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Plasmacytoid dendritic cell distribution and maturation are altered in lupus prone mice prior to the onset of clinical disease

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Abstract

Plasmacytoid dendritic cells (pDCs) and their production of type I interferons (IFN) are key pathogenic mediators of systemic lupus erythematosus (SLE). Despite the key role of pDCs in SLE, the mechanism by which pDCs promote disease is not well understood. The first objective for this study was to assess the number and maturation state of pDCs in pre-disease NZM2410 lupus prone mice compared to control mice. Second, we sought to identify mechanisms responsible for the alteration in pDCs in NZM mice prior to onset of clinical disease. We compared the number and percent of pDCs in the spleens and bone marrow (BM) of pre-disease NZM24010 (NZM) mice to C57BL/6 (B6) control mice. In the spleens of pre-disease NZM mice, pDC percent and number were increased. This increase occurs in parallel with a decrease in BM pDC number and percent in the NZM mice. The decrease in BM pDC number suggests the increase in spleen pDCs is a result of altered pDC distribution and not increased production of pDCs in the BM. To determine if pDC developmental potential is altered in lupus prone mice, we cultured BM from NZM and B6 mice *in vitro*. We found a reduced percentage/number of pDCs developing from the BM of NZM mice compared to B6 mice, which further supports that the increase in pDC number is a result of altered pDC distribution rather than increased pDC production. To better characterize the pDC population, we compared the percentage of mature pDCs in the spleens and BM of NZM mice to controls. In the NZM mice, there is a dramatic reduction in the number of mature pDCs in the BM of NZM mice, suggesting that mature pDCs exit the BM at a higher rate/earlier maturation time compared to healthy mice. We conclude that pDCs contribution to disease pathogenesis in NZM mice may include the alteration of pDC distribution to increase the number of pDCs in the spleen prior to disease onset.

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Keywords

Rodent; Dendritic cells; Autoimmunity; Systemic lupus erythematosus

1. Introduction

Plasmacytoid dendritic cells (pDCs) play a key role in the immune response to viral infections by producing large amounts of type I interferon (IFN) following toll-like receptor (TLR) stimulation [1]. The pDC's produce 200–1000 times more type I IFN than any other cell type and type I IFNs contribute to SLE in both humans and mice [2]. Over half of SLE patients have increased type I IFN activity in their peripheral blood [3]. Further, the IFN activity corresponds with disease activity in some patients [3]. In some of the lupus prone mouse models, the absence of the type I IFN receptor protects mice from disease and the administration of exogenous type I IFN hastens disease progression [4–6]. Regarding pDCs themselves both the haplodeficiency and transient depletion of pDCs protect lupus prone mice from disease [7,8]. Based on these data, pDCs are accepted as integral to the development of SLE.

The mechanism by which pDCs become dysregulated and thus contribute to disease remains unknown. Delineating the number and function of pDCs, in both human and murine lupus, has not yielded a reproducible mechanism, as findings are complicated by differences in methodology and disease models. In human studies, pDCs are reported to be reduced, normal, or increased in the blood of SLE patients [2,9,10]. In mice, early work showed an increase in pDCs in (NZBxNZM)F1 lupus prone mice only after the onset of disease [11]. With improved identification methods, pDCs were found to be increased in the spleens of multiple strains of lupus prone mice prior to development of clinical disease, suggesting an increased number of pDCs contributes to the cell's pathogenic role in SLE [12]. Regarding function, pDCs from lupus prone mice have an altered activation profile expressing increased levels of CD40 and reduced levels of CD54 and CD80 [13]. Together, these findings suggest both pDC number and function likely contribute to SLE pathogenesis.

Due to the importance of pDCs in the pathogenesis of SLE and lack of consensus on the mechanisms by which pDCs impact disease, we measured the number of pDCs in the BM and spleens of pre-disease lupus prone mice and investigated a possible mechanism responsible for the altered number of pDCs.

2. Materials and methods

2.1. Mice

Female NZM2410 and C57BL/6 mice between 10 and 14 weeks of age were used for all experiments. The mice were maintained at the Ralph H. Johnson VAMC Animal Care Facility (Charleston, SC) using Institutional Animal Care and Use Committee approved protocols #421 and #498 originally approved August 2008 and 2011 respectively.

2.2. Spleen pDCs

Spleens were harvested from mice and processed as previously described [14]. Briefly, spleens were processed into a single cell suspension, counted and stained for flow cytometry. The flow cytometry protocol used was previously described [14]. Briefly, cells were stained with LIVE/DEAD Fixable Dead Cell Stain (Life Technologies), blocked with antiCD16/CD32, and surface antigens were stained with fluorochrome-conjugated antibodies. Cells were acquired on the LSRFortessa cell analyzer (BD Biosciences, San Jose, CA). Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OR). The flow cytometry data was analyzed as previously described [14]. pDCs were identified as CD11b⁻, B220⁺, SiglecH⁺ using a serial gating strategy. Maturation markers were measured on the preselected pDC populations as indicated. Fluorescence minus one controls were used as indicated to select gates.

2.3. Bone marrow DCs

Bone marrow (BM) was flushed from the bilateral femurs and tibias of mice and processed into a single cell suspension as described previously [14]. After a single cell suspension was obtained, the cells were either stained for flow cytometry or cultured in Flt3L. The BM cells were stained and analyzed as described for the spleen cells.

BM derived pDCs (BMDC) were cultured from total BM. The BM was plated at a concentration of 1.5×10^6 cells/mL in RPMI-media with 10% supernatant from a Flt3L-producing cell line (a kind gift of Dr. Stephania Gallucci, Temple University). On day 7, the BMDCs were harvested and stained for flow cytometry. The staining protocol used is the same described in spleen cells. In the BMDCs, pDCs were identified from a population of singlets and live cells using the markers CD11c and B220.

2.4. Antibodies and reagents

Fluorescent-conjugated antibodies including anti-mouse CD11c, CD11b, B220, SiglecH, mPDCA1, and MHCII (I-A/I-E) were purchased from Biolegend (San Diego, CA). PE-conjugated anti-mouse Ly49Q was purchased from MBL International (Woburn, MA). LIVE/DEAD fixable near-IR dead cell staining kit was purchased from Life Technologies (Grand Island, NY).

2.5. Statistics

Statistical analysis was performed with GraphPad prism software (GraphPad). Data with normal distribution, as assessed with GraphPad software, was analyzed by a Student *t*-test. Data that was not normally distributed was assessed with a Mann-Whitney analysis. Data expressed as mean and SE.

3. Results

3.1. Pre-disease lupus prone mice have increased numbers of spleen pDCs and reduced numbers of BM pDCs

The absolute number of pDCs are elevated in spleens of multiple strains of pre-disease lupus prone mice, suggesting an increased number of pDCs contributes to these cells' pathogenic

role in SLE [12]. The mechanism by which pDCs are increased in these murine lupus models is unknown. In this study, we hypothesized that the increase in spleen pDC number occurs as a result of an increase in BM pDC production rather than a prolonged life span or altered distribution. To test this hypothesis, we compared both the percent and absolute number of pDCs (CD11b⁻B220⁺, SiglecH⁺) in the spleens and bone marrow of pre-disease (10–14 week old) female NZM2410 lupus prone mice to age and sex matched B6 controls. We studied only female mice based on the female/male 9:1 ratio in lupus. We studied 10–14 week old post-pubertal, pre-disease mice. Studying mice prior to disease onset increases the likelihood of identifying a change in the pDC population that represents a mechanism of disease development rather than a result of disease. The addition of SiglecH, a pDC specific surface marker, improved the specificity of identifying pDCs by flow cytometry. We confirmed that the population of cells expressing SiglecH also expressed the marker PDCA1, as inflammation may alter the expression of these pDC markers [14,15].

We found a significant increase in both the percent and absolute number of spleen pDCs in pre-disease NZM mice compared to controls (Fig. 1A, F). The increased percent of pDCs suggests the increase is partially pDC specific and not due to changes in total spleen cellularity, which is not significantly different between the strains (Fig. 1D). To determine the role of the BM pDC production on spleen pDC number, we measured the percent and absolute number of pDCs in the BM, as pDCs reach full maturity in the BM prior to migrating to the periphery. In the BM, both pDC percent and absolute number were significantly decreased in NZM mice compared to controls, which contradicted our original hypothesis that increased BM pDC production is responsible for the increase in spleen pDC number (Fig. 1B, F). This finding suggests the increase in pDC number in the spleen is not due to increased BM production of pDCs, but a change in distribution of pDCs. To further test the developmental potential of pDCs from the BM of pre-disease NZM mice, we cultured BM from pre-disease lupus prone mice and B6 controls in the pDC growth factor FMS-like tyrosine kinase 3 ligand (Flt3L). Using this culture system, the percent of pDCs developing from the BM of NZM mice was reduced compared to controls (Fig. 1E, H). This finding suggests that BM precursors from NZM mice do not have an increased developmental potential compared to control mice, thereby suggesting the increase in spleen pDC number in NZM mice is not likely due to increased BM production of pDCs. To determine if the reduced percent of pDCs cultured from NZM mice is due to an overall decreased ability of BM precursors to respond to Flt3L or a pDC-specific effect of Flt3L, we compared the number of cells on day 7 to the number of BM cells cultured on day 0 (Fig. 1E). There was no difference in percent yield, suggesting the reduction in pDC percent is not a product of an overall reduction in response to Flt3L (Fig. 1E). Together these data suggest the increased spleen pDC number is not likely caused by increased BM production of pDCs, but rather a change in distribution of mature pDCs. Furthermore, the increased percent of pDCs and unchanged total spleen cellularity suggests the change in distribution is not due to an overall increase in total spleen cell number, but rather a mechanism that is cell specific.

To determine if the increase in pDC number is pDC specific or common to other lineages of Dendritic cells (DCs), we measured the percent and number of conventional DCs (cDCs) in the spleens of NZM and B6 mice. There was no difference in either percent or number of cDCs in the spleens on NZM and B6 mice, suggesting the increase in pDC number is a

result of a pDC specific mechanism. We do however, acknowledge that we did not measure other lymphocyte lineages therefore we cannot rule out alterations in T, B, NK cell and macrophage populations. Given the importance of pDCs in the development of SLE these findings may represent an important mechanism of disease pathogenesis.

3.2. Mature pDCs are dramatically reduced in the BM of pre-disease lupus prone mice

To investigate the mechanism responsible for the change in pDC distribution, we measured the maturation state of pDCs in the BM and spleen using the marker Ly49Q. PDCs acquire Ly49Q expression as a final step of maturation in the BM [16]. The expression of this marker is required for pDCs to exit the BM and produce inflammatory cytokines [16,17]. In the BM of NZM mice, Ly49Q⁺ mature pDCs are dramatically reduced in both percent and number compared to B6 controls (Fig. 2A, C). In the spleen, the percent of pDCs expressing Ly49Q was unchanged and there was a trend towards an increase in absolute number of Ly49Q⁺ pDCs (Fig. 2B, C). The lack of increase in Ly49Q⁺ pDCs in the spleen is likely due to the lack of a distinct mature and immature pDC population in the spleen (Fig. 2E). However, since the total spleen pDC percent and number are increased in NZM mice, these data suggest mature pDCs from NZM mice exit the BM at a higher rate or have altered survival compared to healthy controls. Since lupus prone mice have a significantly altered population of mature pDCs in the BM we also looked at the maturation state of the spleen pDC population using another marker of maturation, MHCII expression. MHCII is expressed on pDCs after stimulation with toll-like receptor (TLR) ligands and its presence signifies a maturation event in the pDC lifecycle [14]. In our previous work we showed an increase in a different TLR mediated pDC activation marker, PDC-TREM, in the lupus prone mice. Since PDC-TREM expression is induced by TLR stimulation, we hypothesized there is an increase in the percent of MHCII⁺ pDCs in the spleens of NZM mice. To test this hypothesis we measured the percent of spleen pDCs (CD11b⁻B220⁺SiglecH⁺) expressing MHCII in NZM and control mice. Interestingly, NZM mice had a reduced percent of pDCs expressing MHCII. Like the BM pDC population, this finding will be important to consider in future functional studies, as the spleen pDC population in lupus prone mice differs in maturation state compared to control mice, thereby making comparison of pDCs identified by traditional pDC markers difficult.

4. Discussion

We found that pDC distribution and maturation is altered in pre-disease NZM2410 (NZM) mice. PDC numbers are increased in the spleen and reduced in the BM of NZM mice compared to controls. The altered distribution may be due to increased migration of mature pDCs from the BM, altered pDC survival, or DC population plasticity. Further, spleen pDCs from NZM mice have an altered TLR-induced maturation profile, with a reduced percent of MHCII⁺ pDCs. Together these findings suggest that pDCs from NZM mice have both alterations in the distribution and maturation patterns prior to the development of clinical disease. Given the importance of pDCs in lupus pathogenesis these findings may represent a mechanism by which these cells contribute to disease. Further, these findings will be important to consider when evaluating and executing functional pDCs studies in NZM animals, as the BM and spleen pDC populations expressing the traditional pDC surface

markers may represent a heterogeneous population of pDCs, which differ between healthy and NZM mice.

Early human studies reported reduced levels of pDCs in the peripheral blood of SLE patients compared to controls [18]. The reduced levels of blood pDCs are purported to be due to tissue localization of pDCs, as pDCs are present in the skin and kidneys of SLE patients [2,10]. In contrast, recent studies report normal to increased pDC levels in the peripheral blood of SLE patients [9]. The differences in these findings are likely due to challenges in identifying pDCs and differences in disease pattern and progression. In (NZMxNZB)F1 mice, Gleisner et al. reported increased numbers of spleen pDCs in lupus prone mice after onset of clinical disease, with normal pDC numbers in the spleens of pre-disease mice [11]. A more recent study by Zhou et al. found increased numbers of pDCs in the spleens of multiple strains of pre-disease lupus prone mice [12]. Our study agrees with the latter study and expands this data by reporting an increase in spleen pDC percent along with the decrease in BM pDCs number and percent. The differences between pDC number among mouse studies is almost certainly due to use of different pDC identification markers. In Gleisner et al. pDCs were identified as CD11c^{int}B220⁺ cells. The use of B220 as a pDC marker results in contamination with T, B and NK cells. Both Zhou et al. and our study used pDC specific markers [15,19]. Zhou et al. used PDCA1 and our study used SiglecH to identify the pDCs. Additionally we confirmed that SiglecH⁺ cells and PDCA1⁺ cells represented an overlapping population of cells in predisease NZM mice, as PDCA1 expression can change under inflammatory conditions [14,15]. This finding suggests the markers PDCA1 and SiglecH are equally useful in identifying pDCs in pre disease NZM mice. Thus, our study in combination with previous work supports the hypothesis that pDCs are increased in number and percentage in the spleens of NZM2410 lupus prone mice prior to disease development, thereby representing an early mechanism contributing to disease pathogenesis.

Despite similar findings in the spleen, our results in the BM are in disagreement with Zhou et al. Zhou et al. did not report a difference in BM pDC number between B6 and NZM2410 pre-disease mice, however we found a significant decrease in both the percent and number of pDCs in the BM [12]. This difference may be attributed to the larger sample size in our study. We believe the decrease to be a true finding, as there is a profound reduction in the percent and number of mature Ly49Q⁺ pDCs in the BM of NZM mice.

This is the first report, to our knowledge, of a reduction of mature Ly49Q⁺ pDCs in the BM of NZM2410 mice or any other lupus prone strain. Given that Ly49Q expression is involved in pDC migration and function, we hypothesize this finding to be significant to disease development [16,17]. Ly49Q expression is required for pDCs to exit the bone marrow and produce type I IFNs. Functionally, DC migration is implicated in lupus pathogenesis in (NZMxNZB)F1 mice as DCs (CD11c⁺) cells from lupus prone mice have increased spleen homing capacity [11]. Additionally, the DC migration receptor CCR2 is implicated in the pathogenesis of SLE, as MRL/lpr mice lacking CCR2 are protected from disease [20]. Further, type I IFNs are critical in SLE pathogenesis. Deficiency of the IFN receptor protects mice NZM2328 mice from disease [5]. Additionally, IFN α treatment can produce SLE like symptoms as a side effect in humans. Considering that DC migration and IFN

production contribute to SLE pathogenesis and Ly49Q plays a role in both pDC migration and IFN production, it is likely that our findings represent a possible mechanism by which pDCs contribute to disease.

Further, we report a reduction of the spleen MHCII⁺ pDCs. Initially, we hypothesized MHCII⁺ pDCs are increased in the spleens of lupus prone mice, as expression of MHCII is dependent on TLR stimulation and other TLR mediated pDC activation markers are elevated in NZM2410 mice [14]. Our findings did not support this hypothesis, as we found a decrease in MHCII⁺ pDCs, suggesting that TLR induced pDC maturation in NZM mice plays a role in SLE pathogenesis through a mechanism not explained by an increased number of antigen presenting MHCII⁺ pDCs. TLR induced pDC maturation and antigen presentation is a multifaceted process. Antigen targeting to pDCs via SiglecH in mice and BDCA2 in humans, results in the induction of a tolerogenic state [21,22]. This is in contrast to antigens targeting to PDCA1, which results in increased response to viral infection [23]. These findings suggest MHCII⁺ pDCs may act as either a pro-inflammatory or tolerogenic cells depending on the environment. The reduction of MHCII⁺ pDCs in pre-disease NZM mice may represent a reduction in tolerogenic pDCs. Further, we previously reported an increase in PDC-TREM⁺ pDCs in pre-disease NZM mice. PDC-TREM expression is also induced by TLR stimulation, and the expression of PDC-TREM is required for pDCs to acquire type I IFN producing capacity [24]. The increase in PDC-TREM⁺ pDCs in combination with the reduction in MHCII⁺ pDCs suggest an alteration in the ratio of type I IFN producing to antigen presenting pDCs in NZM mice. The preference for the PDC-TREM expressing pDC may represent a mechanism of disease pathogenesis in NZM mice, as IFN production is a major driver of lupus like disease in this model. Taken in the context of previous work our findings provide novel insight into pDCs abnormalities in to SLE.

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Abbreviations:

ERα	Estrogen receptor alpha
ERαKO	Estrogen receptor alpha knock out
SLE	Systemic lupus erythematosus
pDC	Plasmacytoid dendritic cell
DC	Dendritic cell
mDC	Myeloid dendritic cell

LDC	Lymphoid dendritic cell
cDC	classical dendritic cell
TLR	Toll-like receptor
IFN	Interferon
WT	Wild-type
BM	Bone marrow
NZM	NZM2410

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Fig. 1.

pDC and cDC percent and number and total cellularity in the BM and spleens and pDC percent in Flt3L driven BMDC cultures from the BM of B6 and NZM2410 10–14 week old female mice. Percent and number of pDCs (B220⁺CD11b⁺SiglecH⁺) in spleen and BM of B6 and NZM mice (A, B). Percent and number of cDCs (SiglecH⁺, CD11c⁺, MHCII⁺) in the spleens of B6 and NZM2410 mice (C). Spleen and BM cellularity in B6 and NZM2410 mice (D). Percent of pDCs (CD11c⁺B220⁺) in Flt3L driven BMDC culture from the BM of B6 and NZM mice and % yield of cells from cultures (E). Flow cytometry plots represent data from at least 3 separate experiments, which are summarized in the graphs (F, G & H), B6 (n = 5) and NZM (n = 7). Results are expressed as the mean ± SD. Statistical analysis performed with Mann-Whitney analysis or student's *t*-test depending on normality of data. *P* < 0.05 was considered significant.

Fig. 2. Percent of Ly49Q⁺ and MHCII⁺ pDCs in the BM and Spleens of B6 and NZM2410 10–14 week old female mice. Percent of Ly49Q⁺ pDCs (B220⁺CD11b⁺SiglecH⁺) in BM of B6 and NZM2410 mice (A). Percent of Ly49Q⁺ pDCs (B220⁺CD11b⁺SiglecH⁺) in the spleens of B6 and NZM2410 mice (B). Absolute number of Ly49Q⁺ pDCs in the spleen and BM of B6 and NZM2410 mice (C). Percent of MHCII⁺ pDC in the spleens of B6 and NZM mice (D). Flow cytometry plots represent data from at least 4 separate experiments, which are summarized in the graphs (E & F), B6 (n = 5) and NZM (n = 7). Results are expressed as the mean ± SD. Statistical analysis was performed with Mann-Whitney analysis. P < 0.05 was considered significant.