\textsubscript{FH} cells, and increased IL-6 levels (Swaak et al., 1989).

MATERIALS AND METHODS

Mice. Blimp-1\textsuperscript{+} mice were provided by K. Calame (Columbia University, NY, NY) and backcrossed with C57BL/6 for eight generations. CD11c-CRE mice were generated in the Reizis laboratory. DC-Blimp-1\textsuperscript{−/−} and control mice were bred in the animal facility of The Feinstein Institute for Medical Research (FIMR) in specific pathogen-free conditions. IL-6 mice were provided by K. Calame (Columbia University, NY, NY) and backcrossed with C57BL/6 for eight generations. CD11c-KO mice and CRE mice were generated in the Reizis laboratory. DCBlimp-1\textsuperscript{−/−} mice were bred with DCBlimp-1\textsuperscript{−/−} mice in the animal facility of FIMR.

Purification of splenic DCs and in vitro generation of BM-DCs. CD11c\textsuperscript{+} splenic DCs were enriched with an EasySep kit (STEMCELL technologies) according to the manufacturer’s protocol. Then, CD11c\textsuperscript{+} Siglec-H\textsuperscript{−} DCs were further purified by cell sorter (FACSaria; BD). Cell purity was routinely >95%.

To generate BM-DCs, BM cells were harvested from the femur with PBS. T cells and B cells were depleted by incubation with antibodies from hybridoma cell lines (American Type Culture Collection; TIB-120, TIB-211, TIB 207, and TIB-146) with rabbit complement (Pel-Freeze Biologicals). The remaining cells were cultured in RPMI 1640 with 10\% FCS and 200 ng/ml Flt3L (PeproTech) for 8 d. The nonadherent cells were collected. The remaining cells were cultured in RPMI 1640 with 10\% FCS and 200 ng/ml Flt3L (PeproTech) for 8 d. The nonadherent cells were collected. To measure cytokines in the supernatants, 10\(^5\) DCs/ml were cultured overnight in medium with or without 1 µg/ml LPS (Sigma-Aldrich).

Histology of spleen and kidney. Spleens from 6–10-wk-old DCBlimp-1\textsuperscript{−/−} and control mice were fixed with 4\% PFA and transferred to a 30\% sucrose solution. The fixed spleens were snap frozen in Tissue-Tek O.C.T. compound (Sakura) and sliced to 7 µm. On the day of staining, sections were fixed with ice-cold acetone and blocked with blocking buffer. After blocking, samples were incubated with fluorochrome-conjugated antibodies diluted in dilution buffer for 1 h at room temperature. After incubation with antibodies, slides were washed with PBS three times.

Kidneys were harvested from 8-mo-old mice and fixed with formaldehyde and 70\% ethanol. Fixed tissues were paraffin embedded and sliced to 5-µm thickness. Tissue was stained with standard hematoxylin and eosin. Images were visualized using a fluorescence microscope (AxioCam II; Carl Zeiss) and analyzed by OpenLab software (PerkinElmer).

qPCR. Total RNA was extracted from purified DCs with RNeasy kit (Invitrogen) according to the manufacturer’s instructions and subjected to reverse transcription with iScript cDNA synthesis kit (Bio-Rad Laboratories). cDNA was analyzed by qPCR using LightCycler 480 probes master with various primers (Applied Biosystems). Relative induction of each gene of interest was calculated by \(\Delta\Delta Ct\).

Additional methods. Information on serum immunoglobulin ELISA, Western blotting, ANA, antibodies, immunization with NP(16)-CGG, proteiniura and IgG deposition in kidney, hybridoma generation, and Ig sequencing are available in the supplemental Materials and methods.

Statistics. Unpaired two-tailed Student \(t\) tests were used for statistical analysis with Prism software (GraphPad Software). \(P < 0.05\) was considered to be significantly different.

Online supplemental material. Fig. S1 shows Blimp-1 expression in hematopoietic lineages and additional phenotypes of DCBlimp-1\textsuperscript{−/−} mice. Fig. S2 shows gender-dependent serology and secretion of IL-6 from B cells. Fig. S3 shows enhanced antibody and GC response in DCBlimp-1\textsuperscript{−/−} mice. Fig. S4 shows analysis of CD4\textsuperscript{+} T cells in the spleen of control and DCBlimp-1\textsuperscript{−/−} mice. Fig. S5 shows blocking of T\textsubscript{FH} by anti–IL-6 antibodies in vitro. Additional information is provided in the supplemental Materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110658/DC1.

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