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Phenotypic and functional alterations of pDCs in lupus-prone mice

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Plasmacytoid dendritic cells (pDCs) were considered to be the major IFN α source in systemic lupus erythematosus (SLE) but their phenotype and function in different disease status have not been well studied. To study the function and phenotype of pDCs in lupus-prone mice we used 7 strains of lupus-prone mice including NZB/W F1, NZB, NZW, NZM2410, B6.NZM^{Sle1/2/3}, MRL/lpr and BXSB/Mp mice and C57BL/6 as control mice. Increased spleen pDC numbers were found in most lupus mice compared to C57BL/6 mice. The IFN α -producing ability of BM pDCs was similar between lupus and C57BL/6 mice, whereas pDCs from the spleens of NZB/W F1 and NZB mice produced more IFN α than pDCs from the spleens of C57BL/6 mice. Furthermore, spleen pDCs from MRL-lpr and NZM2410 mice showed increased responses to *Tlr7* and *Tlr9*, respectively. As the disease progressed, IFN signature were evaluated in both BM and spleen pDC from lupus prone mice and the number of BM pDCs and their ability to produce IFN α gradually decreased in lupus-prone mice. In conclusion, pDC are activated along with disease development and its phenotype and function differ among lupus-prone strains, and these differences may contribute to the development of lupus in these mice.

Systemic lupus erythematosus (SLE) is the most common autoimmune disease in young women. The course of SLE varies greatly among individuals, ranging from mild to rapidly progressive and even fatal disease. Patients with SLE usually present with high interferon α (IFN α) levels in peripheral blood cells¹. Moreover, patients with chronic hepatitis who receive IFN α therapy can develop lupus-like symptoms². Recent studies have found that neutralizing IFN α can decrease the autoantibody titer and relieve clinical symptoms in both human SLE patients and lupus-prone mice^{3–6}. Therefore, the abnormal activation of the IFN α pathway is considered one of the important mediators of SLE pathogenesis^{7,8}.

New Zealand Black (NZB) \times New Zealand White (NZW) F1 (NZB/W F1) mice represent the first strain developed to exhibit symptoms that mimic lupus, including high titers of autoantibodies and severe nephritis⁹. This strain has been widely used in studies of lupus pathogenesis. High IFN levels have also been detected in the NZB/W F1 strain and its sibling strains, such as NZB, New Zealand Mixed (NZM) 2328, NZM2410, B6.NZM^{Sle1/2/3} and B6.Nba2 mice^{10–13}. Similarly, other lupus models, such as BXSB/Mp and pristane-induced lupus models, also include expression of high levels of IFN α ^{14,15}. Although the role of IFN α in MRL-lpr mice is controversial, IFN inducible genes have been upregulated in response to disease progression in MRL-lpr mice¹⁶. The abnormal activation of the type I IFN pathway has been detected in both human SLE patients and lupus-prone mice, although the IFN source remains unclear.

Almost all immune cells can produce IFN α , and plasmacytoid dendritic cells (pDCs) are by far the most important producers of IFN α . pDCs selectively express high levels of *Tlr7* and *Tlr9* when stimulated with viral microbes, bacterial DNA or *Tlr7/9* ligands¹⁷. Human pDCs consist of CD11c⁺ BDCA2⁺ BDCA4⁺ CD123⁺ cells¹⁸, whereas mouse pDCs consist of CD11c^{int} mPDCA1⁺ B220⁺ cells^{19,20}, i.e., mPDCA1, which are also known as BST2 or CD317. mPDCA1 could be recognized by the antibody 120G8. Notably, DNA- or RNA-containing immune

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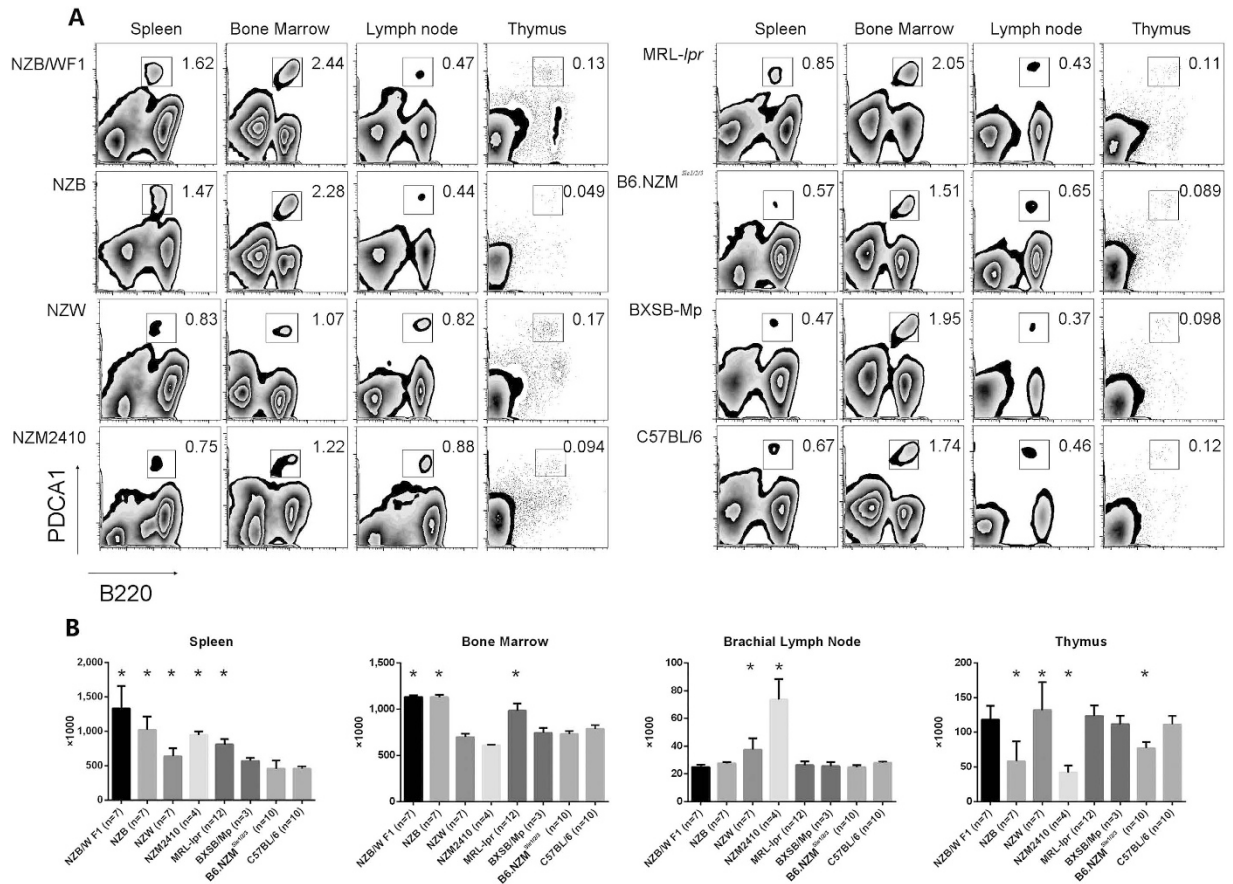


Figure 1. Numbers of pDCs in different lymphoid organs of different strains. Analysis of pDCs from different lymphoid organs in 6-week-old lupus-prone mice (8-week NZB mice). C57BL/6 mice were used as the control strain. LN-pDCs were isolated from 1 brachial LN, and BM pDCs were isolated from 2 tibiae and 2 femurs in each mouse. The total pDC numbers were calculated by multiplying the organ pDC percentage by the total cell numbers. A: pDC percentages in different organs among different strains. B: pDC numbers among different organs. * $p < 0.05$ compared with the C57BL/6 strain.

complexes (ICs) from SLE patients trigger pDCs from healthy donors to produce $\text{IFN}\alpha$ via the TLR9 or TLR7 pathways, which suggests that $\text{IFN}\alpha$ is linked to SLE pathogenesis^{21–23}. Lupus-prone mice, such as NZB, NZW and NZB/W F1 mice, exhibit higher spleen pDC numbers than C57BL/6 mice^{24,25}. Moreover, NZB mice show increased $\text{IFN}\alpha$ secretion following *in vivo* CpG injection²⁶. Hence, we hypothesize that pDCs may represent an important $\text{IFN}\alpha$ source in both SLE patients and lupus-prone mice. The main function of pDCs is the efficient production of $\text{IFN}\alpha$, which has become the focus of intense investigation^{18,27}. Unfortunately, the availability of human pDCs is limited because pDCs account for only 0.1% of human PBMCs. Therefore, here, we studied pDCs derived from lupus-prone mice to illuminate the pathogenesis underlying SLE.

The aim of this study was to analyze the pDC phenotype and its $\text{IFN}\alpha$ -producing ability by following *Tlr7* and *Tlr9* stimulation in different lupus-prone mouse strains. Here, we studied 7 lupus strains and found increased pDC cell counts and function in the NZB/W F1, NZB, NZM2410 and MRL-*lpr* strains. In the advanced lupus stage, the number and function of pDCs changed according to the development of the disease.

Results

NZB/W F1, NZB and MRL-*lpr* mice had larger pDC counts. We first hypothesized that the increased numbers of pDCs were responsible for the high IFN expression in lupus-prone mice. Here, we used only female mice for the experiments, for all strains except BXSB/Mp. The total cell numbers of different organs are given in Supplementary Table 1. The spleen pDC numbers were the highest in NZB/W F1 and NZB mice among all tested strains (Fig. 1A), followed by NZW, NZM2410 and MRL-*lpr*, whereas the numbers in B6.NZM^{Sle1/2/3} and BXSB mice were not significantly different. Moreover, the (bone marrow) BM was also found to contain large numbers of pDCs in mice. Specifically, higher BM pDC levels were found in NZB, NZB/W F1 and MRL-*lpr* mice (Fig. 1B). The numbers of BM pDCs in NZW, NZM2410 and B6.NZM^{Sle1/2/3} mice and male BXSB/Mp mice compared to that in C57BL/6 mice were not significantly different. Both the lymph nodes (LNs) and thymus glands contained fewer pDCs, suggesting that the LNs and thymus may contribute relatively little to the high IFN expression (Fig. 1C,D). However, the increased numbers of pDCs in the NZB/W F1, NZB and MRL-*lpr* strains suggested that

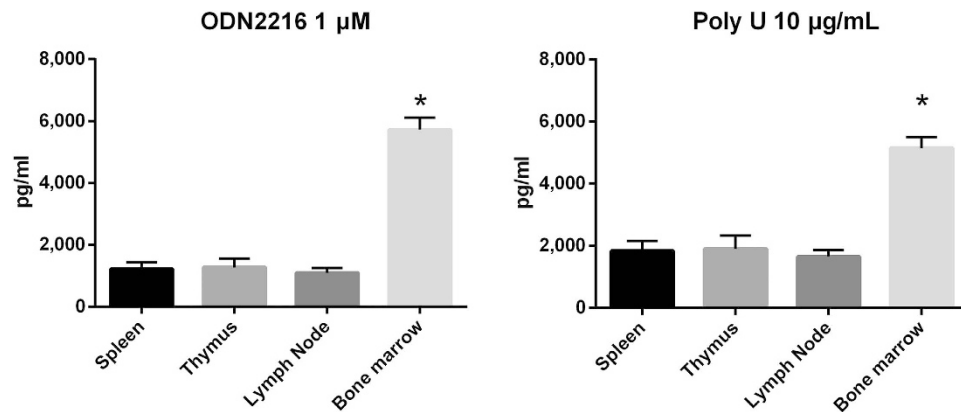


Figure 2. IFN α production by pDCs in different organs of C57BL/6 mice. IFN α produced by pDCs in different organs (BM, spleen, LNs, thymus) from C57BL/6 mice that were treated with Tlr ligands (ODN2216 for *Tlr9*, Poly U for *Tlr7*) for 48 h. Each well contained 50k cells. Cells from the inguinal, axillary, brachial and cervical superficial LNs were pooled together for LN-pDC isolation. *p < 0.05 compared with spleen pDCs

pDCs may contribute to the high IFN expression. The IFN expression in the B6.NZM^{Slc1/2/3} and BXSB/Mp strains was attributed to high pDC function, which prompted further investigation.

BM pDCs produced higher levels of IFN α than pDCs from other lymphoid organs. Previous studies have indicated that the ability of pDCs from diverse lymphoid organs to produce IFN α differed in C57BL/6 mice^{28,29}. Therefore, we first compared the abilities of pDCs from the spleen, BM, LN and thymus of C57BL/6 mice to produce IFN α to assess the necessity of comparing pDC function separately in various lupus-prone mouse strains. The results suggested that BM pDCs could produce far more IFN α upon stimulation with both *Tlr7* and *Tlr9* than spleen pDCs, whereas the IFN α -producing abilities of thymus and LN pDCs were similar to that of spleen pDCs (Fig. 2). Thus, spleen and BM pDCs were selected for subsequent studies.

Strong IFN α producing abilities of spleen pDCs from NZB/W F1, NZB, NZM2410 and MRL-*lpr* mice upon *Tlr7* or *Tlr9* stimulation. The strong IFN α -producing abilities of pDCs from lupus-prone mice are also likely to contribute to the mechanism underlying the high IFN expression level. A comparison of the IFN α levels in response to stimulation with both *Tlr9* and *Tlr7* was necessary because either DNA- or RNA-containing ICs from lupus patients may stimulate pDCs to produce IFN α ^{21,22,30}. Here, we used ODN2216 and poly U as *Tlr9* and *Tlr7* ligands, respectively. Upon ODN2216 stimulation, spleen pDCs from NZB/W F1, NZB and NZM2410 produced higher levels of IFN α than spleen pDCs from C57BL/6 mice. BM pDCs from NZB and B6.NZM^{Slc1/2/3} mice produced higher and lower levels of IFN α , respectively, than BM pDCs from C57BL/6 mice. In contrast, the production of IFN α by BM pDCs from other strains did not significantly differ from that of BM pDCs from C57BL/6 mice. The poly U stimulation results showed that spleen pDCs from NZB/W F1 and NZB mice also produced higher levels of IFN α . However, strikingly, spleen pDCs from MRL-*lpr* mice produced higher IFN α levels than those from control mice. Upon poly U stimulation, the BM pDCs from the various lupus strains did not differ (Fig. 3). Together, these results suggest that the overactivity of pDCs in some lupus strains may result in high IFN expression.

pDCs from NZB and NZB/W F1 mice had higher survival ratios in *in vitro* stimulation. Zhan *et al.* have found that lower pDC death rates are linked to high levels of IFN α production *in vitro*²⁵. To identify the reasons underlying the high IFN α production by BM pDCs and the strain differences in the pDC IFN α -producing ability, we analyzed the pDC survival rates in response to *Tlr7* or *Tlr9* stimulation *in vitro*. Previous studies have found that all pDCs died within 48h *in vitro*. Therefore, we collected the cells after 24 h and calculated the number of surviving pDCs. All BM pDCs showed higher survival rates than spleen pDCs after 24 h *in vitro*. Among the various strains, both spleen and BM pDCs from NZB and NZB/W F1 had the highest surviving cell proportions, regardless of the stimulator. Although pDCs from MRL-*lpr* mice produced higher levels of IFN α in response to *Tlr7* stimulation, their pDC survival rates were the lowest (Fig. 4), which suggested that in addition to increased pDC survival, other mechanisms may affect the IFN α -producing ability.

BM pDCs exhausted in advanced lupus stage. Studies of the phenotype and function of pDCs in the advanced stages of lupus have been limited³¹. Therefore, we sought to determine how pDCs behave in the advanced stage of lupus in mouse models. We divided all lupus-prone mice into 3 different groups as follows: 6-week-old (or 8-week-old for NZB, owing to its physical retardation) mice were regarded as the pre-lupus stage, at which point neither proteinuria nor autoantibody is detectable. Here, we defined ANA titers “1:100 < ANA < 1:320” and proteinuria “+ ≤ protein ≤ 2+” as cut-off values for the early lupus stage, whereas ANA ≥ 1:320 or urine protein ≥ 3+ were used to define the advanced stage. To date, few studies have focused on the change in the total cell numbers in the BM of lupus mice. Our data showed that the total BM cell numbers were slightly

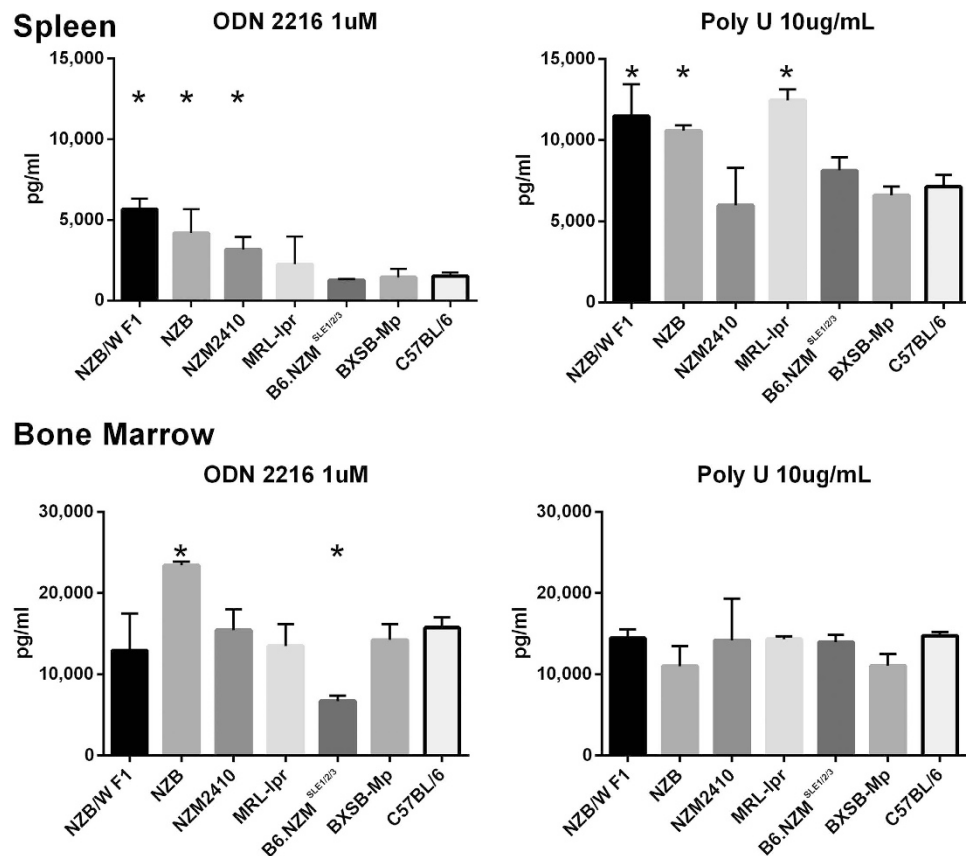


Figure 3. IFN α produced by spleen and BM pDCs from lupus-prone mice. Spleen and BM pDCs from different strains were stimulated for 48 h with TLR ligands. Each well contained 100 k cells. The figure shows the results of one of three independent experiments. * $p < 0.05$ compared with C57BL/6 mice.

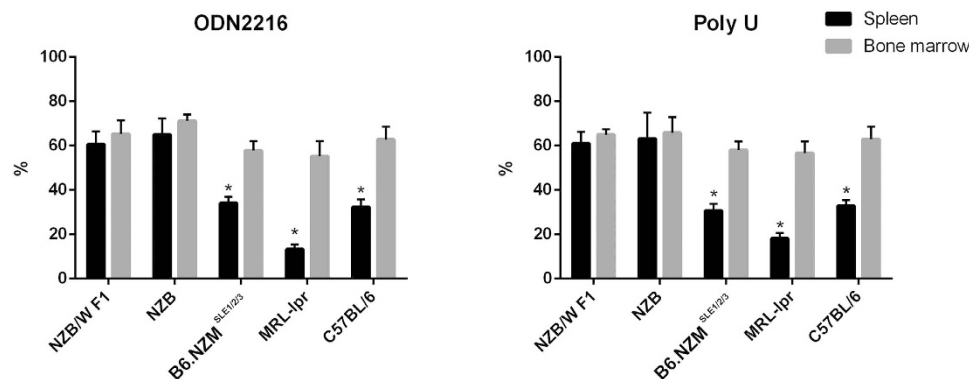


Figure 4. pDC survival rate after 24 h of stimulation with *Tlr7* or *Tlr9* *in vitro*. Spleen and BM pDCs from six-week-old mice were isolated and stimulated according to the protocols mentioned above. Previous studies already demonstrated that almost all pDCs die within 48h. Therefore, we compared the survival rates at 24 h. The pDC survival rate at 24 h in 5 tested strains. BM pDCs exhibited higher survival rates in B6.NZM^{Sle1/2/3}, MRL/*lpr* and C57BL/6 strains in response to both ODN2216 and poly U stimulation. The survival rates of BM and spleen pDCs did not significantly differ in NZB and NZB/W F1 mice.

increased in NZB/W F1 mice, but this increase was not observed in MRL-*lpr* and B6.NZM^{Sle1/2/3} mice (Table 1). The BM pDC numbers significantly decreased as the disease progressed in all 3 tested strains (see Table 2). Elderly C57BL/6 mice did not exhibit changes in pDC numbers in either the spleen or the BM.

Disease-induced pDC migration may decrease the number of pDCs in the BM. We also measured the number of pDCs in the peripheral organs, including the spleen, LN and kidney. NZB/W F1 mice exhibited slightly enlarged spleens, results corroborating the findings of a previous study³². In addition, MRL-*lpr* mice exhibited marked spleen enlargement, and the total number of spleen cells was up to 20-fold higher in the advanced stage

Strains	Spleen (Mean \pm SD) $\times 10^6$			BM (Mean \pm SD) $\times 10^6$		
	Pre-lupus	Early lupus	Advanced lupus	Pre-lupus	Early lupus	Advanced lupus
NZB/W F1	109.9 \pm 8.1	123.8 \pm 6.9*	155.8 \pm 24.8*	42.1 \pm 2.1	48.3 \pm 3.5*	46.8 \pm 3.6*
MRL- <i>lpr</i>	106.7 \pm 1.5	345.0 \pm 22.6*	2175.0 \pm 473.8*	43.3 \pm 4.0	45.5 \pm 5.2	47.6 \pm 7.2
B6.NZM ^{<i>Sle1/2/3</i>}	109.2 \pm 4.0	312.7 \pm 72.7*	763.6 \pm 141.9*	43.0 \pm 6.3	44.7 \pm 7.1	39.5 \pm 6.8
C57BL/6	93.4 \pm 5.1	95.8 \pm 6.2	101.9 \pm 6.4	47.3 \pm 6.1	47.9 \pm 5.5	46.4 \pm 7.1

Table 1. Total cell numbers in spleen and BM (2 femurs and tibias) in lupus-prone mice at different disease stages. Age-matched C57BL/6 were used as a control. *P < 0.05 compared with the pre-lupus stage.

Strains	Spleen pDC (Mean \pm SD) $\times 10^6$			BM pDC (Mean \pm SD) $\times 10^6$		
	Pre-lupus	Early lupus	Advanced lupus	Pre-lupus	Early lupus	Advanced lupus
NZB/W F1	1.502 \pm 0.45	1.158 \pm 0.75*	1.498 \pm 0.28	1.07 \pm 0.12	0.476 \pm 0.05*	0.432 \pm 0.10*
MRL- <i>lpr</i>	0.841 \pm 0.11	1.051 \pm 0.75	3.238 \pm 1.46*	0.551 \pm 0.07	0.349 \pm 0.09*	0.227 \pm 0.05*
B6.NZM ^{<i>Sle1/2/3</i>}	0.460 \pm 0.12	0.720 \pm 0.14	1.294 \pm 0.40*	0.571 \pm 0.10	0.482 \pm 0.13*	0.391 \pm 0.05*
C57BL/6	0.455 \pm 0.03	0.452 \pm 0.04	0.427 \pm 0.05	0.773 \pm 0.08	0.662 \pm 0.04	0.653 \pm 0.17

Table 2. The change in the pDC numbers in the spleen and BM as a function of disease stage. Age-matched C57BL/6 were used as a control. *P < 0.05 compared with the pre-lupus stage.

lupus mice than in pre-lupus mice. Furthermore, the total spleen cell population of B6.NZM^{*Sle1/2/3*} mice was up to 4–6 times larger in the advanced stage than in the pre-lupus stage (see Table 1). The absolute spleen pDC numbers were higher in MRL-*lpr* and B6.NZM^{*Sle1/2/3*} mice than in NZB/W F1 mice (Table 2).

Moreover, lymphomegaly is common in lupus mouse models, and all tested strains exhibited various degrees of LN enlargement. Specifically, the LN in MRL-*lpr* strains was about 10 times larger in mice with advanced disease than in pre-lupus mice. However, the number of LN pDCs showed limited increases in the NZB/WF1 and B6.NZM^{*Sle1/2/3*} strains (Supplementary Table 2). pDC infiltration in organs has also been reported to decrease the number of BM pDCs. The kidney is one of the most common organs affected by lupus. Fiore *et al.* have found BDCA4-positive cells in type III and type IV lupus nephritis renal tissue³³, but related studies in mouse models have not been reported. The total renal pDC numbers were calculated on the basis of a FACS analysis. However, pDC infiltration in the kidney was not detectable in the pre-lupus stage, whereas the renal pDC numbers were slightly increased in response to disease development in all tested strains. Although the total renal pDC numbers were very low and almost undetectable, the total number of renal B220+ cells was significantly increased (Supplementary Fig. 3), which implied that pDCs expressing B220+ did not significantly infiltrate the kidneys of lupus-prone mice. At the advanced lupus stage, the number of renal pDCs greatly declined, which may have been the result of tissue fibrosis (Supplementary Table 2 & Supplementary 3).

Based on these results, the increased total pDC numbers in peripheral organs were not sufficient to compensate for the decreased pDC numbers in the BM. In fact, the lower number of BM pDCs may be caused by disease progression instead of pDC migration.

Decreased IFN α -producing ability of BM pDCs upon ODN2216 simulation in advanced-stage lupus. The effect of disease status on the IFN α -producing ability has not yet been studied. Therefore, we collected spleen and BM pDCs from NZB/W F1, B6.NZM^{*Sle1/2/3*} and MRL-*lpr* mice in different disease stages and then challenged them with ODN2216. BM-pDCs from NZB/W F1 mice lost their responses to stimulation in the pre- and advanced lupus stages. Similarly, spleen pDCs from F1 mice in the advanced lupus stages also revealed decreased responses (Fig. 5). The loss of IFN α production was also observed in BM pDCs from MRL-*lpr* and B6.NZM^{*Sle1/2/3*} mice but not in their spleen pDCs. The pDC function of age-matched C57BL/6 mice remained unchanged.

pDC was activated in lupus-prone mice in the advanced lupus stage. The upregulation of both MHC-II and CD80 in pDCs is a hallmark of pDC activation. The levels of both MHC-II and CD80 in spleen and BM pDCs in all 3 tested strains were significantly upregulated in the advanced lupus stage compared with the pre-lupus stage, whereas this difference was not observed in control C57BL/6 mice (Fig. 6). The IFN inducible genes is considered to be another marker of pDC activation. To date, more than 30 genes have been reported to be induced by type I interferon. Because only limited numbers of pDCs can be obtained from individual mice, we analyzed the pDC activation status of 3 genes: MX1, IFIT2 and CXCL10, we also added pan-IFN α genes to further test the activation status. In all 3 tested strains, both IFN α genes and IFN inducible genes expression levels were elevated in both the spleen and BM pDCs in the advanced lupus stage but not in elderly C57BL/6 controls (Fig. 7).

Taken together, these data indicate that pDCs are activated at the advanced lupus stage in lupus mouse models.

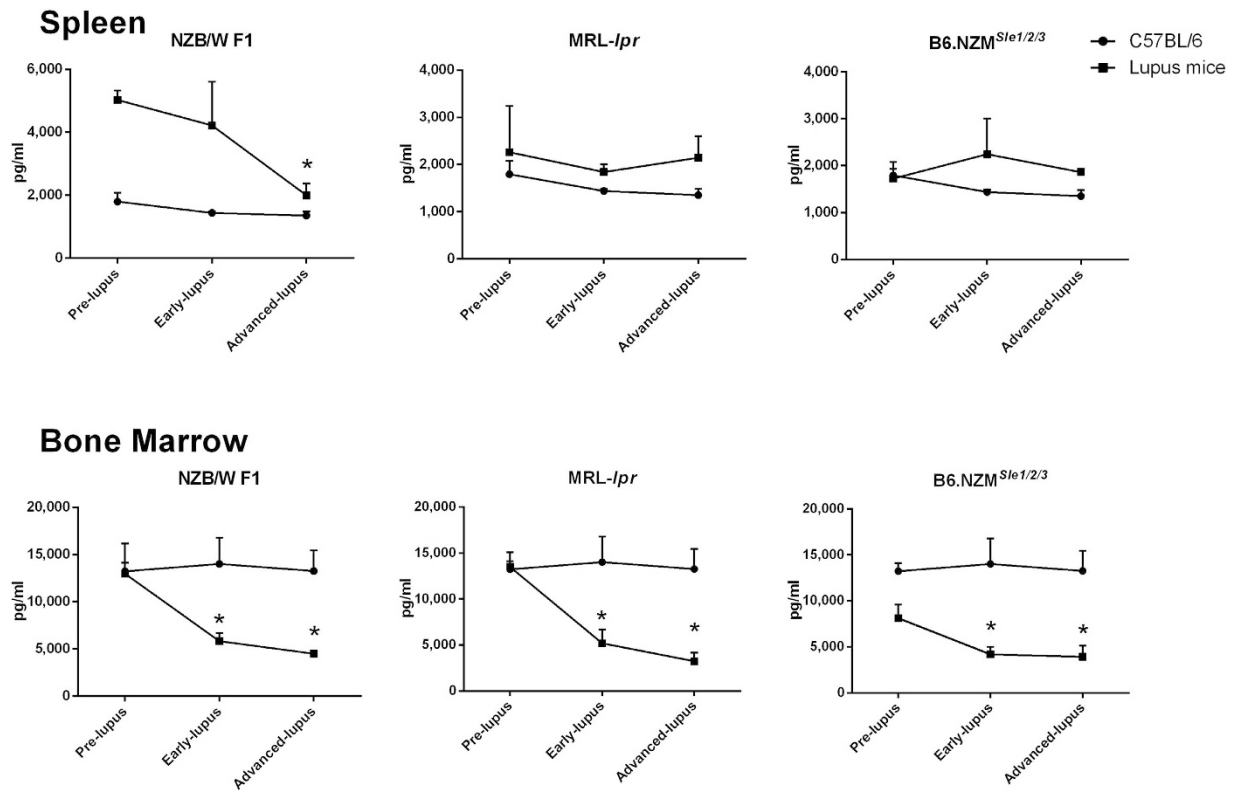


Figure 5. IFN α -producing abilities of pDCs from different disease stages. Only spleen pDCs from NZB/W F1 mice exhibited decreases in IFN α production upon ODN2216 stimulation in the advanced lupus stage. Decreases in the IFN α -producing abilities of BM pDCs were observed in all 3 tested strains during the advanced lupus stage. * $p < 0.05$ compared with the pre-lupus stage

Discussion

The underlying pathogenesis of SLE is elusive and complex because of a wide range of potential disease mechanisms among individuals. To mimic these different mechanisms, we used 7 different lupus-prone mouse strains^{34,35}. NZB/W F1 mice, the first model lupus-prone strain, presented with a high titer of autoantibodies and severe nephritis. NZB/W F1 mice were generated by mating female NZB mice and male NZW mice. Both NZB and NZW mice develop nephritis and autoantibodies at the later stages of the disease, but unlike NZB/W F1 mice, lupus symptoms in these 2 strains are often mild. The life span of NZW mice is similar to that of C57BL/6 mice, whereas NZB mice have a shorter life span. In NZB mice, B cells are unusually mature, hyperactivated and resistant to apoptosis. CD4⁺ T cells also contribute to the disease of NZB mice because of the types of MHC-II molecules expressed by these mice. Naturally, hemolytic anemia instead of lupus is a major cause of death in NZB mice²⁶. NZB/W F1 is the only heterozygous strain that inherits disease-related gene loci from both NZW and NZB mice and develops severe lupus symptoms. A previous study has observed polyclonal B cell activation with the help of both α/β T and γ/δ T cells in NZB/W F1 mice. The functional impairment of regulatory cells, including CD4⁺CD25⁺, CD8⁺, NK T, and B-1 B cells, has been found in NZB/W F1 mice²⁶. The NZM2410 strain, which was generated by repeated backcross mating of NZB/W F1 mice to NZW mice, was discovered to have *Sle1*, *Sle2* and *Sle3* lupus-related gene loci. These 3 disease related loci also exist in NZW mice. Each of these three gene loci was able to induce autoimmunity in C56BL/7 mice and B6.NZM^{Sle1/2/3} mice that bear all three loci; these mice exhibited hyperproliferative and hyperactive T and B cells, which caused severe nephritis and high titers of autoantibodies³⁶. Although NZM2410 and B6.NZM^{Sle1/2/3} mice have the same disease-related gene loci, they present with different clinical symptoms. Previous studies have found that NZM2410 mice develop glomerulosclerosis at the early stage of nephritis, whereas other mice develop diffuse proliferative nephritis²⁶. In our work, B6.NZM^{Sle1/2/3} mice also presented with chondritis, as evidenced by ear collapse, conjunctivitis and dermatitis, which might be the result of different immune microenvironments. MRL/Mp mice, which were generated by backcrossing 4 different strains, including LG, AKR, C3H/Di and C57BL/6, exhibited dermatitis, lower titers of autoantibodies and mild nephritis in later life²⁶. MRL/Mp mice with the *lpr* mutation developed Fas deficiency, resulting in higher titers of autoantibodies and severe nephritis. Autoreactive T and B cells that fail to undergo apoptosis are considered to be characteristic of pathogenesis in the MRL-*lpr* strain²⁶. The BXSb/Mp strain, which was generated by backcrossing SB/Le mice and C57BL/6 mice³⁷, carries the *Yaa* mutation originating from the SB/Le strain. This mutation results in the insertion of 17 genes from the X chromosome into the Y chromosome. Among the 17 genes in the *Yaa* mutation, Tlr7 is considered to be the major cause of lupus^{38,39}. Because gene imprinting does not silence the Y chromosome, all male mice carrying this mutation express high levels of Tlr7 in B cells and pDCs³⁸. Intriguingly, C57BL/6 mice with only the *Yaa* mutation do not develop clinical lupus, but

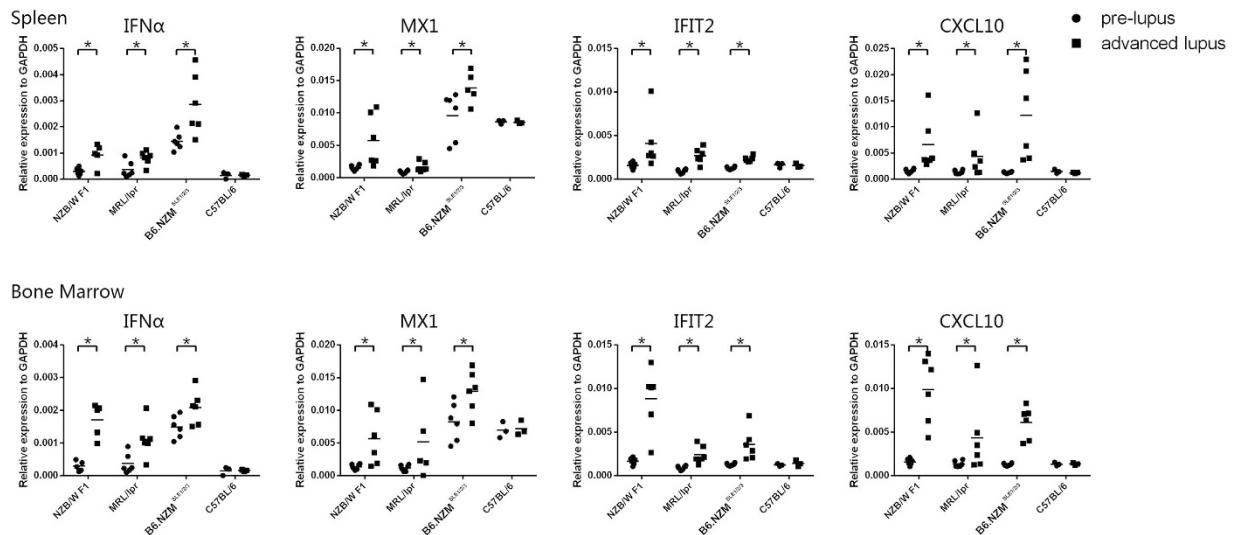


Figure 7. IFN α - and IFN-inducible gene expression levels by disease stage. Here, we used 3 different lupus strains—NZB/W F1, MRL-*lpr* and B6.NZM^{Sle1/2/3}—to test the IFN α gene expression and IFN-inducible gene expression. The pre-lupus and advanced lupus stages are described above. Six-week- and 6-month-old C57BL/6 mice served as controls. In the 3 tested lupus strains, both the spleen and BM IFN α expression levels were elevated. Three IFN-inducible genes, MX1, IFIT2 and CXCL10, were also universally upregulated in both the spleen and BM pDCs in all 3 tested strains. Elderly C57BL/6 mice did not show increases in IFN α expression and IFN-inducible gene expression in the spleen or BM pDCs.

Despite of the large variation in pDC numbers and function among different mouse strains, all lupus-prone mice could develop disease. In our study, NZB mice with mild lupus symptom showed the highest pDC number and strong IFN α producing ability, while B6.NZM^{Sle1/2/3} and BXSB/Mp mice with much more severe lupus symptoms than NZB mice showed similar pDC function and phenotype to C57BL/6 mice. These findings indicate that increased pDC number and enhanced cytokine production by pDC are not directly linked to the development and/or severity of the lupus in mouse models. Since pDC itself is one of the strongest IFN α -producing cell in mouse, thus the activation of pDCs and their cytokine production are expected to influence the disease progression. Indeed, increased pDC number and/or enhanced pDC function in some lupus models would enhance the toxic effect of IFN α like NZB/W F1 mice.

In this study, spleen pDCs from NZM2410 and MRL-*lpr* mice showed inconsistent selective responses to *Tlr9* and *Tlr7* stimulation, but the detailed mechanisms underlying this response remain unclear. On the basis of previous reports, we speculate that this phenomenon may be related to differences in SLE pathogenesis in these models. NZM2410 mice do not generate anti-RNP antibodies²⁶, which suggests that this strain cannot produce RNA-containing ICs. Thus it is likely that pDC activation in the NZM2410 strain *in vivo* relies on sensor DNA containing IC through *Tlr9* pathway, but not the *Tlr7* pathway, which requires the presence of RNA-containing ICs. Conversely, MRL-*lpr* mice produced both anti-dsDNA antibodies and anti-RNP antibodies, suggesting that this strain has both DNA- and RNA-containing ICs. Previous studies have found that *Tlr9* knockout MRL-*lpr* mice do not exhibit ameliorated clinical symptoms of disease, whereas *Tlr7*-deficient MRL-*lpr* mice exhibit decreased IFN signature and the remission of clinical symptoms^{45–49}. This finding implies that pDC activation in the MRL-*lpr* strain may depend on selective *Tlr7* pathways that more actively interact with RNA-containing ICs to release IFN α . The reasons underlying this phenomenon remain unknown, but the genetic background may contribute to this effect. Nevertheless, this inference requires confirmation, and further studies are required to determine whether this phenomenon also exists in human patients.

Additionally, the number of BM pDCs, which produce much higher levels of IFN α than spleen pDCs, was significantly decreased in the advanced stage of lupus. This depletion may result from immune regulation at the advanced lupus stage, at which point a wide range of immune cells are activated, thus resulting in release of large amounts of cytokines, especially type I interferon. Because the immune environment may substantially change throughout disease progression, pDC proliferation might be suppressed by negative immune-regulation mechanisms, which suggests that BM pDC depletion results from a comprehensive immunoreaction negative feedback loop. Moreover, previous studies have demonstrated that activated pDCs do not respond to a second stimulus¹⁷, our data found pDC activation in lupus-prone mice in advanced disease stage, therefore pDCs isolated from mice in advanced lupus stage have reduced IFN α producing ability upon stimulation *in vitro*.

One of the important functions of pDC is to present antigen and elicit subsequent T cell activation and this function maybe enhanced in lupus-prone mice. OT-I and OT-II mice are widely used tools to investigate the antigen presenting function of antigen presenting cells (APCs) to prime CD8+ T cell and CD4+ T cell respectively. Of note, these mice were generated in C57BL/6 background and present H-2b haplotype. The lupus-prone mice used in current study have different genetic background and H-1 haplotypes as follows: NZB/W F1 (H-2^{d/z}), NZB (H-2^{d/d}), NZW (H-2^{z/z}), NZM2410 (unknown, possibly H-2^{z/z}), MRL-*lpr* (H-2^{k/k}), B6.NZM^{Sle1/2/3} (H-2^{b/b})

and BXSb/Mp (H-2^{b/b}). To test the antigen-specific T cell activation, APCs are required to present the same H-2 molecules as T cells. As mentioned above, abnormal T cells were also found in most lupus-prone mice. Therefore current methods and technologies cannot give the answer that APC function is enhanced in lupus-prone mice.

In summary, our data indicate that pDCs are activated in lupus-prone mice. We also found abnormal pDC phenotypes and function in some lupus-prone mice, which may increase the IFN expression in advanced disease. Our findings provide information that may guide future studies of SLE patients, but they will require validation in humans.

Materials and Methods

Ethics approval. All the animal experiments in this study were approved by the Ethic Committee of Renji Hospital, School of Medicine, Shanghai Jiao Tong University, and were performed in accordance with the guidelines for animal experimentation of Shanghai Jiao Tong University.

Mice. NZB, NZW, NZM2410, MRL-*lpr*, MRL/Mp, B6.NZM^{Sle1/2/3}, C57BL/6, BXSb/Mp, and BXSb.B6-*Yaa* mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were bred at the animal facility of Renji Hospital, School of Medicine, Shanghai Jiao Tong University. The NZB/W F1 strain was generated by breeding male NZW and female NZB mice. We used the commonly used C57BL/6 mice as a reference strain, and all statistical analyses were based on comparisons with C57BL/6 mice in this study unless otherwise indicated.

Mouse pDC isolation and phenotypic analysis. pDCs were isolated from the spleen, LNs, thymus and BM of individual mice as described elsewhere in detail⁵⁰. Briefly, the organs were minced and digested with DNase/collagenase solution. The BM cells were flushed from 2 femurs and 2 tibias per mouse with PBS. To isolate the pDCs, the total cells obtained from the spleen, LNs, thymus and BM were resuspended in Nycodenz (Nycomed Pharma AS, Oslo, Norway) medium (1.077, 1.082, 1.076 and 1.080 g/mL, respectively) and centrifuged at 1700 × g for 10 min. Cells in the light density fraction were then harvested and labeled with CD11c, mPDCA-1, B220 and aqua for sorting. The final pDC purity exceeded 95% (Supplementary Fig. 1). PerCP.Cy5.5-, PE- and APC-labeled isotypes were introduced as negative controls. To analyze the pDC numbers and phenotypes, 1×10^7 cells from organs were used and labeled with CD11c, mPDCA-1, B220, CD80 and MHC-II. The pDC gating strategies are provided in Supplementary 3A. The pDC numbers were calculated by multiplying the total organ cell number by the percentage of CD11c^{int}mPDCA-1⁺B220⁺ cells. The mean fluorescence intensities (MFIs) of MHC-II and CD80 of pDC were calculated by FlowJo software (Treestar Inc., Ashland, OR, USA). The living cell rates were detected by using Annexin V and PI staining. 100K of pDC were stimulated with ODN2216 or Poly U for 24 h and then performed Annexin V and PI staining follow the protocol from the manufacture (BD Pharmagen, San Jose, CA, USA). The gating strategy could be found in supplementary fig. 2B. Anti-mouse CD11c, mPDCA-1, B220, CD80, MHC-II and isotypes were purchased from ebioscience (San Diego, CA, USA). Aqua was purchased from ThermoFisher Scientific (Waltham, MA, USA).

In vitro Tlr9 and Tlr7 stimulations. Isolated spleen or BM pDCs (100k) were resuspended in 100 μL of complete 1640 medium and then stimulated in a 96-well U-bottom plate with 1-μM ODN2216 or 10-μg/mL poly U (Invivogen, San Diego, CA, USA) supplemented with Lipofectamine 2000 (ThermoFisher Scientific) (1.5 μL: 1000 μL) for 48 h. The supernatants were collected and stored at -80 °C for enzyme-linked immunosorbent assay (ELISA). Each group consisted of at least 3 mice, and each experiment was repeated at least 3 times.

ELISA. A mouse IFN α ELISA (eBioscience) was performed according to the manufacturer's protocol. We used a QUNTA Lite (Inova Diagnostic, San Diego, CA, USA) kit by switching the 2nd antibody to FITC-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Dallas, Texas, USA).

Quantitative polymerase chain reaction (qPCR) analysis. The pDCs were lysed in TRIzol (ThermoFisher Scientific) and then maintained at -80 °C for RNA extraction. The total RNA was extracted following the manufacturer's protocol. cDNA was synthesized by using TaqMan Reverse Transcription Reagents (ThermoFisher Scientific), and 1:100-diluted cDNA was used to perform qPCR using SYBR[®] Premix Ex Taq (TAKARA BIO INC., Shiga, Japan). The primer information is provided in the supplementary material. Relative expression levels were calculated by using the delta-delta CT method. A P value < 0.05 was considered to indicate a significant difference.

Statistics. The data were analyzed with Student's t test by using GRAPHPAD PRISM V6 (Graphpad Software, San Diego, CA, USA).

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Author Contributions

Z.Z, X.F. and N.S. conceived and designed the experiments. Z.Z., X.H., R.Q., C.X., Y.Z., Y.W. and J.M. performed the experiments. Z.Z. analyzed the data and prepared all the figures and tables. Z.Z., X.F., L.W. and N.S. wrote the main manuscript text. All authors reviewed the manuscript.

Additional Information

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