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Dendritic and stromal cells from the spleen of lupic mice present phenotypic and functional abnormalities

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ABSTRACT

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the increase in the percentage of autoreactive B and T lymphocytes. Since dendritic cells (DCs) are essential for B cell and T cell function, we hypothesized that changes in DC biology may play a critical role in the pathogenesis of the disease. We analyzed the phenotype and distribution of two main DC subsets, conventional (cDC) and plasmacytoid (pDC), in lupus prone (NZW×NZB)F1 (BWF1) mice and age-matched NZW×BALB/c control mice. Our results show that both subsets of lupic DCs displayed an abnormal phenotype, characterized by an over-expression of the co-stimulatory molecules CD80, CD86, PD-L1 and PD-L2 compared with control mice. Accordingly, spleen $CD4^+$ T cells from lupic mice exhibit an activated phenotype characterized by a higher expression of PD-1, CD25, CD69 and increased secretion of IFN-γ and IL-10. Interestingly, lupic mice also present an increase in the percentage of cDC in peripheral blood and an increase in the percentage of pDCs in spleen and mesenteric lymph nodes (MLNs) compared with control and pre-lupic mice. Homing experiments demonstrate that lupic and pre-lupic DCs migrate preferentially to the spleen compared to DCs from control mice. This preferential recruitment and retention of DCs in the spleen is related to an altered expression of different chemokine and chemokine receptors on both, DCs and stromal cells from lupic mice. Our results suggest that this altered phenotype and migratory behavior shown by DCs from lupic mice may account for the abnormal T cell and B cell responses in lupus.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease that predominantly affects young females and is associated with significant morbidity and mortality. SLE is characterized by the loss of tolerance to self-antigens, deregulated autoreactive T cell and B cell activation, the production of autoantibodies and altered cytokine production. The serological hallmark of SLE is the prominent elevation of pathogenic autoantibodies against nuclear antigens including double-stranded DNA (dsDNA), nucleosomes and various small nuclear ribonucleoproteins (snRNPs), which causes the formation and deposition of immune complexes leading to tissue inflammation and damage in kidneys, skin, joints and the central nervous system (Rahman and Isenberg, 2008).

Numerous immunological alterations have been reported in patients with SLE. Studies targeting B cells, both in mice and in humans, have demonstrated the importance of B cells in promoting immune activation and tissue damage in lupus, partly through antibody-independent mechanisms (Chan et al., 1999a, 1999b). Activated T cells also play an important role, indicated by the fact that the disease is substantially reduced when T cells are absent or when their function is inhibited (Jevnikar et al., 1994; Jabs et al., 1992). Additionally, decreased numbers, and/or impaired function of $CD4^+$ regulatory T cells (Treg), have also emerged as an important pathogenic mechanism in SLE (Valencia et al., 2007; Scalapino et al., 2006; Miyara et al., 2005; Liu et al., 2004; La Cava, 2008; Horwitz et al., 2004; Crispin et al., 2003). However, as the complexity of the immune system has gradually been unveiled in the past few decades, the paradigm has shifted the focus toward dendritic cells (DCs), as the key participant in the initiation, amplification and perpetuation of the disease.

Abbreviations: SLE, systemic lupus erythematosus; NZW, New Zealand white; NZB, New Zealand black; BWF1, (NZW×NZB)F1; Treg, regulatory T cell; DCs, dendritic cells; cDC, conventional dendritic cell; pDC, plasmacytoid dendritic cell; APCs, antigen presenting cells; MLN, mesenteric lymph node; PLN, peripheral lymph node.

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DCs comprise several subsets that are classified according to different criteria including maturation status (Banchereau and Steinman, 1998; Steinman and Idoyaga, 2010; Shortman and Liu, 2002). Immature, Ag-capturing DCs can sense diverse environmental and endogenous stimuli that induce them to undergo a maturation process. When DCs mature in response to microbial products, the expression of hundreds of genes is altered, which regulate important DC functions such as antigen processing, migration, and antigen presentation (including expression of co-stimulatory and adhesion molecules) (Steinman and Banchereau, 2007). Chemokines and chemokine receptors play a crucial role in the initiation of adaptive immune responses, by directing the trafficking and homing of immature and mature Ag-presenting DCs. DC migration into lymphoid organs can induce either T cell tolerance or a strong innate and adaptive immunity toward a specific antigen. In general, tolerance is produced when immature DCs interact with T cells, whereas the induction of immunity requires effective DC maturation signals while interacting with T cells (Banchereau and Steinman, 1998; Stoll et al., 2002; Angelini et al., 2002; Sallusto and Lanzavecchia, 2002).

In addition to their maturation status, DCs can be subdivided into plasmacytoid DC (pDC) and myeloid or conventional DC (cDC) subsets (Banchereau and Steinman, 1998). cDCs present the features for effective induction of adaptive immunity. On the other hand, pDCs are capable of rapidly producing large amounts of type-I IFNs (particularly IFN-α and -β) in antiviral defense against infection. These DC subsets have different migration programs and are likely to have divergent roles in the induction and regulation of the immune response (Penna et al., 2002; Pulendran et al., 1999). Evidence from patients with SLE and mice with lupus-like syndromes suggest that DCs play an essential role during the development and progression of the disease (Crispin and Alcocer-Varela, 2007; Kis-Toth and Tsokos, 2010). Other studies indicate that lupus patients have a reduced (Scheinecker et al., 2001; Migita et al., 2005; Fiore et al., 2008; Jin et al., 2008) or normal (Tucci et al., 2008; Henriques et al., 2012) mDC frequency when compared with healthy controls. Although two earlier reports indicated impairment in the T cell stimulatory capacity of lupus mDCs, several recent studies have revealed the presence of activated mDC phenotypes with increased expression of the co-stimulatory molecules CD80, CD86 and/or B lymphocyte stimulator BLyS. mDC functional enhancement in IL-6 and IL-8 production and T cell stimulatory capacity has also been reported (Decker et al., 2006; Ding et al., 2006; Gerl et al., 2010).

Currently, the prevailing concept is that DCs from patients with SLE are abnormally activated and contribute to the disease, probably by promoting T cell and B cell activation through antigen presentation and cytokine secretion. Interestingly, DCs accumulate both in T cell (Fields et al., 2001) and in B cell areas (Ishikawa et al., 2001) in the lymph nodes and spleen of lupus-prone MRL/lpr (Fas-deficient) and BWF1 mice. In the latter strain, the accumulation of DCs has been attributed to high levels of Flt-3L (Adachi et al., 2002), which is also increased in patients with lupus (Gill et al., 2002). Moreover, several lines of evidence have suggested a pathogenic role for IFN-α/β during SLE pathogenesis. First, sera from patients with lupus have been shown to induce differentiation of peripheral blood mononuclear cells into DCs, through a mechanism that is dependent on IFN-α (Blanco et al., 2001), which is a cytokine produced in large amounts by pDCs (Cella et al., 1999). Second, long term IFN-α treatment for the hepatitis C virus (HCV) or leukemia patients sometimes generates severe lupus-like symptoms (Ho et al., 2008; Tolaymat et al., 1992). Third, elevation of serum IFN-α has been observed in a positive correlation with the anti-dsDNA autoantibody level and disease activity index (SLEDAI) in some SLE patients (Bengtsson et al., 2000; Dall'era et al., 2005). Fourth, peripheral leukocytes of lupus patients often exhibit a signature of IFN-inducible genes up-regulation, which correlates positively with disease severity (Bennett et al., 2003; Baechler et al., 2003; Han et al., 2003; Tang et al., 2008). Finally, in the BWF1-related strains of lupus-prone mice, type-I IFN receptor deficiency ameliorates the disease while the over-expression of IFN-α accelerates the disease (Santiago-Raber et al., 2003; Agrawal et al., 2009; Liu et al., 2011).

Recruitment of leukocytes is a characteristic feature of tissue injury in SLE, including nephritis. Analysis of lupus-prone mice revealed that kidney cells can produce inflammatory chemokines like CCL2/MCP1, CCL4/MIP1, CCL5/RANTES and CXCL10/IP10 after stimulation with TNF-α or IL-1β (Vielhauer et al., 2007; Segerer et al., 2000). Recently, lymphoid organ stromal cells that produce chemokines such as CCL21 and CCL19 (Luther et al., 2000), which are crucial for delineating the T cell zone, have been described to support the survival of resting T cells (Zhou et al., 2003), and control immune responses through changes in chemokine production and lymphocyte traffic (Mueller and Germain, 2009). In spite of the influence of stromal cells in the initiation and maintenance of the immune response, their role in the pathogenesis of SLE has not been directly addressed.

The aim of this study was to analyze the distribution and phenotype of cDCs and pDCs in spleen and other lymphoid tissues of BWF1 mice before and after the onset of the disease, and determine their migration capacity. In addition, we investigate the contribution of the spleen microenvironment in the pathogenesis of SLE. We demonstrate that lupic mice present an increased percentage of pDCs in the spleen and MLN, and an increased percentage of cDC in the blood compared with control mice. Moreover, both subpopulations of lupic DCs show an altered activation state. Furthermore, lupic DCs migrate preferentially to the spleen compared with control DCs. In agreement with this, we found differences in the gene expression of several chemokines and chemokine receptor genes when comparing spleen DCs from lupic and control mice. Additionally, we demonstrate that lupic stromal cells present an altered chemokine and chemokine receptor gene expression pattern when compared to control and pre-lupic stromal cells, a fact that may be related to the preferential recruitment and retention of DCs to the spleen. These differences in turn may be responsible for the abnormal activation state of T cells during the disease.

2. Materials and methods

2.1. Mice and SLE evaluation

Female lupus-prone BWF1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the animal facility of Fundacion Ciencia & Vida. All procedures were conducted as specified and approved by the institutional guidelines for animal experiments. The experiments were performed with 2- to 10-month old BWF1 female mice or age-matched NZW×Balb/c female mice as controls. Lupic mice were sacrificed at 6–10 months of age or when they became positive for kidney disease by severe proteinuria (*i.e.* ≥500 mg/dl protein) on two consecutive measurements, which predict mortality from renal failure (Rozzo et al., 1996; Vyse et al., 1997). Pre-lupic BWF1 mice were sacrificed at 2–3 months of age and all tested negative for proteinuria and antibodies against dsDNA. Proteinuria was measured on a monthly basis by a standard semi-quantitative test, using a Combur Test N (Roche Diagnostics, Germany). Antibodies against dsDNA were measured in serum samples by a standard ELISA assay using calf thymus DNA, as previously described (Ohnishi et al., 1994). Briefly, 3.1 μg/ml dsDNA was used to coat ELISA plates (NalgeNunc International, USA) during overnight incubation. Antigen-coated plates were subsequently blocked for 1 h with PBS containing 1.5% BSA, then incubated for 1 h at room temperature with test or

standard sera diluted in twofold serial dilutions starting from a 1:100 dilution. Plates were then washed with PBS-0.05% Tween 20, and incubated for 1 h with calf peroxidase-labeled anti-mouse (HRP) antibody. Color was developed by adding the TMB substrate kit (BDBioscience) and absorbance at 450 nm (OD_{450}) was measured using an ELISA plate reader (JENWAY, UK).

2.2. Flow cytometry and antibodies

Spleen and lymph node cells were harvested, Fc receptors were blocked with anti-CD16/CD32 antibody in PBS + 2% FCS for 20 min at 4 °C and then the cells were surface-stained with the relevant antibodies in PBS + 2% FCS for an additional 30 min at 4 °C. The following antibodies were purchased from BioLegend: PerCP anti-CD11c (N418), FITC anti-CD3 (17A2), PE anti-CD4 (RM4-5), PE anti-CCR6 (29-2L17) and PE anti-CCR4 (2G12). Antibodies from eBioscience were: purified anti-mouse CD16/CD32 (2.4G2), FITC anti-CD11c (HL3), APC anti-CD25 (PC61.5), PE anti-CD62L (MEL14), PE anti-CD11c (HL3), APC anti-CD11c (HL3), APC-H7 anti-CD19 (1D3), PECy7 anti-B220 (RA3-6B2), PE anti-CD86 (GL1), FITC anti-PD-1 (J43), PE anti-PD-L1 (MIH5), and PE anti-CCR9 (CW-1.2). The following antibodies were purchased from BD Biosciences: FITC anti-CD79b (HM79b), PE-anti-CD69 (H1.2F3), FITC anti-IA^d (AMS-32.1), APC-anti-PD-L1 (TY25) and FITC anti-CD80 (1610A1). All flow cytometry was conducted on a FACS Canto II flow cytometer (BD) and analyzed with FlowJo software (Tree Star).

2.3. Isolation of splenic dendritic cells

Spleen and lymph node were mechanically disaggregated and the cells incubated for 45 min at 37 °C in a solution containing 100 μg/ml collagenase D (Roche) and 50 μg/ml DNase I (Roche) dissolved in PBS supplemented with 2% FBS. Single cell suspensions were washed in RPMI 1640 and depleted of erythrocytes by incubation for 5 min at room temperature with an ammonium chloride lysis solution. For homing experiments, total $CD11c^+$ DCs were positively selected using anti-CD11c MACS (clone N418; Miltenyi Biotec) following the manufacturer's instructions. To study the expression of chemokines and their receptors, highly purified DCs were obtained by cell-sorting after depletion of T and B cells using antibodies against lineage markers (anti-CD3 for T cells, anti-CD79b for B cells) and Dynabeads coupled with anti-rat IgG and anti-mouse IgG antibodies. The purity of cells was >98% as determined by flow cytometry.

2.4. DC-labeling with Calcein and TRITC

DCs purified from the spleen of control, pre-lupic and lupic mice were resuspended in DMEM supplemented with 2% FBS and 20 mM HEPES. Control cells were incubated with 1 μg/ml Calcein (Molecular Probes) while pre-lupic and lupic cells were incubated with 3.6 μg/ml of TRITC (Molecular Probes) at 37 °C for 20 min. After incubation, the cell suspensions were washed and resuspended in DMEM supplemented with 2% FBS and 20 mM HEPES.

2.5. T cell labeling with CFSE

To evaluate cell proliferation, T cells were labeled with 5 μM carboxy fluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Invitrogen, California, USA) according to the manufacturer's instructions.

2.6. Suppression assays

Spleen regulatory T cells ($CD4^+CD25^{high}$ T cells) and naïve T cells ($CD4^+CD25^-$) were isolated by cell sorting. Naïve T cells were labeled with CFSE and cultured with DCs and soluble anti-CD3 (1 μg/ml) in 96-well plates. For suppression assays, Tregs were added to the cultures at a 1:1 ratio. After 72 h of culture, cells were harvested and proliferation was analyzed by flow cytometry.

2.7. Homing assays

In competitive homing assays, control, pre-lupic or lupic mice were intravenously injected with 1×10^6 TRITC-labeled-DCs from pre-lupic or lupic mice plus 1×10^6 Calcein-labeled-DCs from control mice. The mixture of cells (input) was evaluated by FACS analysis before injection. Mice were sacrificed 2 days later and single cell suspensions from spleen were analyzed by flow cytometry. The homing index (HI) was calculated as previously described (Ho et al., 2008) where:

$$HI = (TRITC^+)_{spleen}/(Calcein^+)_{spleen} : (TRITC^+)_{input}/(Calcein^+)_{input}.$$

2.8. Generation of stromal cells

To generate stromal cells, single-cell suspensions from the spleen were placed into a tissue culture dish with α-MEM (GIBCO) supplemented with 10% FBS and cultured at a concentration of 5×10^6 cells/ml in 24 well plates. During the first week, half of the medium was replaced by fresh medium every 48 h, removing non-adherent cells. After the first week, half of the medium was replaced once a week for an additional 4–5 weeks.

2.9. RNA isolation

Total RNA of DCs or stromal cells grown to confluence was extracted by using Trisure (Bioline, UK) according to the manufacturer's instructions. The RNA was stored at −80 °C until use. For quality control, RNA purity and integrity was evaluated with an agarose gel visualized with ethidium bromide. RNA yield was determined by spectrophotometry.

2.10. Real time PCR array

The expression level of different chemokines and chemokine receptors in DCs and stromal cells was evaluated using the Mouse Chemokines and Receptors RT-PCR Arrays and RT SYBR Green/ROX qPCR Mastermix (SABiosciences, USA), according to the manufacturer's instructions. Results were monitored using different controls available on the plates. Finally, fold-changes compared to control mice were calculated using the manufacturer's software (SABiosciences). To compare results from different PCR arrays, the threshold and baseline values were set manually according to the manufacturer's instructions, and the resulting threshold cycle value (Ct) data was uploaded into the data analysis template on the manufacturer's website (http://www.sabiosciences.com/pcr/arrayanalysis.php). RNA expression of each gene was normalized using five housekeeping genes as controls. The relative expression of each gene compared to the control group, was calculated on the website using the ΔCt method. Differences were considered significant at $p < 0.05$. In the expression studies, a gene was considered to be up-regulated if the difference was >2-fold and down-regulated if the difference was >0.5-fold when compared with the control.

2.11. Statistical analysis

Statistical comparisons of data between control and SLE mice were performed using Student's unpaired *t*-test with two tailed and 95% confidence intervals. Homing indices were analyzed using

a one-sample Wilcoxon-signed rank test. p values <0.05 were considered significant. All statistical tests were performed using Graph Pad Prism program version 4.

3. Results

3.1. Dendritic cells accumulate in lymphoid organs of lupic mice and present an activated state

DCs play a critical role at initiating the immune response (Banchereau et al., 2000; Steinman et al., 2003) and they have been implicated in the pathogenesis of lupus (Crispin and Alcocer-Varela, 2007; Kis-Toth and Tsokos, 2010; Scheinecker et al., 2001). Consequently, we determined the frequency and phenotype of DCs in different secondary lymphoid organs of lupus prone mice BWF1 before and after the development of disease. We compared DCs from lupic and pre-lupic mice to age- and sex-matched non-autoimmune (NZW×Balbc) control mice. Lupic mice from 6 to 10 months of age showed high titers of anti-DNA antibodies in their sera and elevated proteinuria (more than 500 mg/dl) indicating kidney damage. Pre-lupic mice of 2–3 months old showed no sign of autoimmunity (anti-DNA antibodies or kidney damage). As shown in Fig. 1A, we observed significantly higher proportions of DCs (defined by the expression of CD11c and lacking the B cell marker CD79) in the spleen, MLN and blood of lupic mice as compared to pre-lupic and control mice. There was no significant difference in the percentage of $CD11c^+$ cells in peripheral lymph nodes (PLN) among lupic, pre-lupic and control mice (Fig. 1A). We therefore analyzed which DC subset was expanded during the development of lupus. To investigate this point, we determined the absolute numbers of the two main DC subsets, conventional DCs (cDC) and plasmacytoid DCs (pDC), in the spleen, PLN, MLN and blood of lupic, pre-lupic and control mice. We discriminated cDCs from pDCs based on the expression of CD11c and B220, since only pDCs co-express both markers. Fig. 1B shows that the total number of spleen pDCs was significantly increased in lupic mice compared with pre-lupic and control mice, while there were no significant differences in cDCs in this organ. In PLN, pre-lupic and lupic mice exhibit lower numbers of both DC subsets compared to age- and sex-matched control mice. In MLN we found a decrease of cDCs in pre-lupic mice while there was no difference in lupic mice considering that in this organ there is a normal decrease of this subset with age (young control *vs.* old control). In contrast, the absolute number of pDCs in MLN is conserved during the course of the disease. In blood we found a significant increase in cDCs and pDCs compared to pre-lupic and control mice. On the other hand, although we observed a modest splenomegaly in lupic mice, we found no significant differences in total cell numbers between lupic, pre-lupic and control mice (Supplementary Fig. 1), indicating that the increase of pDCs cannot be attributed to a dramatic increase in the overall cellularity. In contrast, PLN and MLN showed a decrease in cellularity compared to pre-lupic and control mice (Supplementary Fig. 1). These data show that during the development of lupus there are significant changes in the composition of the DCs in steady state in different lymphoid organs and blood, which could be associated with the disease.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.01.011.

In light of these results we studied the activation status of the different DC subsets during the development of lupus. For this, we analyzed the expression levels of the co-stimulatory molecules CD86, CD80, PD-L1 and PD-L2 on cDCs and pDCs from spleen of BWF1 and control mice. As shown in Fig. 2, all of these activation markers were significantly higher in both subsets of lupic DCs compared to pre-lupic or control DCs. These data suggest that lupic DCs have enhanced T cell co-stimulatory capacity. In agreement with this observation and previous reports (Kasagi et al., 2010), we found that spleen $CD4^+$ T cells from lupic mice express significantly higher levels of PD-1, CD69 and lower levels of CD62L compared to pre-lupic and control mice (Supplementary Fig. 2A and B). Moreover, the frequency of $IFN\gamma^+$- and $IL10^+$-producing $CD4^+$ T cells was also significantly increased in lupic mice compared to pre-lupic and control mice (Supplementary Fig. 2C). In spite of this, we found no significant differences in the percentages of $CD4^+$ and $CD8^+$ T cells in the spleen of lupic mice compared to control mice (Supplementary Fig. 2D). Altogether, these results suggest that during the development of lupus there is an accumulation of activated pDCs in the spleen that present an enhanced T cell co-stimulatory capacity, which may contribute to inducing the activation of $CD4^+$ T cells.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.01.011.

3.2. Lupic and pre-lupic dendritic cells have an enhanced capacity to migrate to the spleen

The capacity to migrate is crucial for the function of DCs and allows them to travel among blood and tissues. In order to explain why DCs are accumulated in the spleen, we performed competitive homing experiments by injecting into control, pre-lupic or lupic mice recipients a mixture of control and pre-lupic or control and lupic DCs stained with Calcein and TRITC, respectively.

The spleens of the recipient mice were analyzed 24 h (data not shown) and 48 h after the adoptive transfer of Calcein- or TRITC-labeled $CD11c^+$ DCs. Flow cytometric analysis of DCs 48 h after the adoptive transfer showed that a larger number of lupic DCs (Calcein) compared to control DCs (TRITC) localize to the spleen of the different animals, irrespective of whether the recipient was normal or lupic (Fig. 3A). Pre-lupic DCs also showed a preferential migration to the spleen of control and pre-lupic recipients compared to control DCs (Fig. 3B). The homing indices indicate that DCs from pre-lupic and lupic mice migrate into the spleen of the different recipients ninefold more efficiently compared to control DCs. Additionally, pre-lupic and lupic DCs migrate more efficiently to the spleen of pre-lupic and lupic recipient mice than control recipient mice, indicating that the spleen microenvironment of the recipient mice also has an effect on DC migration (Fig. 3A and B). Although we cannot rule out a direct or indirect effect of the spleen microenvironment in DC recruitment or phenotype, these data suggest that lupic and pre-lupic DCs have a higher capacity to migrate to the spleen.

3.3. Lupic dendritic cells present an altered pattern of expression of chemokines and chemokine receptors

In order to migrate to a specific organ, DCs need to encounter the appropriate chemokine microenvironment and express the corresponding receptors. In view of our results showing the preferential migration of lupic DCs to the spleen, we decided to study by PCR-array the chemokine and chemokine receptors on DCs isolated from the spleen of control and lupic mice. Our results show that the genes for CCL8, CXCL10 and CCL2 were significantly up-regulated in DCs derived from lupic mice compared to controls ($p < 0.05$ in all cases), while genes for CCR2, CCR3, CCR4, CSF2, CCR1, CCR1L1, CCR9, CCL17, CCR1L1, CXCR7, and IL8RB were all found to be significantly down-regulated. The gene expression for CCR6 did not vary between lupic and control DC (Fig. 4). To confirm our PCR-array results, we determined by flow cytometry the expression of CCR4, CCR6 and CCR9 on spleen cDCs and pDCs obtained from control, pre-lupic and lupic mice. Fig. 5 shows that control pDCs express CCR4, CCR6 and CCR9. Although CCR4 expression is reduced in pDCs

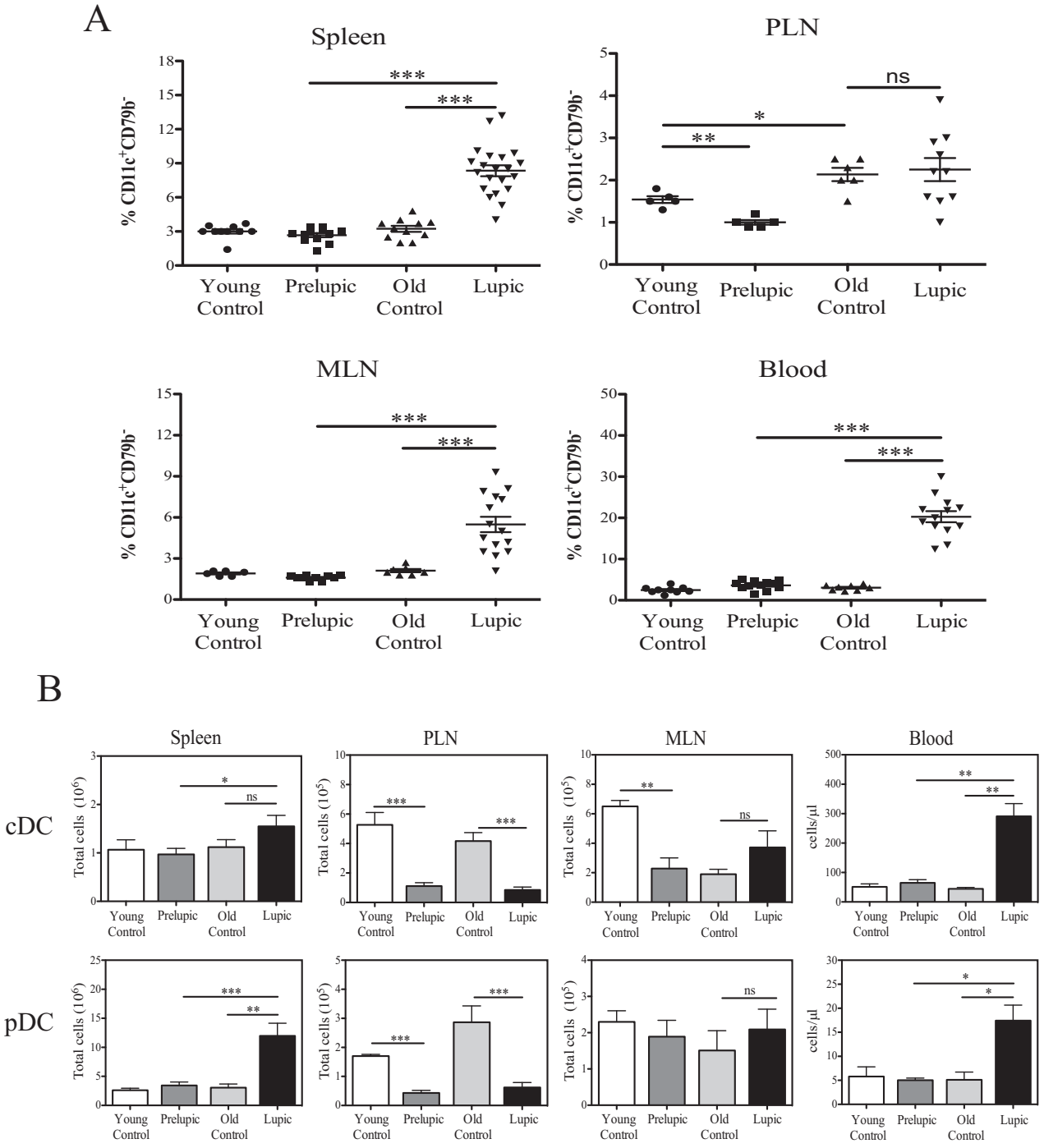

Fig. 1. Lupic mice exhibit an increased percentage and number of DCs in the spleen, MLN and Blood. (A) Percentages of $CD11c^{+}CD79b^{-}$ cells in the organs of control, pre-lupic and lupic mice. Single-cell suspensions were obtained from indicated organs, as described in Section 2. Cells were stained with PE-labeled anti-CD11c and FITC-labeled anti-CD79b. Data are expressed as the mean ± S.E.M. ($^{*}p < 0.05$ and $^{***}p < 0.001$). (B) Total numbers of pDCs ($B220^{+}CD11c^{int}$) and cDCs ($B220^{-}CD11c^{high}$) from spleens, PLN, MLN and blood of control, pre-lupic and lupic mice were determined by flow cytometry. Data are expressed as the mean ± S.E.M. for nine mice per group. Statistical differences between lupic, pre-lupic and control mice are denoted by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$.

from pre-lupic and old mice compared to young controls, there is a further and significant reduction in CCR4 expression in pDC from lupic mice. We also found a significant reduction in CCR9 expression in pDC from pre-lupic and lupic mice compared to their controls. In agreement with our RNA data, we found no significant changes in CCR6 expression during the development of lupus. Conventional DCs on the other hand express low levels of CCR9 compared to pDCs (40% *vs.* 6% respectively) and this receptor is also down-regulated in lupic cDCs as it is in pDCs. Recently, CCR9 expression on pDCs has been associated with induction of tolerance (Hadeiba et al., 2012) and the loss of CCR9 expression by pDCs could be responsible for the loss of tolerance in lupus.

3.4. Stromal cells from lupic mice have an altered gene expression profile of chemokines and chemokine receptors

Increasing evidence demonstrates that stromal microenvironment may regulate the development and function of immune cells

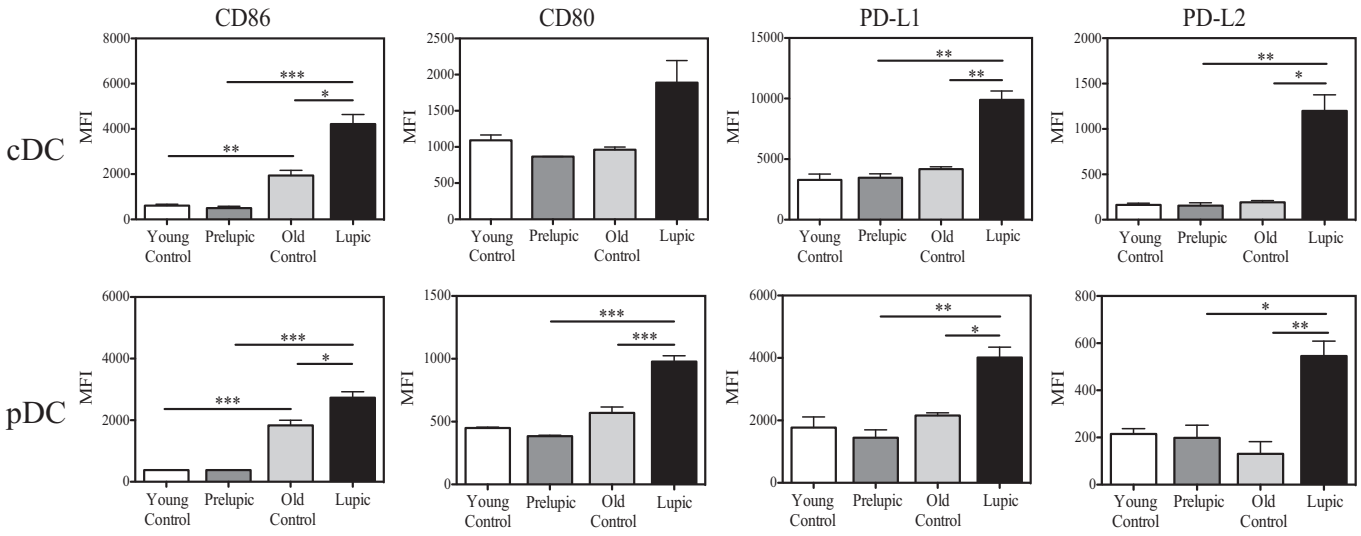

Fig. 2. Lupic dendritic cells from the spleen show an altered activation state. cDCs ($B220^{-}CD11c^{high}$) and pDCs ($B220^{+}CD11c^{int}$) from the spleen of control, pre-lupic and lupic mice were analyzed by flow cytometry for the expression of indicated activation markers. Results show the mean fluorescence intensity (MFI) for each population. Statistical differences in the MFI of a marker between lupic, pre-lupic and control mice are denoted by $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$. Data shown represent five independent experiments.

in the lymphoid and non-lymphoid organs. The microenvironment in parenchymatous organs contributes to the regulation of local immune responses (Turley et al., 2010; Roozendaal and Mebius, 2011). The spleen is an important secondary lymphoid organ where mature DCs present antigens to T cells and initiate T cell response. However, it is not known how splenic stromal cells can regulate T cell responses. Our results suggest that the spleen microenvironment of lupic mice may affect the distribution and migration of different cells allowing for the accumulation of phenotypically abnormal DCs and T cells. To test this hypothesis we evaluated by

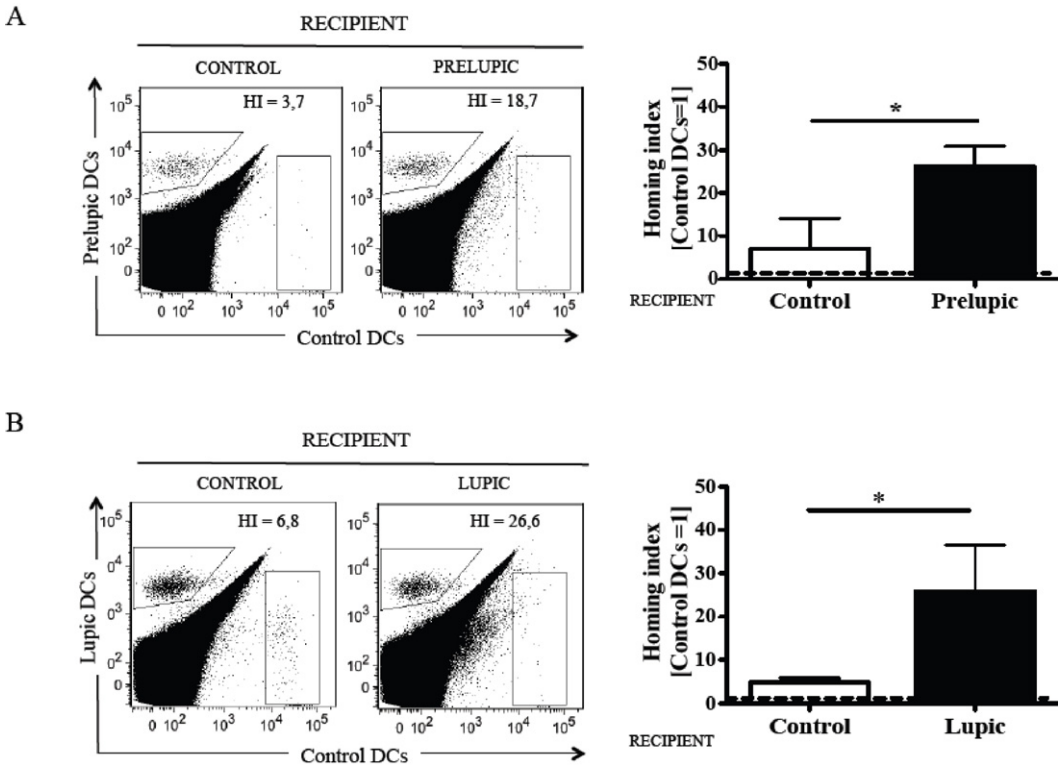

Fig. 3. Lupic and pre-lupic DCs migrate preferentially to the spleen. Spleen DCs from control, pre-lupic and lupic mice were isolated by immunomagnetic beads coupled to anti-CD11c. (A) Control DCs were labeled with Calcein and mixed with an equal number of TRITC-pre-lupic DCs, and the homing index (HI, the ratio of [TRITC cells] to [Calcein cells] in recipient spleen divided by the input ratio) was determined in recipient spleens 48 h after adoptive transfer. Bar graphs (right) show the homing indices in control and pre-lupic recipients and represent the mean ± S.E.M. of three experiments ($^{*}p < 0.05$ compared with control recipients). (B) Control DCs were labeled with Calcein and mixed with an equal number of TRITC-lupic DCs, and the homing index (HI, the ratio of [TRITC cells] to [Calcein cells] in recipient spleen divided by the input ratio) was determined in recipient spleens 48 h after adoptive transfer (left). Bar graphs (right) show the homing indices in control and lupic recipients and represent the mean ± S.E.M. of three experiments ($^{*}p < 0.05$ compared with control recipients).

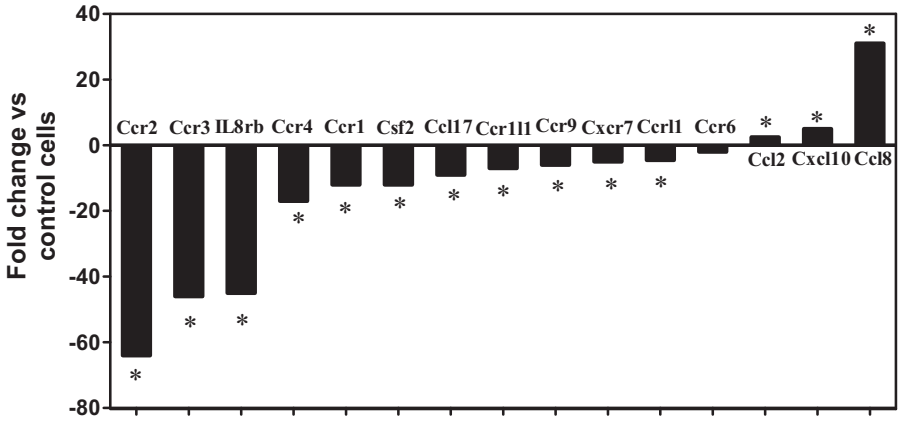

Fig. 4. Dendritic cells from the spleen of lupic mice have differential gene expression of chemokines and chemokine receptors compared to control dendritic cells. Total RNA isolated from lupic, pre-lupic and control DCs was analyzed using an RT2 profiler chemokine and chemokine receptor PCR array. Data are presented as the fold difference relative to the control, calculated from the average ΔCt normalized to housekeeping genes. The transcripts displaying a twofold or greater difference in expression are shown. Gene expression was detected by quantitative RT-PCR in triplicate (*$p < 0.05$).

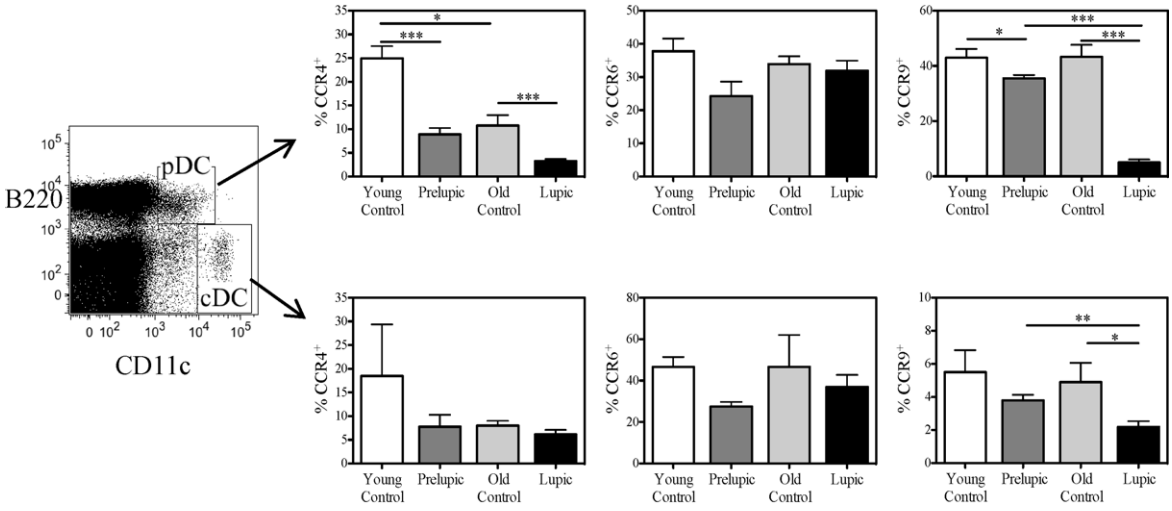

Fig. 5. Lupic DCs from the spleen show decreased expression of chemokine receptors compared with pre-lupic and control DCs. Spleen cells from control, pre-lupic and lupic mice were analyzed by flow cytometry for the expression of CCR9, CCR6 and CCR4 on cDCs ($B220^{-}CD11c^{high}$) and pDCs ($B220^{+}CD11c^{int}$). Results show the percentage of positive cells for each chemokine receptor. Data shown represent the average of six independent experiments.

PCR-array the expression of a set of chemokines and chemokine receptors on spleen stromal cells from control, pre-lupic and lupic mice. As shown in Fig. 6, stromal cells from lupic mice have a different expression pattern of chemokines and chemokine receptors compared to pre-lupic and control stromal cells. The figure shows genes that present at least a twofold change compared to control mice. The mRNA expression levels of CCL19, CX3CL1, CXCL13, and IL-16 in lupic mice were significantly higher than those found in

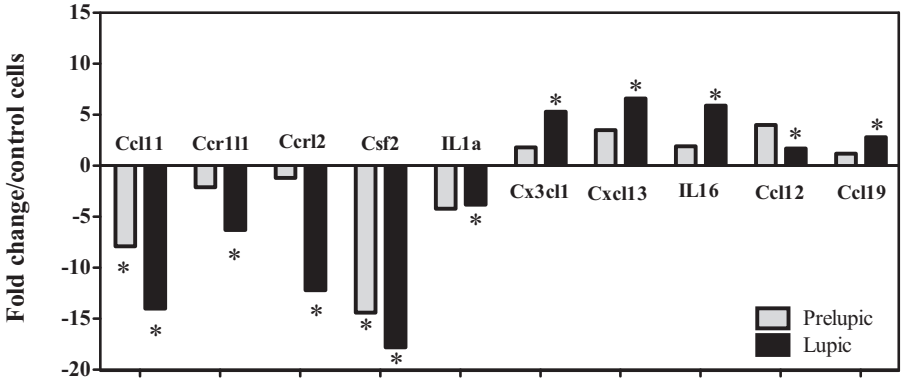

Fig. 6. Expression of chemokines and chemokine receptor genes in lupic stromal cells. Total RNA isolated from lupic, pre-lupic and control stromal cells was analyzed using an RT2PCR array profiler for chemokines and chemokine receptors. Data are presented as the fold difference relative to controls, calculated from the average ΔCt normalized to housekeeping genes. The transcripts displaying a twofold or greater difference in expression are shown. Gene expression was detected by quantitative RT-PCR in triplicate (*$p < 0.05$).

the pre-lupic mice (all $p < 0.05$). Collectively, these data demonstrate that the spleen microenvironment of lupic mice is abnormal compared to pre-lupic and control mice, and suggest that these differences could contribute to the attraction of DCs and activation of T cells.

3.5. Lupic mice present higher numbers of functional regulatory T cells

Regulatory T cells play an important role in T cell homeostasis and are critical regulators of immune tolerance (Sakaguchi, 2008; Horwitz, 2008). Quantitative and/or qualitative deficiencies in Treg cells could lead to the development of autoimmune diseases (Sakaguchi et al., 1995; Bonelli et al., 2008; Buckner, 2010). To further elucidate the role of Tregs in SLE, we compared the percentage of these cells in the spleen of lupic, pre-lupic and control mice. Our results show that the percentage of $CD4^+Foxp3^+$ T cells was significantly higher in lupic mice compared to control and pre-lupic mice (Fig. 7A). When we analyzed $CD4^+FoxP3^+$ T cells on the basis of CD25 expression, there were considerably higher numbers of $CD4^+FoxP3^+$ cells that do not express CD25 in lupic mice compared to pre-lupic and control mice (Fig. 7A). This result is in agreement with a report in humans where this subpopulation of Treg ($CD4^+Foxp3^+CD25^-$) appears in active SLE (Zhang et al., 2008; Bonelli et al., 2009).

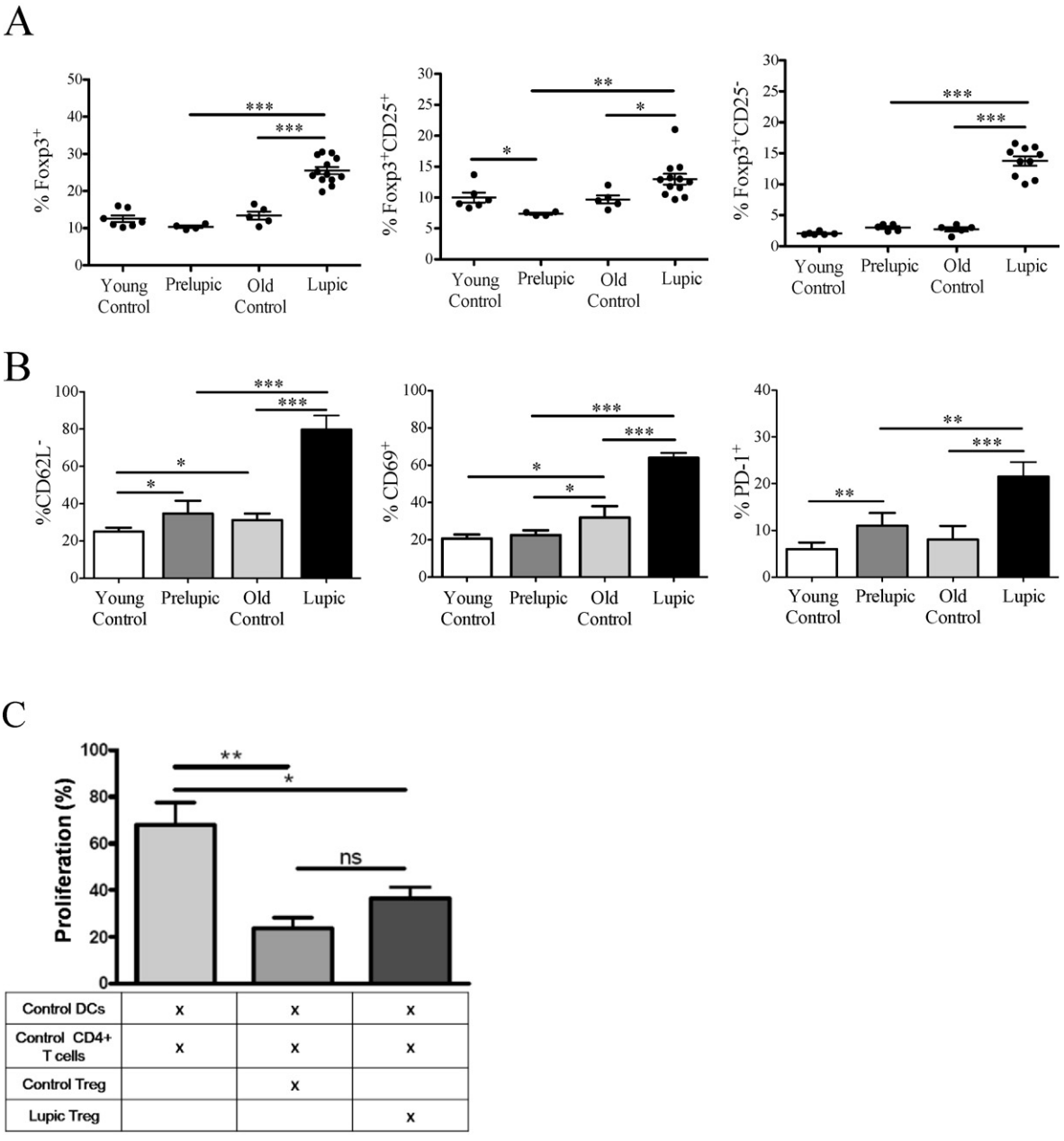

Fig. 7. Lupic mice exhibit an increased percentage of functional Treg in the spleen compared with pre-lupic and control mice. (A) Percentages of total, $CD25^-$ and $CD25^+$ regulatory $CD4^+$ T cells in the spleen of control, pre-lupic and lupic mice. Single-cell suspensions were obtained from the spleen, as described in Section 2 and analyzed by flow cytometry. Data are expressed as the mean ± S.E.M. ($^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$). (B) Bar diagrams show the average percentage of $CD69^+$, PD-1^+ and $CD62L^-$ cells among $Foxp3^+$ T cells in spleen. Data represent the mean ± S.E.M. ($n = 8$ per group). (C) *In vitro* suppression assay comparing the suppressive capacity of $CD4^+CD25^+$ Treg from lupic and age-matched control mice. The proliferation of CFSE labeled responder cells in the presence of Treg was analyzed by flow cytometry and is presented as the percentage of proliferating responder cells. Error bars indicate SD from triplicate cultures from 6 experiments.

In patients with active and inactive SLE, T cells have been reported to display an activated phenotype, which is characterized by the expression of various T cell activation markers (Gerli et al., 2009). To investigate on the activation status of Treg in our model, we analyzed the expression of three well-known activation markers: CD69, PD-1 and CD62L. As shown in Fig. 7B, we found that lupic mice have an increased percentage of $CD69^+$, $PD\text{-}1^+$ and $CD62L^-$ Tregs compared with pre-lupic and control mice, which is consistent with a Treg-activated phenotype. To test the suppressive capacity of lupic Tregs *in vitro*, we isolated $CD4^+CD25^+$ regulatory T cells from control, pre-lupic or lupic mice, cultured them with $CD4^+CD25^-$ T cells and the proliferation of the effector cells was assessed by CFSE dilution. Our results showed that $CD4^+CD25^+$ Treg cells from lupic mice or control mice displayed similar suppressive activity *in vitro*, indicating an intact suppressive capacity, even though they showed an unusual activated phenotype.

4. Discussion

SLE is an autoimmune disease characterized by the presence of autoantibodies and the formation of immune complexes that target multiple organs. Although SLE is mainly a B cell-mediated disease, T cell hyperactivity has been correlated with the development of lupus as a consequence of abnormalities in antigen-presenting cells and Treg deficiency (Gerli et al., 2009; Zhu et al., 2005). On the other hand, since DCs play a central role in the initiation of the immune response and they are responsible for the balance between immunity and tolerance, in this report we decided to evaluate different subsets of DCs in the BWF1 mice model. We also inquired as to the role of stromal cells and Treg cells during the development of the disease in the same mice model.

Dendritic cells have an essential role in maintaining peripheral tolerance. As a consequence, depletion of DCs breaks self-tolerance and produces spontaneous autoimmunity (Fransen et al., 2010). Although they share some common features, such as being antigen-presenting cells (APCs) and having the capability to regulate T cell and B cells, pDC and cDC subsets have unique functions and thus may play distinct roles in SLE immunopathogenesis. Of the two DC subsets, pDCs are thought to have a central role in SLE pathogenesis through the production of IFN-α, which has a pivotal role in inducing SLE (Blanco et al., 2001; Bengtsson et al., 2000; Dall'era et al., 2005; Liu et al., 2011). Although controversial, the number of pDCs in peripheral blood is altered when compared to normal controls (Migita et al., 2005; Jin et al., 2008; Farkas et al., 2001). Conventional DCs have also been found to be abnormal in SLE (Crispin and Alcocer-Varela, 2007; Jin et al., 2008; Monrad et al., 2008). Patients with this condition have deficient numbers of cDCs (Migita et al., 2005) and monocyte-derived DCs from blood of patients with SLE exhibit abnormal phenotypes and functions (Koller et al., 2004).

In the present study we demonstrate that lupic mice have an alteration in the percentage of pDC and cDC subsets in the spleen, peripheral blood and PLN compared with control and pre-lupic mice (Fig. 1). pDCs were markedly increased in the spleen in BWF1 mice developing lupus nephritis. However, we observed a decrease in pDC numbers in PLN and blood in lupic mice compared with pre-lupic and control mice. In our data, an increased number of cDCs in peripheral blood was observed in lupic mice, but they were decreased in PLN. The origin of DCs that accumulate in secondary lymphoid organs could be an important factor to consider in the development of SLE, since cDCs and pDCs present different characteristics concerning the ability to intake apoptotic and necrotic cell material that is presented to T cells, an element that has to be considered for the induction of T cell tolerance (Banchereau and Steinman, 1998).

DCs can induce either T cell tolerance or strong innate and adaptive immunity to specific antigens. In general, tolerance is initiated when DCs that interact with T cells are in an immature state, whereas the initiation of immunity requires that this interaction takes place in the presence of activated DCs (Banchereau et al., 2000). Increased differentiation from monocytes (or potentially other myeloid precursors) to the DC stage, and up-regulation of maturation/activation markers could promote and enhance the capabilities of lupus DCs to prime and activate T cells in the spleen and other lymphoid organs, further contributing to the T cell hyper-responsiveness and enhanced activation described in SLE. Certainly, the up-regulation of activation molecules in DCs and the increased migration to lymphoid organs during SLE could be essential features that promote stimulation and priming of T cells in these organs.

Here, we show an up-regulation of CD80 and CD86 on both subsets of spleen DCs from lupic mice compared with DCs from pre-lupic and control mice. Therefore, increased DC maturation in SLE in the absence of extrinsic danger signals suggests that these cells can become very efficient autoantigen-presenting cells and drive autoimmune responses. In addition, the role of co-stimulatory molecules is well documented in murine models of lupus, including the observation that treatment with a combination of blocking mAbs to CD80 and CD86, before the onset of murine lupus, significantly improves survival and diminishes the severity of the disease (Nakajima et al., 1995; Kinoshita et al., 2000).

As a consequence of higher expression of co-stimulatory molecules by lupic DCs, we found that $CD4^+$ T cells from lupic mice are in a hyper-reactive state, characterized by an increased expression of CD69, CD25, PD-1 and decreased expression of CD62L compared to $CD4^+$ T cells from pre-lupic and control mice (Supplementary Fig. 2). Recent reports in BWF1 mice have shown that PD-1 is predominantly expressed on $CD4^+$ T cells that infiltrate the kidney, and that $CD4^+PD\text{-}1^{high}$ T cells produced higher levels of IFN-γ than $CD4^+PD\text{-}1^{low/-}$ T cells. Stimulation with PMA/ionomycin caused splenic $CD4^+PD\text{-}1^+$ T cells to secrete high levels of IFN-γ, IL-10, low levels of TNF-α, low levels of IL-2, IL-21, and no IL-4, IL-17 (Kasagi et al., 2010). In human autoimmune diseases, a similar type of $CD4^+PD\text{-}1^+$ T cell that produces IFN-γ has been shown to accumulate at inflammatory sites (Hatachi et al., 2003). In addition, the IFN-γ produced by $CD4^+$ T cells is responsible for IgG class switching, IgG2a anti-ds-DNA Ab production, and mesangial cell hyperplasia in BWF1 mice (Jacob et al., 1987; La Cava et al., 2004). In our study, the percentage of $CD4^+PD\text{-}1^+$ T cells among splenic lymphocytes was significantly higher in lupic than pre-lupic or control mice (Supplementary Fig. 2). In addition, we found that $CD4^+$ T cells from lupic mice produce significantly more IFN-γ and IL-10 than $CD4^+$ T cells from pre-lupic and control mice. It is still uncertain whether the IL-10 produced by $CD4^+$ T cells has a pathogenic or a regulatory role during the development of nephritis in BWF1 mice; further investigation will be needed to clarify this issue.

Cumulative data suggests a distinct role of PD-1 and its ligands PD-L1 (B7-H1) and PD-L2 (B7-DC) in T cell regulation. Studies in mouse models of autoimmunity and tolerance have revealed that PD-1:PD-L1/2 interactions are not only important in the initial phase of activation and expansion of self-reactive T cells, but also influence self-reactive T cell effector function upon antigen re-encounter. A recent report demonstrated that IFN-γ-producing $CD4^+PD\text{-}1^{high}$ T cells play a key role in the progression of murine lupus nephritis (Kasagi et al., 2010). Recently, several studies showed that tissue-specific expression of PD-L1 is functionally important and plays a key role in protecting against pathogenic self-reactive T cells (Ansari et al., 2003; Keir et al., 2006). Nevertheless, several reports showed that up-regulated expression of PD-L1 in inflamed tissue failed to suppress the disease (Ansari et al.,

2003; Bertsias et al., 2009). Here, we show that PD-L1 and PD-L2 expression was increased on splenic cDCs and pDCs from lupic mice in comparison with pre-lupic and control mice. Given the direct correlation between PD-L1/PD-L2 and SLE disease activity, future investigations may reveal a role for PD-L1/PD-L2 fusion proteins or other molecules capable of ligating PD-1 in the treatment of SLE or other autoimmune diseases.

The proper localization of DCs in secondary lymphoid organs and their recruitment at sites of inflammation in response to chemotactic stimuli are critical for an optimal immune response (Sallusto and Lanzavecchia, 2000; Randolph et al., 2008). Any impairment of migratory capacity could lead to SLE-related changes, such as altered numbers of DCs, DC occurrence in the skin, impaired clearance of apoptotic material or decreased induction of self-tolerance. In the current study, we report that that lupic and pre-lupic DCs have distinctive characteristics that allow them to migrate and to remain in the spleen significantly more efficiently than control DCs (Fig. 3). The higher frequency of injected DCs found in the spleen of pre-lupic and lupic mice compared to the spleen of control mice suggests that the spleen microenvironment in lupic mice may play an important role in the localization of DCs. It remains to be elucidated what kind of stimuli could induce marked recruitment of DCs into organs or resistance to enter apoptosis at this environment.

Chemokines direct the migration of leukocytes throughout the body and thus orchestrate the inflammatory response. The up-regulation of chemokines may result in the inappropriate recruitment of autoreactive lymphocytes to sites of inflammation (Tang et al., 2008; Vielhauer et al., 2007; Segerer et al., 2000). Alternatively, systemic elevation of chemokines may desensitize chemokine receptors expressed on activated leukocytes, resulting in the loss of normal homing mechanisms and consequently global inflammatory responses. In this study, we provide experimental evidence that lupic DCs constitute an important source of chemokines, allowing for the selective and directional migration of DCs and T lymphocytes.

In agreement with previous studies in humans that implicate CCL2 (MCP-1) and CXCL10 (IP10) with SLE disease activity (Vielhauer et al., 2007; Bauer et al., 2009; Fu et al., 2008), we found that CCL2, CCL8 (MCP2) and CXCL10 levels are increased in lupic DCs *versus* pre-lupic and control DCs (Fig. 4). CCL2 and CCL8 are inflammatory chemokines that attract neutrophils, monocytes and immature DCs. Additionally, CXCL10 has been found to participate in the recruitment of pDCs, the major source of type I IFN (Wenzel and Tuting, 2007). This correlates with the fact that the majority of lupus patients exhibit increased serum type-I IFN and this has been correlated with activity and disease severity. Moreover, these chemokines are IFN-inducible since they have consensus sequences for IFN-responsive elements in their promoter sequence, including ISRE (IFN-stimulated responsive element) and IRF (interferon regulatory factor) (Fu et al., 2008).

In addition, we demonstrated that the expression of chemokine receptors CCR2, CCR3, CCR4, CCR1, CCR9, CXCR7 and CCR1L1 was reduced in lupic DCs compared with pre-lupic and control mice. This observation is in agreement with previous findings demonstrating that mature DCs down-regulate their responsiveness to inflammatory chemokine pathways, such as CCR2–CCL2 (Geissmann et al., 2003; Merad et al., 2002), CCR5–CCL5 (Stumbles et al., 2001), and CCR6–CCL20 (Merad et al., 2002). Recent data have shown that tolerogenic pDCs in lymphoid tissues express the chemokine receptor CCR9 (Hadeiba et al., 2008), which is a chemoattractant receptor involved in homing of memory and effector lymphocyte populations to the small intestines (Kunkel et al., 2000) and also in progenitor T cell homing to the thymus (Uehara et al., 2002). $CCR9^+$ pDCs efficiently induce Treg cells from peripheral T naïve cells, inhibiting Ag-specific immune responses and graft-*versus*-host disease (GVHD). Furthermore, plasmacytoid dendritic cells that express CCR9 transport peripheral antigens to the thymus to promote central tolerance (Hadeiba et al., 2012). Thus, it is possible that lupic mature DCs, which have a decreased expression of CCR9, are intrinsically inefficient at inducing central tolerance. More importantly, we found that lupic pDCs had a diminished expression of CCR9 and that both subsets of lupic DCs had decreased expression of CCR4. These results suggest that the production of chemokines in the spleen microenvironment and down-regulation of chemokine receptors in dendritic cells present in the spleen of lupic mice would allow a preferential localization of DCs.

There is some evidence that stromal cells can control immune responses through changes in chemokine production controlling cell entry or egress from the tissues (Mueller and Germain, 2009), but it is not known how stromal cells participate in the pathogenesis of SLE. Our data indicate that the stromal cells from lupic mice show significant differences in their chemokine and chemokine receptor expression when compared with stromal cells from pre-lupic or control mice, which may explain the differential behavior of DCs in lupic mice. In agreement with results from other groups, we found an increased expression of CXCL13 and IL-16 in stromal cells from lupic mice (Bauer et al., 2009; Wong et al., 2010; Lynch et al., 2003; Xue et al., 2009), as well as increased expression of CX3CL1 and CCL19. Collectively, these results suggest that stromal cells from lupic mice may be involved in the accumulation of DCs and consequently on an enhanced T cell activation, although the exact mechanism remains to be elucidated. Additionally, a differential pattern of expression for chemokines and their receptors has been linked to the migration of B lymphocytes and the induction of nephritis in lupic mice (Penna et al., 2002; Vielhauer et al., 2007; Segerer et al., 2000).

Another specialized $CD4^+$ T cell subset implicated in the appearance of systemic autoimmune syndromes is regulatory T cells (Sakaguchi and Sakaguchi, 2005). Our results revealed an increased frequency of Treg cells (as determined by Foxp3 intracellular expression) compared to Tregs from control and pre-lupic mice. Unexpectedly, $CD4^+CD25^+$ Tregs from lupic mice express T cell activation markers, although they exhibit normal suppressive function (Fig. 7). Indeed, when we closely investigated the phenotype of lupic Tregs we discovered that a large proportion of these cells were CD25-negative. Interestingly, these results agree with recent results from four separate groups indicating that there is a considerable proportion of $CD4^+CD25^-Foxp3^+$ T cells present in peripheral blood of SLE patients (Bonelli et al., 2008, 2009; Zhang et al., 2008; Suen et al., 2009). However, the nature of the $CD4^+CD25^-Foxp3^+$ T cell subset and the clinical significance of their increased number in SLE are still unknown. As mentioned, regardless of the unusual phenotype of these Tregs, they have a normal suppressive function. Interestingly, similar to that seen in other lupus prone mice (Monk et al., 2005), in the lupus model used in this study we found that effector T cells from lupic mice not only show hyperactivity as mentioned before, but they are also resistant to the suppressive activity of Tregs (data not shown).

5. Conclusion

Collectively, our results demonstrate that the microenvironment of the spleen of lupic mice is characterized by a particular pattern of expression of chemokines that may be involved in the homing of DCs to the spleen which in turn should maintain T cells in an activated state over time, making them resistant to normal suppression mechanisms leading to the production of an autoimmune response. An increase in regulatory T cells could be a compensatory mechanism, which is unable to control autoimmunity.

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Authors' contribution

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published.

Study conception and design: M.A. Gleisner, P. Reyes, M.R. Bono and M. Rosemblatt; acquisition of data: M.A. Gleisner, P. Reyes, V. Simon; analysis and interpretation of data: M.A. Gleisner, P. Reyes, D. Sauma, J. Alfaro, P. Solanes and N. Crisostomo.

Conflict of interest

The authors declare no conflict of interest.

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