

# Treatment of Systemic Lupus Erythematosus by Nutrition and Dendritic Cell Targeting

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## Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease involving the inflammatory damages of multiple organs. Lupus nephritis (LN) as the manifestation in the kidney occurs in more than 50% of SLE patients and is a major cause of morbidity and mortality. Current treatments consist of immunosuppressants that always lead to compromised immune responses with increased risks of infections as the major side effect. To minimize this side effect, it is crucial to develop new treatments that are more natural and specific.

Vitamin A, particularly in the form of its functional metabolite, retinoic acid, has shown some beneficial effects against LN in both lupus-prone mouse models and clinical cases. However, a more systemic evaluation of vitamin A treatment in lupus had not been investigated. In our study, we found paradoxical effects of all-*trans*-retinoic acid (tRA) on lupus-like disease in MRL/lpr lupus-prone mice. Starting at 6 weeks old when the inflammatory environment had been established in MRL/lpr mice, tRA administration reduced immune cell numbers in the secondary lymphoid organs and improved glomerulonephritis. However, circulating autoantibodies and inflammation in renal tubulointerstitium and other organs were increased. The detrimental effects of tRA were not present in MRL control mice, which didn't have an established inflammatory environment at 6 weeks old as shown in MRL/lpr mice, suggesting that the pro-inflammatory effects of tRA are dependent on the pre-existing inflammatory environment. Therefore, to successfully apply vitamin A-based treatment, it is important to avoid

the detrimental effects of tRA on lupus by identifying and then specifically eliminating the critical pro-inflammatory immune cell types in lupus. As treatments usually start after the onset of apparent symptoms in patients at the effector stage of autoimmune responses, targeting the inflammatory contributors at this stage appears to be more practical and critical.

Among different types of leukocytes, we chose to focus on dendritic cells (DCs), because they are highly diverse and critical in the immune responses as a bridge between the innate and adaptive immune systems. Plasmacytoid DCs (pDCs) as a candidate target have been demonstrated to be crucial for the initiation of lupus development by producing IFN $\alpha$ . However, we demonstrated that although pDCs produced a large amount of IFN $\alpha$  during disease initiation, those from late-stage lupus mice were found to be defective in producing IFN $\alpha$ , suggesting that pDC-targeted treatments should be performed at the initiation stage. This will depend on the progress in early diagnosis in the future. Besides pDCs, we identified a CD11c<sup>+</sup> cell population absent at the early-stage but gradually accumulating at the late-stage in the kidneys of lupus mice. These cells have a phenotype of mature monocyte-derived DCs, with particularly high CX3CR1 expression on the surface. Consistent with their pathogenic cytokine profile, *in vivo* administration of anti-CX3CR1-saporin conjugates to dysfunction these cells in MRL/lpr mice significantly reduced proteinuria scores. *Ex vivo* activation of renal-infiltrating CD4<sup>+</sup> T cells showed increased survival rate, proliferation and IFN- $\gamma$  production of activated CD4<sup>+</sup> T cells when they were cultured with these renal-infiltrating CD11c<sup>+</sup> cells. These results suggest that the renal-infiltrating CD11c<sup>+</sup> cells are pathogenic and promote inflammation in the kidney at the later effector stage of lupus by interacting with renal-infiltrating CD4<sup>+</sup> T cells.

In conclusion, although vitamin A showed anti-inflammatory effects on reducing glomerulonephritis, its use in lupus treatment should be guarded due to the other potential pro-inflammatory effects induced by the pre-existing inflammatory environment. IFN $\alpha$ -producing pDCs and CX3CR1<sup>high</sup>CD11c<sup>+</sup> monocyte-derived DCs could be specific therapeutic targets to reduce the established inflammation at the early stage and late stage of LN, respectively. Therefore, it is worthwhile to further investigate the comprehensive effects of combination therapy on lupus, with vitamin A administration and pDCs-specific depletion at the early stage, and CX3CR1<sup>high</sup>CD11c<sup>+</sup> monocyte-derived DCs-specific depletion at the late stage.

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## General Audience Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease involving the inflammatory damages of multiple organs. Lupus nephritis (LN) as the manifestation in the kidney occurs in more than 50% of SLE patients and is a major cause of morbidity and mortality in this disease. Current treatments consist of immunosuppressants that always lead to compromised immune responses with increased risks of infections as the major side effect. To minimize this side effect, it is crucial to develop new treatments that are more natural and specific. My first project was to determine whether vitamin A as a supplement could ameliorate SLE. It turned out to be effective at attenuating LN, but at the same time the nutrient caused massive inflammation in other peripheral organs such as the brain and lungs. This suggests that we need to be cautious when recommending vitamin A supplementation to lupus patients. In order to identify more specific targets in the treatment of SLE, my second and third projects focused on dendritic cells (DCs) that are essential for lupus pathogenesis. I found that plasmacytoid DCs (pDCs), known to be pathogenic in SLE, were in fact defective at promoting inflammation at the late stage of disease, suggesting that pDCs might not be a good target of intervention. In contrast, monocyte-derived conventional DCs turned out to be highly pathogenic especially for the development of LN and could be a potential therapeutic target. Altogether, my investigations have increased our understanding of the pathogenesis of SLE.

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## Table of Contents

<b>Acknowledgements .....</b>	<b>vi</b>
<b>Chapter 1. Introduction.....</b>	<b>1</b>
References.....	8
<b>Chapter 2. Breakdown of Immune Tolerance in Systemic Lupus Erythematosus by Dendritic Cells (Literature Review).....</b>	<b>13</b>
Abstract.....	14
Introduction .....	15
<i>In vivo</i> DC depletion studies: indication of DC involvement in lupus.....	17
<b>Breakdown of immune tolerance to self in SLE by cDC.....</b>	<b>18</b>
Changes of cDC number and phenotype in lupus.....	18
MoDC in lupus.....	20
Regulation of cDC activation in lupus.....	21
Activation of T cells and B cells by cDC in lupus.....	23
Potential treatment strategies of lupus by targeting cDC .....	25
<b>Breakdown of immune tolerance to self in SLE by pDC .....</b>	<b>25</b>
Changes of pDC number and phenotype in lupus.....	25
Critical roles of IFN $\alpha$ in lupus development.....	27
Regulation of IFN $\alpha$ production from pDC in lupus.....	29
IFN $\alpha$ -producing ability of pDC in lupus .....	34
Possible IFN $\alpha$ production from cells other than pDC in lupus .....	35
Potential treatment strategies of lupus by targeting pDC and IFN $\alpha$ .....	36
<b>Open questions .....</b>	<b>37</b>
<b>Summary .....</b>	<b>39</b>
References.....	40
<b>Chapter 3. Chemokines and Chemokine Receptors in the Development of Lupus Nephritis (Literature Review).....</b>	<b>53</b>
Abstract.....	54
Introduction .....	55
<b>Key chemokines and chemokine receptors in LN.....</b>	<b>57</b>
CXCL13 and CXCR5.....	58
CXCL12 and CXCR4.....	60
CXCL9/10/11 and CXCR3.....	63
CCR1, CCR5 and CCL3/4/5 .....	66
CCL2 and CCR2 .....	69
CX3CL1 and CX3CR1 .....	71
Other T helper cell-associated chemokine receptors/chemokines.....	72
<b>Mechanisms of chemokine induction in lupus nephritic kidney .....</b>	<b>74</b>
<b>Chemokines as biomarkers for LN.....</b>	<b>77</b>
<b>Potential role of chemokine-based drugs to treat LN .....</b>	<b>79</b>
<b>Summary .....</b>	<b>82</b>
<b>TABLES.....</b>	<b>83</b>
<b>Disclosure .....</b>	<b>86</b>
References.....	87
<b>Chapter 4. Paradoxical effects of all-<i>trans</i>-retinoic acid on lupus-like disease in the MRL/lpr mouse model.....</b>	<b>97</b>

<b>Abstract.....</b>	<b>98</b>
<b>Introduction .....</b>	<b>99</b>
<b>Materials and Methods.....</b>	<b>101</b>
<b>Results .....</b>	<b>107</b>
Identifying an appropriate age of female MRL/lpr mice for intervention .....	107
Inflammation of the skin, brain and lung with tRA.....	108
Differential effects of tRA on glomeruli and tubulointerstitium of the kidney.....	109
Reduced lymphadenopathy with tRA .....	110
Increased circulating autoantibodies with tRA and VARA.....	111
Minimal induction of inflammation with tRA in the absence of an immunogenic environment.....	112
<b>Discussion .....</b>	<b>114</b>
<b>Acknowledgments.....</b>	<b>119</b>
<b>FIGURES .....</b>	<b>120</b>
<b>TABLES.....</b>	<b>133</b>
<b>References.....</b>	<b>134</b>
<b>Chapter 5. <i>Cutting Edge</i>: Plasmacytoid Dendritic Cells in Late-Stage Lupus Mice Defective in Producing IFN<math>\alpha</math>.....</b>	<b>139</b>
<b>ABSTRACT .....</b>	<b>140</b>
<b>INTRODUCTION .....</b>	<b>141</b>
<b>Materials and Methods.....</b>	<b>143</b>
<b>Results and Discussion.....</b>	<b>144</b>
IFN signatures in lupus pDCs.....	144
PDCs from late-disease lupus mice unable to produce IFN $\alpha$ upon CpG stimulation.....	145
Defect of IFN $\alpha$ production in pDCs independent of the transcriptional repressor Foxj2 .....	146
<b>FIGURES .....</b>	<b>149</b>
<b>References.....</b>	<b>155</b>
<b>Chapter 6. Renal-infiltrating CD11c<sup>+</sup> cells are pathogenic in murine lupus nephritis through promoting CD4<sup>+</sup> T cell responses.....</b>	<b>157</b>
<b>SUMMARY.....</b>	<b>158</b>
<b>Introduction .....</b>	<b>159</b>
<b>Materials and methods .....</b>	<b>161</b>
<b>Results .....</b>	<b>169</b>
Renal accumulation of CD11c <sup>+</sup> myeloid cells as LN progresses.....	169
Renal-infiltrating CD11c <sup>+</sup> cells exhibiting a mature monocyte-derived dendritic cell phenotype...170	
High expression of CX <sub>3</sub> CR1 on kidney-infiltrating CD11c <sup>+</sup> cells that is dispensable for renal homing .....	171
The pathogenic role of renal-infiltrating CD11c <sup>+</sup> cells in vivo .....	172
Promotion of renal-infiltrating CD4 <sup>+</sup> T cell response by syngeneic renal-infiltrating CD11c <sup>+</sup> cells .....	174
<b>Discussion .....</b>	<b>176</b>
<b>Acknowledgments.....</b>	<b>182</b>
<b>Conflict of interest .....</b>	<b>182</b>
<b>FIGURES .....</b>	<b>183</b>
<b>TABLES.....</b>	<b>194</b>
<b>References.....</b>	<b>196</b>
<b>Chapter 7. Discussion.....</b>	<b>200</b>



## Chapter 1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease with persistent inflammation that damages multiple organs including kidney, skin, lung, heart, joints and brain [1]. A majority of patients are women of childbearing age [1, 2]. SLE is initiated by a breach of immunotolerance to self, which promotes the generation of high affinity autoantibodies primarily against nuclear components and phospholipids [3, 4]. The autoantibodies recognize and bind self antigens, forming immune complexes (IC) that deposit in the peripheral tissues. The complement system is subsequently activated by IC *in situ* and induces inflammation, which amplifies itself by recruiting inflammatory leukocytes [5, 6]. Commonly-used drugs for the treatment of SLE include nonsteroidal anti-inflammatory drugs, antimalarial medicine, glucocorticoids, and immunosuppressive drugs [1]. Recently, several antibody products specifically perturbing autoimmune reactions have been developed to replace the traditional, more toxic chemical agents [1, 7, 8]. However, they may compromise the normal immune response to infections [9, 10]. In addition, some SLE patients are resistant to current standard of care treatments or experience relapses of symptoms [11]. Therefore, there is a need for more natural and more precise interventions with minimal side effects.

Nutrition is not only the natural supply for the maintenance of an organism but also is a critical determinant of a proper immune system development. The changes of particular nutrients have been linked to certain abnormal immune responses. Therefore, exploring the possible beneficial effects of more natural nutritional therapies on lupus could be a new strategy to fine-tune the immune responses back to a balanced status without severe compromises. Vitamin A as a

necessary nutrient plays an important role in the development of a balanced immune system [12, 13]. All-*trans*-retinoic acid (tRA), a predominant vitamin A metabolite, exerts most of the functions attributed to vitamin A [14]. Recent studies of intestinal mucosa have shown that tRA secreted by gut-specific CD103<sup>+</sup> dendritic cells can modulate the T helper (Th)17-regulatory T cell (Treg) balance [15-17]. tRA has also been shown to induce gut-tropic, IgA-producing B cells [18]. Systemically, tRA is known to regulate Th1-Th2 balance [19, 20] and increase antigen-specific antibody response by promoting the activation and the differentiation of B cells into plasma cells [21-23]. More recently, tRA was shown to be essential for the differentiation of conventional dendritic cells [24]. These evidences imply that tRA may affect autoimmunity but whether and how tRA, or vitamin A in general, may do so is not clearly understood.

A beneficial effect of tRA, alone or in combination with low-dose immunosuppressive drugs, on lupus nephritis (LN), which is the inflammation of the kidney, has been reported in both mouse models and SLE patients [25-29]. However, SLE is a systemic autoimmune disease involving many other organs besides the kidney. Evidence is lacking on how tRA affects other SLE-manifested organs, such as the brain and lung. We herein demonstrate the complex effects of tRA on different peripheral tissues using the classical lupus-prone MRL/lpr mouse model, with ameliorated glomerulonephritis but increased tubulointerstitial nephritis and inflammation in other organs. However, tRA administration in MRL control mice didn't show the similar increased inflammation, suggesting the detrimental effects of tRA in MRL/lpr mice were associated with the established inflammatory environment that may enhance the pro-inflammatory effects of tRA.

Among different manifestations, LN occurs in about 50% of SLE patients and is a major cause of morbidity and mortality in SLE patients [30]. In our study, the effects of tRA on LN are different between the glomerular region and tubulointerstitial region, suggesting that different inflammatory mediators may contribute to the damages of different regions by distinct mechanisms. LN is known to be initiated by renal deposition of high-affinity autoantibodies generated by activated autoreactive B cells with the help from activated autoreactive T cells [1, 5]. However, the downstream immune mechanisms causing the progression of LN are not well understood. Studies have shown that different types of leukocytes including various T cell subsets, B cells, plasma cells, natural killer cells, monocytes/macrophages, dendritic cells (DCs) and neutrophils are accumulated in the kidneys of both patients and lupus-prone mice with active LN [31, 32]. Using lupus-prone mice, one study further demonstrated that LN could still develop in the absence of humoral immune responses, and such development was associated with leukocyte infiltrations in the kidney [33]. This highlights the critical roles of cellular immune responses in LN progression.

Among different renal-infiltrating cell types, DCs are of interest because there are many subtypes of DCs and that each of them has specific and diverse immune functions through interacting with other immune cells [34]. CD11c as a general surface marker for DCs has been utilized in lupus-prone mice to demonstrate the pathogenic roles of CD11c<sup>+</sup> cells in SLE, particularly in the development of LN [35-40]. However, as CD11c is also expressed on some macrophages [34], it is unclear whether the pathogenic CD11c<sup>+</sup> cells belong to DCs; and if they do, which subpopulation(s) of DCs are more important for LN progression.

Plasmacytoid dendritic cells (pDCs) were discovered as professional interferon (IFN)-producing cells[41, 42]. These cells are poor antigen-presenting cells, but they can produce a large amount of IFN $\alpha$  in response to toll-like receptor (TLR)-7/9 ligation[43]. It has been long recognized that type I IFNs, especially IFN $\alpha$ , facilitate the progression of SLE. Based on large amounts of supporting evidence obtained with human patient samples[44-49] and murine models[50-54], three anti-IFN $\alpha$  monoclonal antibodies have been tested in human clinical trials for SLE[55-59]. All of them were shown to be relatively safe, and one has shown a promising efficacy (12 May 2014 press release on [www.astrazeneca.com](http://www.astrazeneca.com)). Studies of SLE patient samples have revealed that immune complexes in the patient sera are capable of inducing IFN $\alpha$  production from pDCs[44, 60], a process where neutrophil extracellular traps are involved[61, 62]. However, as virtually all cells can produce IFN $\alpha$  upon stimulation[63], which one(s) play a predominant role in driving early- and late-stage SLE has become an interesting question.

Recently, two groups of researchers have independently shown that depletion of pDCs from mouse models of SLE ameliorates lupus-like disease[64, 65], indicating that pDCs indeed play an important pathogenic role in SLE. It was shown that early deletion of pDCs in pre-disease lupus-prone mice significantly reduced lymphadenopathy and improved kidney pathology later in life. Strikingly, the benefit was sustained even after the pDC population recovered[65]. This suggests that pDCs contribute to disease early in SLE pathogenesis, and that the recovered pDCs present in late-stage lupus mice might be less pathogenic. This is consistent with results of previous studies using cells from SLE patients with active disease, where pDC depletion did not decrease the capacity of the remaining patient cells to produce IFN $\alpha$  upon viral stimulation[45]. It was hypothesized that pDCs from patients with active SLE became tolerant due to repetitive

exposure to TLR9 ligands such as CpG[66], so that they produced less IFN $\alpha$  while becoming better antigen-presenting cells than normal pDCs[67]. Similar results have been reported in murine lupus models, where pDCs from late-stage lupus-prone mice of New Zealand Black (NZB) background expressed higher co-stimulatory molecules such as CD80/86[68], and lost the ability to produce IFN $\alpha$  after repeated CpG treatment[69].

We explored the mechanisms behind pDC abnormalities in late-stage lupus mice. We used a classical mouse model of SLE, MRL/lpr, as well as its parent strain MRL, and showed that pDCs isolated from late-stage lupus mice were indeed defective of producing IFN $\alpha$  upon CpG stimulation. Using RNA sequencing, we found that sorted pDCs from lupus mice possessed IFN signatures like SLE patient cells, and that an altered gene expression profile was already present in pDCs prior to the onset of lupus-like disease. As pDCs appear to contribute to disease early [65], we also analyzed pDCs in pre-disease lupus mice and identified a couple potentially interesting markers. These results provide the first demonstration of genetic abnormalities in pDCs isolated from lupus mice, and layout a platform for future studies using SLE patient pDCs. They also suggest that IFN $\alpha$  itself, rather than pDCs, might be a better therapeutic target in the treatment of moderate-to-severe SLE.

Unlike pDCs, the role of conventional DCs (cDCs) subsets in LN remains unclear. Functionally, cDCs can both induce immune tolerance to self and initiate immune responses to foreign pathogens [70]. Immature cDCs in the absence of foreign antigen stimulation express low level MHC-II on the surface, and act to maintain immune tolerance to self. Upon activation by foreign stimulators in tissues, however, cDCs capture surrounding antigens and mature with up-

regulation of MHC-II and activation markers (CD40, CD80, CD86, PD-L1, PD-L2, etc.), becoming professional antigen presenting cells to facilitate inflammation through initiating adaptive immune response by priming naïve T cells and promoting the existing immune response. Three signals are needed for cDCs to prime naïve T cells. The first signal involves the interaction between MHC-II-peptide complex on cDCs and T-cell receptor on T cells; the second signal comes from the ligation of co-stimulatory molecules, with CD80 and CD86 on cDCs and CD28 on T cells; and the third signal is provided by the cytokines that cDCs secrete, which induce the differentiation of naïve T cells into different effector helper T cell subsets.

The efficient interactions between cDCs and T cells also requires the proper positioning of cDCs in the T cell-enriched area, which depends on chemokine-chemokine receptor-guided migration of cDCs.

Chemokines are a group of cytokines with small molecular weight whose main action is the recruitment of leukocyte subsets under homeostatic or pathological conditions. Through interacting with chemokine receptors that are expressed on the cell surface as 7-transmembrane proteins coupled with G-protein for signaling transduction, chemokines can induce firm adhesion of targeted cells to the endothelium and direct the movement of targeted cells to their destination according to the concentration gradient of a given chemokine [71]. Unlike adhesion molecules broadly shared by different types of immune cells, chemokines are selectively used by specific cell populations and have been found to be involved in the migration of leukocytes to nephritic kidney of both SLE patients and lupus-prone mice.

Here, using lupus-prone mice, we show that a subpopulation of CD11c<sup>+</sup> cells with surface markers representing mature monocyte-derived cDCs accumulates in lupus nephritic kidneys. Although highly expressing the chemokine receptor CX3CR1 on the surface, these cDCs are still able to infiltrate into nephritic kidneys with CX3CR1 deficiency, suggesting that CX3CR1 is dispensable for their recruitment and that some other chemokine receptors may be required. However, through targeting CX3CR1 by using anti-CX3CR1-saporin conjugates to directly disrupt the functions of these cDCs, we have demonstrated that these renal-infiltrating cDCs have a pathogenic role in promoting LN. Further *ex vivo* co-culture studies suggest that the pathogenic role of these renal-infiltrating cDCs is due to, at least partially, enhancing kidney-infiltrating T cell responses.

In the following chapters, I have included literature reviews to summarize current research progresses related to the roles of DCs and chemokines/chemokine receptors in the development of SLE. This is followed by three original research studies that showed the effects of vitamin A treatment on lupus, the functional changes of pDCs in early-stage versus late-stage lupus, and the pathogenic role of a newly identified renal-infiltrating cDCs in LN, respectively. Finally, based on these three research studies, a possible combined treatment strategy with tRA administration and DC-targeting is proposed and discussed as a further research direction in the future.

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## **Chapter 2. Breakdown of Immune Tolerance in Systemic Lupus Erythematosus by Dendritic Cells (Literature Review)**

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## Abstract

Dendritic cells (DC) play an important role in the pathogenesis of systemic lupus erythematosus (SLE), an autoimmune disease with multiple tissue manifestations. In this review, we summarize recent studies on the roles of conventional DC and plasmacytoid DC in the development of both murine lupus and human SLE. In the past decade, studies using selective DC depletions have demonstrated critical roles of DC in lupus progression. Comprehensive *in vitro* and *in vivo* studies suggest activation of DC by self-antigens in lupus pathogenesis, followed by breakdown of immune tolerance to self. Potential treatment strategies targeting DC have been developed. However, many questions remain regarding the mechanisms by which DC modulate lupus pathogenesis that require further investigations.

## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease that causes damage of multiple organs.[1] Disease activity and stages can be generally divided into three patterns—the remitting relapsing pattern, chronically active disease, and long quiescence—based on various clinical manifestations that include, but not limited to, skin rash, arthritis, nephritis, hematological disorders and neurological disorders.[2] During SLE pathogenesis, autoreactive T cells are activated that in turn activate autoreactive B cells to produce high affinity autoantibodies against self-antigens.[3] Immune complexes (ICs) formed by aggregation of autoantibodies and self-antigens circulate in the blood and eventually deposit in peripheral tissues where the complement system is activated, ultimately inducing the release of signals that further recruit and activate autoreactive cells to feed forward a vicious cycle of chronic inflammation. Different innate and adaptive immune cell populations, including monocytes/macrophages, neutrophils, dendritic cells (DC), and lymphocytes, are recruited into peripheral tissues following the inflammatory signals to amplify inflammation and cause tissue damage.[1, 4-6]

DC were discovered as the professional antigen-presenting cells (APC) with a primary function of priming naïve T cell activation.[7] Since their discovery, our understanding of how DC contribute to immune responses has much expanded, and DC have been divided into many subpopulations with distinct phenotypes and functions.[8] Two main subpopulations are conventional DC (cDC) and plasmacytoid DC (pDC). cDC originate from common dendritic cell progenitors (CDP) and monocytes generated in bone marrow.[8] Murine cDC is characterized by high expression of CD11c and MHC-II on surface, while human cDC also express nonoverlapping markers CD1c (blood dendritic cell antigen 1, or BDCA1) or CD141 (BDCA3) on different subsets besides CD11c and MHC-II. pDC, on the other hand, are derived only from

CDP. Different from cDC, murine pDC express low level CD11c and MHC-II but are positive for B220 and Siglec-H on surface, and human pDC are defined by the expression of MHC-II, BDCA2 and BDCA4.

Functionally, cDC in tissues capture surrounding antigens and become professional APC that initiate adaptive immune response by priming naïve T cells and promote the existing immune response. Three signals are needed for cDC to prime naïve T cells. The first signal involves the interaction between MHC-II-peptide complex on cDC and T-cell receptor on T cells; the second signal comes from the ligation of co-stimulatory molecules, with CD80 and CD86 on cDC and CD28 on T cells; and the third signal is provided by the cytokines that cDC secrete, which induce the differentiation of naïve T cells into different effector helper T cell subsets. pDC, on the other hand, are professional interferon (IFN) $\alpha$ -producing cells that, through producing a high level of IFN $\alpha$ , activate multiple immune cell populations that express type I IFN receptor (IFNAR).[9] Interestingly, pDC can also up-regulate MHC-II upon activation and act like cDC to activate T cells.[10]

Both cDC and pDC are important for immune tolerance to self.[8] Immature cDC in the absence of foreign antigen stimulation express low level MHC-II on the surface, and act to maintain immune tolerance to self. Upon activation by foreign stimulators, however, cDC mature with up-regulation of MHC-II and activation markers (CD40, CD80, CD86, PD-L1, PD-L2, etc.) to facilitate inflammation. For pDC, while their primary function is to control infections, pDC in thymus are involved in the negative selection to maintain the central tolerance. Not surprisingly, studies have shown that both cDC and pDC play important roles in the development of autoimmune diseases, such as SLE.[11]



Peripheral blood mononuclear cells (PBMC) from SLE patients can be used to study *in vitro* DC responses. Whilst important, information obtained from blood cells is limited. To this end, lupus-prone mouse models that develop lupus-like symptoms spontaneously or artificially can be used to better understand DC-mediated mechanisms of lupus progression under both *in vivo* and *in vitro* conditions. In this review, we summarize recent results obtained from studies of SLE patients and lupus-prone mice on the roles of cDC and pDC in lupus development.

### ***In vivo* DC depletion studies: indication of DC involvement in lupus**

A direct strategy to study whether a cell population is critical for the development of a disease is to deplete the population *in vivo*. Depletion of DC in wild-type mice and lupus-prone mice shows differential contributions of DC to immune homeostasis, with a tolerogenic role of DC in wild-type mice *vs.* an immunogenic role of DC in lupus-prone mice. In wild-type mice, constitutive depletion of CD11c<sup>high</sup> cDC showed normal development of regulatory T (Treg) cells and normal negative selection of CD4<sup>+</sup> T cells in the thymus without an autoimmune response.[12]

Constitutive depletion of both cDC and pDC in wild-type mice, however, led to increased autoimmune inflammation with elevated autoantibodies, increased IFN $\gamma$ /IL-17-secreting T cells in peripheral tissues, and abnormal negative selection of CD4<sup>+</sup> T cells in the thymus.[13] This suggests that pDC, or the combination of pDC and cDC, may contribute to immune tolerance to self. Interestingly, regardless of the presence of pDC, the absence of cDC consistently resulted in dramatic expansion of myeloid cells, particularly neutrophils and macrophages.[12, 13]

In MRL/lpr lupus-prone mice, constitutive depletion of cDC and pDC did not influence the negative selection of T cells in the thymus. However, it led to fewer splenic Treg cells and less CD25 expression on the surface of these cells, suggesting compromised immune tolerance in

MRL/lpr mice in the absence of DC.[14] Importantly, even though myeloid cells expanded dramatically as in wild-type mice, ADDIN EN.CITE [ glomerulonephritis and dermatitis were significantly reduced with DC depletion in MRL/lpr mice, which was accompanied by a significant decrease of the proliferation of total T cells and IFN $\gamma$ -producing effector T cells. The lack of DC also led to significantly fewer plasmablasts and impaired autoantibody production and class switching to IgG, the primary autoantibody isotype in lupus.[14] These results demonstrate a critical role of DC in promoting lupus-like disease in MRL/lpr mice. Interestingly, the initiation of T cell activation in lupus may be DC-independent, as the ratio of naïve to activated T cells in the spleen did not changed with DC depletion. It appears that autoreactive B cells, instead of DC, initiate the activation of autoreactive T cells through antigen presentation in MRL/lpr mice.[15] These data suggest that, although DC can maintain immune tolerance to self in wild-type mice, overall their functions have switched to promoting autoimmune responses in lupus-prone mice.

For pDC, early transient depletion of these cells from BXSB (Yaa) lupus-prone mouse model inhibited type I IFN signature, reduced T and B cell activation, decreased autoantibody production, and improved lupus nephritis.[16] While pDC reappeared later on, the effect of early depletion sustained, suggesting that pDC contribute to lupus disease at the initiation stage. This observation has been confirmed by another study using B6.Nba2 lupus-prone mice.[17]

## **Breakdown of immune tolerance to self in SLE by cDC**

### **Changes of cDC number and phenotype in lupus**

Changes of cell number and phenotype may reflect changes of the cells' activation status and/or their dynamic trafficking into different tissues. In SLE patients, a general sense is that cDC number and frequency in the blood are lower with higher disease activity.[18-23] The decrease of blood cDC may be due to increased migration of cDC into peripheral tissues. For example, more cDC were found to infiltrate the tubulointerstitial region in the kidney biopsy of SLE patients with proliferative or active nephritis than the healthy control (HC) or patients with non-proliferative nephritis, and the increase in renal infiltration was accompanied by a decrease of cDC number in the peripheral blood.[20, 24] Murine cDC, particularly those expressing CD11b, also accumulated in the kidney of various types of lupus-prone mice as lupus nephritis progressed.[25-27] In addition, we and others showed increased cDC accumulation in the spleen and lymph nodes of lupus-prone mice.[28-32] How cDC infiltrated inflamed tissues is unclear, but studies have shown that chemokine receptors chemR23 and CCR7 may be important for cDC migration into the kidney and secondary immune tissues, respectively. [32-35] Renal expression of chemerin—the chemokine ligand of chemR23—and increased chemR23<sup>+</sup> DC in the kidney of SLE patients suggest chemerin-dependent migration of cDC into inflamed kidney in lupus.[34] CCR7, on the other hand, mediates migration of cDC to lymph nodes. Upon IFN $\alpha$  priming and lipopolysaccharide (LPS) stimulation, monocyte-derived cDC (moDC) from SLE patients expressed a significantly higher level of CCR7.[35] Besides IFN $\alpha$  and LPS, ICs can also induce the migration of moDC towards CCR7 ligands both *in vitro* and *in vivo*. [32]

The phenotype of cDC is different between tolerogenic cDC, which suppress inflammation, and immunogenic cDC that stimulate inflammation. cDC in the blood of SLE patients or secondary immune tissues of lupus-prone mice have been shown to exhibit elevated expression of CD40, CD80, CD86, PD-L1 and PD-L2, suggesting that cDC in lupus may be

activated and immunogenic.[18, 36-38] However, *in vitro* studies using moDC from SLE patients or lupus-prone mice have shown inconsistent results regarding the activation phenotype of cDC.[18, 36, 39-41] Some showed higher activation state of moDC and enhanced T cell activation with lupus, while others showed either comparable activities or reduced moDC and T cell activation. The inconsistency may be due to different methods used for moDC differentiation, maturation and activation, as different amounts of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 were used to generate immature moDC, and different stimuli (e.g., LPS, TNF $\alpha$ , CpG or IFN $\alpha$ ) were used to mature or activate moDC in different studies.

### MoDC in lupus

Monocytes can differentiate into cDC under both steady state and inflammatory state *in vivo*. [8] GM-CSF and IL-4 induce moDC *in vitro*. [42] However, whether monocytes are a precursor of cDC in lupus is still an open question. Monocytes incubated with sera from SLE patients could differentiate into cDC, but the differentiation depended on the presence of IFN $\alpha$  in the serum. [43] Later studies also showed that IgG-containing ICs in the serum, tumor necrosis factor (TNF) receptor I on monocytes and the interaction between monocytes and T cells are all important for the differentiation of monocytes into cDC in lupus. [35, 43, 44] Regarding the function of differentiated moDC, only those generated in the presence of SLE sera, rather than moDC generated by IFN $\alpha$ /GM-CSF alone, could promote differentiation of IgG- and IgA-producing plasmablasts from B cells. This suggests that factors other than IFN $\alpha$  in the SLE patient sera affect the function of moDC in lupus. [45]

## Regulation of cDC activation in lupus

*In vitro* studies suggest that self-DNA and/or self-RNA containing antigens could activate cDC.[46-48] *In vitro* generated moDC from both healthy human PBMC and wild-type mouse bone marrow can be activated by necrotic or apoptotic cell particles containing self-DNA and self-RNA to produce inflammatory cytokines (IL-6, TNF $\alpha$ ), upregulate MHC-II and costimulatory molecules (CD40, CD80, CD86), and activate allogeneic T cells that in turn produce IL-2, IFN $\gamma$  and IL-17. It has been demonstrated that cDC generated *in vitro* or isolated directly from human or mouse could be activated by DNA- and RNA-containing self-antigens through the signaling of toll-like receptor (TLR)9 and TLR7/8, respectively.[49-54] However, it is still unclear whether cDC can be activated by nucleic acid-containing self-antigens *in vivo*, because natural IgM antibodies and complement C1q-opsonized apoptotic particles, both present *in vivo* but not necessarily in *in vitro* experiments, have the ability to suppress cDC activation.[55-57] The suppression of p38 MAPK phosphorylation by MAPK phosphatase-1 appears to be important for cDC tolerance induced by natural IgM.[56]

Studies using gene knockouts in mice have shown that TLR7, MyD88 and interferon regulatory factor (IRF)5 are important for cDC activation in lupus, and TLR8, A20, Lyn, B lymphocyte-induced maturation protein-1 (Blimp1) and Bim can downregulate cDC activation.[58-65] While TLR7 promotes cDC activation in lupus, TLR8 downregulates TLR7 expression and TLR7-dependent cDC activation.[58] IRF5-deficient cDC exhibited a reduced ability to produce TNF $\alpha$ , IL-6 and IL-10 in lupus-prone mice.[61] DC-specific deficiency of A20, Lyn or Blimp1 led to lupus-like disease in mice.[60, 62-64] cDC isolated from Bim<sup>-/-</sup> mice compared to wild-type mice induced higher T cell proliferation *in vitro*, and autoantibodies can be generated in non-lupus-prone mice upon transfer of Bim-deficient cDC.[65] The role of

MyD88 in lupus cDC is debated. One study using MyD88-deficient MRL/lpr mice showed no obvious change of lupus nephritis,[59] while another study using DC-specific MyD88 and Lyn double-deficient mice showed attenuated lupus disease compared to DC-specific Lyn-deficient mice.[60] Interestingly, polymorphisms within *TLR7*, *IRF5*, *TLR8*, *A20*, *Lyn* and *Blimp1* gene loci have all been shown to be associated with SLE.[66-70]

Activation of cDC can be regulated by several additional factors according to studies of SLE patient samples. Expression of immunoglobulin-like transcript (ILT)3, an inhibitory receptor, was found to be decreased on circulating cDC of SLE patients, and the decrease was correlated with higher levels of proinflammatory cytokines (type I IFN, TNF $\alpha$ ) in the plasma of these patients.[71] Not surprisingly, SLE-susceptible single nucleotide polymorphisms were identified in the *ILT3* gene locus. Sex hormones may also affect the activation of cDC. In a minichromosome maintenance protein (MCM)6-dependent manner, 17 $\beta$ -estradiol, a female hormone, could induce upregulation of CD40 on *in vitro*-generated moDC that in turn increased T cell activation.[72] cDC purified from SLE patients compared to HC expressed a higher level of MCM6, and MCM6 expression was positively correlated with the level of 17 $\beta$ -estradiol in the sera of SLE patients.[72] Moreover, cDC activation is affected by complement C1q, although the effect of complement C1q on cDC is still unclear. One study showed that immobilized C1q coated on plates induced maturation of immature moDC differentiated *in vitro* from healthy PBMC by GM-CSF/IL-4.[73] Mature moDC, compared to immature moDC, had increased production of IL-12, TNF $\alpha$ , and IL10, and enhanced T cell proliferation and secretion of IFN $\gamma$ . However, another study showed that, when immobilized C1q was added concurrently with GM-CSF/IL-4 during moDC differentiation from PBMC, moDC stayed at immature state.[74] Upon LPS or LPS/IFN $\gamma$  stimulation, these moDC did mature, but they produced less IL-12, TNF $\alpha$ ,

IL-6 but more IL-10.[74] Mature cDC generated by LPS or LPS/IFN $\gamma$  also had reduced ability to activate T cells. The timing of C1q addition appears to be important, and further studies are required to uncover the roles of C1q in regulating cDC maturation and activation.

Apoptosis of activated cDC is important for immune tolerance to self. Under normal condition, activated cDC undergo apoptosis through either Fas-dependent or mitochondria-dependent pathways, the latter by interacting with activated Treg cells that express lymphocyte-activation gene (LAG)3. [75, 76] DC-specific deficiency in either Fas-dependent or Fas-independent apoptosis in mice could induce lupus-like symptoms, suggesting that abnormal accumulation of activated cDC may contribute to breakdown of self-tolerance and lupus development.[75-77]

### **Activation of T cells and B cells by cDC in lupus**

While *in vivo* studies of how cDC affect autoreactive T cells are still lacking, *in vitro* evidence suggests that moDC derived from the bone marrow of lupus mice or from PBMC of SLE patients, upon activation, can promote T cell activation and hamper Treg response.[39, 52, 78-80] It is demonstrated in both mouse and human cell studies that moDC activated by apoptotic cells or cytosolic dsDNA could induce the activation of T cells, including that of autoreactive T cells.[52, 79] In addition, compared to bone marrow-derived macrophages, bone marrow-derived cDC (BMDC) from lupus-prone mice possessed higher ability to activate autoreactive T cells, suggesting that cDC rather than macrophages are the APC for autoreactive T cell activation.[78] Moreover, *in vitro* generated tolerogenic BMDC from SLE patients were less capable of generating Treg cells *in vitro* than HC BMDC. [80] Furthermore, LPS-activated BMDC from

lupus-prone mice suppressed Treg function by producing more IL-6, which indirectly promoted proliferation of CD4<sup>+</sup> T cells.[39]

Several studies using *in vitro* systems have indicated possible roles of cDC in promoting autoreactive B cell activation.[45, 52, 81-83] A couple of them have shown that GM-CSF/IL-4-induced BMDC from B6.Sle1.Sle2.Sle3 lupus-prone mice, compared to BMDC from B6 mice, promoted better B cells proliferation and IgM/IgG production in *in vitro* co-culture system upon anti-CD40 ligation.[81, 82] The enhancement was partially dependent on elevated IL-6 and IFN $\gamma$  produced by activated BMDC. In addition, upon i.p. injection of ICs, splenic CD11c<sup>+</sup> DC from B6.Sle1.Sle2.Sle3 mice produced more IL-6 and IFN $\gamma$  than those from B6 mice. In human cell studies, moDC derived from healthy PBMC *in vitro* activated by either the sera from SLE patients or cytosolic dsDNA promoted B cell antibody class switch to IgG and IgA.[45, 52] Contradictory to these observations, however, one study showed that BMDC from several lupus-prone mouse models, when activated by LPS, possessed reduced IL-6-producing ability compared to BMDC from B6 mice.[83] Due to the decrease of IL-6 production, LPS-activated BMDC from MRL/lpr mice failed to suppress autoreactive IgM production by B cells. The discrepancy may have been due to different lupus-prone mouse models used or different activation methods (anti-CD40 vs. LPS), although another study has shown that LPS could increase IL-6 production from BMDC of B6.Sle1.Sle2.Sle3 mice.[39]

Besides activating T cells and B cells, cDC may also promote lupus development by producing high-mobility group box 1 protein (HMGB1) that not only binds nucleosomes to facilitate activation of cDC as a positive feedback, but also enhances IFN $\alpha$  production by pDC, the later of which will be discussed below.[46, 49, 84]



## Potential treatment strategies of lupus by targeting cDC

To target innate immune cells such as cDC, nanogel-based immunosuppressive drugs have been tested in lupus-prone mice that led to prolonged survival and reduced lupus nephritis.[85, 86] The lipid coating of nanogel enables better uptake of the drug by cDC, thus increasing the amount of immunosuppressive drug inside the cells. In addition, *in vitro* studies have shown that BMDC incubated with immunosuppressive drug-containing nanogel had lower production of inflammatory cytokines compared to cells incubated with free drug. The ability of pDC to produce IFN $\alpha$  was also suppressed, with less IFN $\alpha$  produced in the presence of nanogel.[85] It appears that cDC-targeted therapies may benefit from nanogel-based delivery with minimal side effects.

Efforts have been made to induce the generation of tolerogenic cDC to ameliorate lupus. Several studies have shown that tolerogenic cDC generated by transgenic method or induced *in vitro* can rebuild immune tolerance to self after adoptive transfer to lupus-prone mice.[87-89] Tolerogenic cDC can also be induced from PBMC of SLE patients *in vitro* to suppress T cell activation.[18, 90]

## Breakdown of immune tolerance to self in SLE by pDC

### Changes of pDC number and phenotype in lupus

Human studies of pDC frequency and number in the blood of SLE patients have shown inconsistent results.[19-21, 91-95] The inconsistency may reflect the dynamic change of cell number and migration of pDC corresponding to different disease stages and/or treatments. The decrease of pDC in the circulation of some SLE patients may indicate increased migration of the cells into peripheral tissues. Notably, increased infiltration of pDC to the kidney of SLE patients

has been confirmed by several studies,[20, 24, 95] although the location of the infiltrate is still a matter of debate. It has been suggested that pDC may use IL-18 receptor and chemR23 to migrate into the inflamed kidney that expresses IL-18 and chemerin, respectively.[33, 34, 95] In mice, however, one study showed no change of pDC in the kidney as lupus progressed.[27] pDC can also accumulate in the skin of SLE patients and lupus-prone mice.[96, 97] In MRL/lpr lupus-prone mice, UVB irradiation induces skin infiltration of pDC, while IFN $\alpha$  response in the skin has been shown to be positively correlated with the level of chemerin that can attract pDC through chemR23.[97]

Conversely, the increase of pDC in the circulation of some SLE patients may be due to increased generation and emigration of pDC from the bone marrow. Our study using MRL/lpr mice demonstrated that the number of pDC was increased in the bone marrow compared to MRL control mice.[28] A higher percentage of pDC was also found in the bone marrow of SLE patients compared to HC.[98] It is worth noting that phenotypic identification of pDC varies from one study to another, and that the surface markers used to define pDC in healthy individuals may not be appropriate under the disease environment.[99]

However, we and others have consistently observed the expansion of pDC in secondary immune tissues during lupus progression. We have found that pDC are increased in the MLN of young MRL/lpr mice compared to age-matched MRL controls.[28] Others using NZB/W F1 mice and NZM2328 mice have found similar results in MLN and renal lymph nodes.[38, 100] pDC also accumulate in the spleen of lupus-prone mice, particularly in the marginal zone (MZ) of the spleen.[30, 38, 82, 101, 102] The increase of pDC in secondary lymph tissues on one hand may be caused by inflammation-induced migration and/or self-expansion *in situ*, as will be discussed later. On the other hand, pDC appear to be able to survive better in lupus,[102-104] as

their expression of anti-apoptotic Bcl-2 was found to be increased.[102] Survival signal in pDC from both humans and lupus-prone mice is activated by TLR7/9-induced NF $\kappa$ B pathway.[103, 105] pDCs in lupus are constantly stimulated by TLR7/9 ligands, which are known to suppress miR-29b and miR-29c, allowing for upregulation of the target of these microRNAs, including Bcl-2.[104]

Many functional markers expressed on pDC are altered in SLE patients and lupus-prone mice. The expression of three inhibitory receptors, BDCA2, leukocyte-associated immunoglobulin-like receptor 1 (LAIR-1) and ILT3, on human pDC is reduced in SLE patients compared to HC.[94, 106, 107] On the contrary, MHC-II and costimulatory molecules are increased on pDC of both SLE patients and lupus-prone mice, suggesting an increased ability to present self-antigens and activate autoreactive T cells.[28, 37, 38, 101, 108, 109]

### **Critical roles of IFN $\alpha$ in lupus development**

Many studies have shown that type I IFN, particularly IFN $\alpha$ , is critical for lupus development. It is well known that SLE patients have elevated serum IFN $\alpha$  level that is positively correlated with disease severity.[43] Administration of IFN $\alpha$  into humans for anti-virus or anti-tumor treatment, or into preautoimmune lupus-prone mice, can induce or accelerate lupus-like symptoms.[110-112] Deficiency of the receptor of Type I IFN, IFNAR, in several lupus-prone mouse models resulted in ameliorated lupus symptoms.[100, 113, 114] Interestingly, anti-IFNAR treatment transiently ameliorated lupus disease in MRL/lpr mice, but constitutive depletion of IFNAR in the same model deteriorated lupus symptoms.[115, 116] IFN $\beta$  deficiency in BXSB mice failed to modify lupus progression, indicating that the IFN $\alpha$  subtype is the principal Type I IFN important for lupus development.[116] Recent studies have shown that by either depleting pDC or

abrogating IFN $\alpha$  production of pDC, lupus disease is reduced.[16, 17, 117] However, only the depletion of pDC or blockade of IFN $\alpha$  signaling at early stage of disease could prevent lupus development.[17, 116] Together, these studies suggest that through secreting IFN $\alpha$ , pDC may play a critical role in the development of lupus disease at the early initiation stage.

Many types of leukocytes can express IFNAR on the surface and respond to IFN $\alpha$ , including monocytes, cDC, pDC, T cells and B cells.[116] Sera from SLE patients can induce normal monocytes to differentiate into cDC in an IFN $\alpha$ -dependent manner.[43] Differentiated cDC can subsequently activate both allogeneic and autologous CD4<sup>+</sup> T cells. IFN $\alpha$  can also expand splenic cDC, particularly CD11b<sup>+</sup>CX3CR1<sup>+</sup> cDC, that may have been derived from monocytes.[30] In addition, IFN $\alpha$  is able to pre-condition the immunogenic status of monocytes. Without IFN $\alpha$  priming, monocytes incubated with RNA-containing ICs from SLE patients failed to upregulate activation markers.[118] Same phenomenon was observed for moDC differentiated by apoptotic blebs or apoptotic cells, where IFN $\alpha$  priming enabled these moDC, which were tolerogenic without IFN $\alpha$ , to activate T cells.[119, 120] The molecular mechanism of how IFN $\alpha$  activates monocytes is still unclear, but studies have shown increased expression of two IFN $\alpha$  inducible genes, Ifi202 in bone marrow-derived DC from lupus-prone mice and Ifit4 in monocytes from SLE patients.[121, 122] Overexpression of these genes can activate normal moDC with enhanced IL-12 production, which promotes Th1 differentiation. Besides activation, IFN $\alpha$  also affects the migration of moDC. IFN $\alpha$ /GM-CSF-induced rather than IL-4/GM-CSF-induced moDC from healthy human PBMC can upregulate MMP-9 and migrate towards CCL5 and CCL3 that are expressed in inflamed tissues.[123]

IFN $\alpha$  also influences pDC themselves as well as non-monocyte-derived cDC. In lupus-prone mice, IFN $\alpha$ -dependent expansion of splenic pDC has been documented.[30] With IFNAR-

I deficiency, both the cell number and surface activation markers of splenic pDC were reduced.[100] In the case for non-monocyte-derived cDC, studies of IFNAR-I-deficient NZM2328 mice have shown reduced splenic CD8<sup>+</sup> and CD8<sup>-</sup> cDC with decreased activation markers.[100] IL-12- and TNF $\alpha$ -producing ability of CD8<sup>+</sup> cDC was also reduced in the absence of IFNAR-I.[100]

Regarding T cells, an *in vitro* study showed that normal cDC primed by IFN $\alpha$  could promote naïve T cells to differentiate into Th1/Th17 T cells.[124] However, if IFN $\alpha$  was constantly present in the cDC-T cell co-culture system, it had a suppressive effect for Th1/Th17 differentiation. IFN $\alpha$  can also promote inflammatory T cell function by inducing the migration of effector T cells into inflamed tissues in a CXCR3-dependent manner.[125]

Studies on lupus-prone mouse models have shown that IFN $\alpha$ -producing pDC can directly influence autoreactive B cell response. In BXD2 lupus-prone mice, it was demonstrated that the accumulation of activated pDC in the MZ of spleen resulted in the upregulation of CD86 on MZ B cells, which was important for germinal center (GC) formation and autoantibody production.[126] In addition, MZ B cells increased their migration into the follicular region in response to IFN $\alpha$  produced by the accumulated pDC. Such migration of B cells reduced the interaction with MZ macrophages, causing the macrophages to decrease in number in the MZ.[127] This would compromise clearance of apoptotic cells in the spleen of lupus-prone mice, and promote exposure of autoantigens to DC, autoreactive T cells, and B cells.

### **Regulation of IFN $\alpha$ production from pDC in lupus**

pDC produce a large amount of IFN $\alpha$  upon TLR7 and TLR9 stimulation by bacterial or viral nucleic acids.[8] Thus, infections could be a trigger of IFN $\alpha$  production by pDC in lupus. One study showed that Epstein-Barr virus (EBV) infection was associated with lupus.[128] In addition, self nucleic acid antigens and/or nucleic acid-containing ICs are another potential inducer of TLR7/9-dependent IFN $\alpha$  production by pDC in lupus.[128] Self nucleic acid antigens derived from apoptotic or necrotic cells are increased significantly in SLE patients and lupus-prone mice compared to respective controls.[1] When the sera of SLE patients was mixed with healthy PBMC, more IFN $\alpha$  production was induced from pDC.[129] The patient sera contained ICs formed between IgG and apoptotic cells, which were found to activate pDC to produce IFN $\alpha$  through TLR7/9.[53, 130-133] Interestingly, IgG alone or ICs with nucleic acid digestion failed to induce IFN $\alpha$  production by normal pDC, suggesting a critical role of TLR7/9 stimulation by nucleic acids within the ICs. However, DNA/RNA alone or nucleic acid-containing ICs in the presence of Fc $\gamma$ RAII blockade also could not trigger pDC to produce IFN $\alpha$ , indicating that the interaction between IgG in ICs and Fc $\gamma$ RAII on pDC is important for IC-induced IFN $\alpha$  production by pDC.[130, 133] Moreover, it has been shown that CpG motif in dsDNA of DNA-containing ICs is required for IFN $\alpha$  production by normal pDC.[50]

Self nucleic acid antigens can also induce IFN $\alpha$  production by pDC in an Fc receptor (FcR)-independent pathway free from the formation of ICs. LL37, an antimicrobial peptide, has been shown to complex with self-DNA and -RNA to form nanoscale aggregates that trigger IFN $\alpha$  production by normal pDC in a TLR7/9-dependent manner.[54, 134] Neutrophils from SLE patients possess an increased ability to release neutrophil extracellular traps (NETs), which contain LL37.[108, 135] When LL37 was digested, NETs were no longer able to induce IFN $\alpha$  production by pDC, suggesting a critical role for this peptide.[135] IFN $\alpha$  in turn can upregulate

LL37 and HNP (another antimicrobial peptide) on the surface neutrophils as seen in the blood of SLE patients.[108] The levels of anti-LL37 and anti-HNP antibodies in the patient sera are also increased, which, when ligate with transmembrane expressed LL37 and HNP, respectively, can trigger the release of NETs by neutrophils. These results suggest that a positive feedback loop between NETs release by neutrophils and IFN $\alpha$  production by pDC may initiate and/or promote lupus development in SLE patients. Interestingly, LL37 has been found to be also important for Fc $\gamma$ RIIA-dependent IFN $\alpha$  production from pDC, likely through facilitating the internalization of ICs.[135]

Signaling molecules in the TLR7/9 pathway are important for autoantigen-induced IFN $\alpha$  production from pDC. SLC15A4-, MyD88-, IRF8- or IRF5-deficient lupus-prone mice have shown ameliorated lupus symptoms with reduced IFN $\alpha$  protein level in the serum, decreased IFN $\alpha$  transcript level in pDC, downregulation of type I IFN inducible genes, and suppressed activation of both T cells and B cells.[59, 61, 136-138] In addition, pDC from IRF-5- or IRF7-deficient mice failed to produce IFN $\alpha$  upon stimulation with RNA-containing ICs from the sera of SLE patients.[50, 139] Moreover, interleukin-1 receptor-associated kinase (IRAK)1 and IRAK4 are required for IFN $\alpha$  induction from pDC, as their inhibition abrogates the production of IFN $\alpha$  from healthy pDC stimulated with the sera of SLE patients.[140]

The ability of pDC to produce IFN $\alpha$  is also regulated by many other factors that may influence the outcome of lupus development. High Mobility Group Box (HMGB) proteins, for example, function as universal sentinels for nucleic-acid-mediated immune response through both cytosolic receptors and those in endosomes including TLR9 and TLR7.[141] It has been shown that compared to CpG-A alone, HMGB1-bound CpG-A could induce higher IFN $\alpha$  and TNF $\alpha$  production by normal pDC.[142] This is due to increased recruitment of MyD88 to TLR9

in the presence of HMGB1. In addition, HMGB1 can facilitate the formation of CpG-TLR9 complexes and retain the complexes in early endosome rather than lysosome, resulting in sustained IFN $\alpha$  production by pDC.[49] Studies on SLE patient samples have shown that the level of HMGB1 in the circulation was positively correlated with the concentration of IFN $\alpha$ . [46, 107] Moreover, the interaction between HMGB1 and receptor for advanced glycation endproducts (RAGE) is required, as PBMC from HC incubated with the sera of SLE patients produce much less IFN $\alpha$  when the interaction is blocked.[46, 142]

Amyloid fibrils can also regulate IFN $\alpha$  production from pDC by modulating the trafficking of nucleic acid-TLR complexes. These are stable insoluble aggregates of misfolded protein products with extensive  $\beta$ -sheet structure that can facilitate the maintenance of nucleic acid antigens in early endosomes of pDC.[143] Albeit rare, amyloid fibrils have been found to be associated with SLE cases and complicate lupus nephritis.[144] Immunization of healthy mice with DNA-containing amyloid fibrils induces lupus-like disease, promoting autoantibody production and lupus nephritis.[143]

C-reactive protein (CRP), an acute-phase reactant produced by liver in response to inflammation, can suppress IFN $\alpha$  production from normal pDC by increasing the trafficking to ICs into late endosomes in pDC.[132] Therefore, CRP may be beneficial for lupus disease through inhibiting IFN $\alpha$  production. In SLE patients, the elevation of CRP in response to inflammation is modest and much less than expected, suggesting compromised regulation of IFN $\alpha$  production.[145]

Complement C1q is another suppressive factor of IFN $\alpha$  production from pDC. Human individuals with C1q-deficiency can develop SLE.[146, 147] When C1q is added simultaneously, RNA-containing ICs or CpG stimulated less production of IFN $\alpha$ , IL-6, IL-8 and



TNF $\alpha$  from PBMC or purified healthy pDC.[148] The suppressive effect of C1q on IFN $\alpha$  production from pDC has been shown to be dependent on the ligation of C1q to LAIR-1 expressed on pDC.[149]

Sex hormones may also regulate IFN $\alpha$  production from pDC in SLE patients. One study has shown that that TLR7 agonist induced higher IFN $\alpha$  production by PBMC from healthy women than those from healthy men.[150] In addition, 17beta-estradiol, a female hormone, can increase IFN $\alpha$  production from pDC upon CpG stimulation.[151]

pDC may interact with other cell types *in vivo* that affect their ability to produce IFN $\alpha$ . Studies have shown that B cells, platelets, NK cells and monocytes can differentially influence IFN $\alpha$  production by pDC.[152-156] B cells facilitate IFN $\alpha$  production by normal pDC stimulated with RNA-containing ICs or CpG-A.[152] Interestingly, the mechanisms of B cell involvement are different depending on the stimulation. For RNA-containing ICs, the contact between B cells and pDC through adhesion molecule CD31 is required; while the elevation of CpG-induced IFN $\alpha$  production is B cell contact-independent. The latter may be dependent on an unknown secreted molecule, as the supernatant from CpG-A-stimulated B cell culture could promote IFN $\alpha$  production from pDC. In addition, activated platelets, found to be more abundant in the blood of SLE patients, can promote IFN $\alpha$  production from normal pDC stimulated with nucleic acid-containing ICs through interaction between CD154 on platelets and CD40 on pDC.[153] In lupus-prone mice, depletion of platelets improved, while administration of activated platelets worsened, lupus disease, suggesting the involvement of platelets in lupus development. Moreover, CD56<sup>dim</sup>CD16<sup>+</sup> NK cells can promote IFN $\alpha$  production from pDC upon stimulation with RNA-containing ICs in the co-culture of pDC and NK cells through both secreted MIP-1 $\beta$  and CD11a-dependent direct contact between the two cell types.[154, 155] NK

cells isolated from SLE patients, however, produced less IFN $\alpha$  than NK cells from HC, since most of them were CD56<sup>bright</sup>CD16<sup>-</sup> rather than CD56<sup>dim</sup>CD16<sup>+</sup> NK cells. Furthermore, CD14<sup>+</sup> monocytes, contrary to B cell, platelets and NK cells, can suppress IFN $\alpha$  production from pDC through various mechanisms. It has been shown that upon RNA-containing ICs stimulation, CD14<sup>+</sup> monocytes produced TNF $\alpha$ , prostaglandin E2 and reactive oxygen species, all of which suppressed IFN $\alpha$  production from normal pDC in co-culture.[155] Additionally, monocytes can suppress IFN $\alpha$  production from normal pDC through competitive binding of C1q-coated ICs to reduce internalization of ICs in pDC.[156] Monocytes isolated from SLE patients have less suppressive effect on IFN $\alpha$  production from pDC compared to those isolated from HC.[155]

### **IFN $\alpha$ -producing ability of pDC in lupus**

While the essential role of IFN $\alpha$ -producing pDC in lupus is inarguable, questions remain on whether pDC are the major IFN $\alpha$ -producing cells during the entire course of lupus progression. It has been demonstrated in several studies that PBMC or pDC purified from PBMC of SLE patients produced much less IFN $\alpha$  upon TLR9-ligand stimulation compared to HC.[93, 157-159] Similar results have been obtained from lupus-prone mice.[101] We have shown in our recent study that pDC isolated from older MRL/lpr mice in the late stage of lupus development produced significantly less IFN $\alpha$  upon CpG stimulation *in vitro* compared to pDC purified from younger mice in the early stage.[109] The reduced IFN $\alpha$ -producing ability may be due to continuous exposure to self nucleic acid antigens, as pDC from HC produced much less IFN $\alpha$  after repeated stimulation with CpG or DNA-containing ICs.[159] Notably, one study showed comparable IFN $\alpha$  production between pDC from SLE patients vs. healthy individuals.[160] In

their study, however, IL-3 was added in cell culture medium, which may have enhanced IFN $\alpha$  production by pDC from SLE patients. Resting or the addition of IFN $\alpha$ , IFN $\gamma$  and GM-CSF could also recover IFN $\alpha$ -producing ability of pDC from SLE patients to some extent.[157, 159] This suggests that the deficiency of IFN $\alpha$  production from pDC is reversible. Moreover, IFN $\alpha$  production by pDC from SLE vs. HC was comparable upon stimulation with influenza viruses or TLR7 agonist.[43, 158] It is possible that pDC in SLE patients and lupus-prone mice can still produce a normal level of IFN $\alpha$  through the TLR7 pathway. Collectively, the results of these studies have raised two important questions: 1) Do pDC gradually lose the ability to produce IFN $\alpha$  *in vivo* during lupus progression? 2) If pDC fail to produce IFN $\alpha$  in late stage lupus, what is the source of IFN $\alpha$  that stays at a high level in SLE patients and lupus-prone mice?[94]

### **Possible IFN $\alpha$ production from cells other than pDC in lupus**

An early study showed that PBMC from SLE patients could still produce detectable IFN $\alpha$  when pDC were depleted, suggesting that other cell types besides pDC may have the ability to produce IFN $\alpha$  in SLE.[43] Neutrophils isolated from HC, SLE patients and B6 mice were able to do so upon nucleosomes or CpG-B stimulation.[161] Interestingly, neutrophils from TLR9-deficient mice retained their ability to produce IFN $\alpha$  upon nucleosomes stimulation, suggesting that the production IFN $\alpha$  in neutrophils is TLR9-independent. Moreover, neutrophils from both SLE patients and lupus-prone mice possessed increased IFN $\alpha$  transcript level compared to HC, although the protein level of IFN $\alpha$  was not measured in these studies.[162-164] Besides neutrophils, monocytes and cDC can also produce IFN $\alpha$ . With IFN $\beta$  priming, monocytes purified from healthy human PBMC, as well as cDC derived *in vitro* from bone marrow of

normal mice, were shown to produce IFN $\alpha$  through LPS-activated TLR4 pathway.[165] Monocytes from healthy human PBMC also produced IFN $\alpha$  upon stimulation with liposome-coated RNA.[166] In addition, Ly6C<sup>high</sup> monocytes are the primary source of IFN $\alpha$  in pristinely induced lupus-prone mice, as depletion of these monocytes abrogated IFN $\alpha$  production.[167] cDC, on the other hand, have been shown to produce IFN $\alpha$  through a cytosolic pattern recognition pathway via stimulator of interferon genes (STING).[168]

### **Potential treatment strategies of lupus by targeting pDC and IFN $\alpha$**

Due to the critical role of pDC and IFN $\alpha$  in the development of lupus, potential treatment strategies targeting them have been proposed. One example is intravenous immunoglobulin (IVIg) therapy, where IgG, the major antibody in IVIg, inhibits IC- or CpG-A-mediated production of IFN $\alpha$  from pDC.[129] It has been suggested that that Fc fragment of IgG through blocking Fc $\gamma$ RIIA on pDCs directly suppresses the uptake of nucleic acid-containing ICs by pDC.[169] Through the function of IgG glycan hydrolysis, Endoglycosidase S (Endo S) can also inhibit the uptake of ICs.[170] Sialylated subfraction positive (SNA<sup>+</sup>) Fab' fragment of IgG, targeting unknown receptor on monocytes, induces production of PGE<sub>2</sub> by monocytes, which in turn suppresses TLR7/9 agonist-mediated IFN $\alpha$  production by pDC. Another potential treatment targeting pDC and IFN $\alpha$  is DNA-like class R inhibitory oligonucleotides (INH-ODNs), which block TLR7/9-mediated activation of pDC upon stimulation with nucleic acid-containing ICs.[171] Administration of INH-ODN in MRL/lpr lupus-prone mice dramatically ameliorated lupus disease with reduced pathology and autoantibodies. Moreover, proteasome inhibitors have been shown to suppress IFN $\alpha$  production from normal pDC by inhibiting TLR9 translocation from endoplasmic reticulum to endosomes and lysosomes.[172, 173] Furthermore, HMG-CoA

reductase inhibitors (Statins) and histone deacetylases inhibitors can suppress IFN $\alpha$  production by healthy human pDC through inhibiting IRF7 translocation into the nucleus.[174, 175] Lastly, by neutralizing IFN $\alpha$  directly, Sifalimumab, a monoclonal antibody against human IFN $\alpha$ , was able to reduce IFN-signature in a phase I clinical trial.[176]

A strategy to induce tolerogenic pDC has also been proposed. Subcutaneous injection of H471-94 peptide from histone proteins into NSF1 lupus-prone mice at a low dose induced tolerogenic pDC that promoted Treg cells.[177] Adoptive transfer of tolerogenic pDC into lupus-prone mice was able to reduce autoantibodies against DNA-containing antigens, decrease IL-17 production in spleen, and delay the development of lupus nephritis.[177]

### Open questions

Many questions remain regarding the mechanisms by which DC modulate lupus pathogenesis that need to be revealed by additional studies. The first question is whether and how selective depletion of cDC would affect lupus. Many different lupus-prone mouse models have been generated, making it feasible to investigate whether DC are important for lupus development *in vivo*. Depletion studies of whole DC populations, including both cDC and pDC, in MRL/lpr lupus-prone mice suggest the involvement of DC in promoting lupus development, but not activation of naïve T cells. Two additional studies that selectively deplete pDC or abrogate IFN $\alpha$ -producing ability of pDC in lupus-prone mouse models other than MRL/lpr further demonstrate the importance of pDC in lupus pathogenesis. However, selective depletion of cDC populations in lupus-prone mice has not been reported.

The second question is which TLR, TLR9 or TLR7, is critical for the role of pDC in lupus pathogenesis. Studies have shown that the pathogenic role of TLR7 in lupus-prone mice is

partially dependent on IFN $\alpha$  induction, and TLR9 on the contrary can regulate lupus progression by suppressing TLR7 signaling.[178-181] However, pDC-specific TLR7 or TLR9 deficiency in lupus-prone mice has not been reported, as B cells and some other innate immune cell types also express TLR7 and TLR9.

A third question is how to develop new treatment strategies targeting DC populations for lupus. Current treatments for lupus are non-specific immunosuppressive drugs that suppress general immune responses from both innate and adaptive immune system. Side effects, including increases susceptibility to cancers and/or infections, can be severe. Future direction for lupus treatment should be focused on specific targeting with minimal side effects, where DC are a valuable target. New drugs targeting DC should avoid blocking the mechanism by which they defend against pathogens or cancer cells. Therefore, a better understanding of how DC are activated in lupus *vs.* cancer/infection will be particularly useful.

How to translate results obtained from *in vitro* studies is another question. Through either purifying DC directly from PBMC of SLE patients or *in vitro* generating moDC, researchers have investigated activation of DC by self-antigens, activation/maturation markers on DC, cytokine production by DC and the ability of DC to activate T cells. Similar studies have also been done with bone marrow cells or sorted splenic DC from lupus-prone mice. However, the results from different studies are not always consistent or even contradictory to each other, likely due to differences in stimulation protocols. It is also unclear whether *in vitro* stimulation methods would create the actual environment for DC in SLE patients or lupus-prone mice. In many cases, *in vitro* studies have revealed that the stimuli for DC activation have to be of certain concentrations or given at specific time points, making it difficult to translate the results.

## Summary

Based on the reviewed studies above, we summarize how cDC and pDC may be involved in lupus pathogenesis. At the initiation stage of lupus, dysregulated cDC and pDC are activated by accumulated self-antigens (e.g., self-nucleic acids bound with associated molecules) and cytokines in genetically predisposed individuals, and accumulate in peripheral immune and non-immune tissues. Activated pDC through secreting IFN $\alpha$  then provide immunogenic signals to other immune cells including cDC, monocytes, neutrophils, T cells and B cells. These leukocytes further promote the activation of pDC and IFN $\alpha$  production. With increasing inflammation, monocytes differentiate into activated cDC, which, together with CDP-derived activated cDC, sustain and amplify primed adaptive immune responses in both immune and non-immune tissues, thus exacerbating the disease.

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## **Chapter 3. Chemokines and Chemokine Receptors in the Development of Lupus Nephritis (Literature Review)**

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## **Abstract**

Lupus nephritis (LN) is a major cause of morbidity and mortality in the patients with systemic lupus erythematosus (SLE), an autoimmune disease with damage to multiple organs. Leukocyte recruitment into the inflamed kidney is a critical step to promote LN progression, and the chemokine/chemokine receptor system is necessary for leukocyte recruitment. In this review, we summarize recent studies on the roles of chemokines and chemokine receptors in the development of LN, and discuss the potential and hurdles of developing novel, chemokine-based drugs to treat LN.

## Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with manifestations in multi-organs that are induced by the deposition of circulating autoantibody-autoantigen complexes (immune complexes, IC) and amplified by subsequent infiltration of different types of leukocytes promoting the inflammation. [1] Lupus nephritis (LN), a major cause of morbidity and mortality in up to 60% SLE patients, is characterized by inflammation of the kidney. [2] IC and subsequent complement activation both induce the activation and damage of renal cells that further release inflammatory factors leading to the infiltration of leukocytes into glomerular, tubulointerstitial and perivascular regions of the inflamed kidney to amplify the renal inflammation and damage. [3] Therefore, leukocyte recruitment to the inflamed kidney is a critical step in the development of LN.

Chemokines are a group of cytokines with small molecular weight whose main action is the recruitment of leukocyte subsets under homeostatic and pathological conditions. Through interacting with chemokine receptors that are expressed on the cell surface as 7-transmembrane proteins coupled with G-protein for signaling transduction, chemokines can induce firm adhesion of targeted cells to the endothelium and direct the movement of targeted cells to their destination according to the concentration gradient of a given chemokine. [4] Through this mechanism chemokines can induce directed chemotaxis of responsive cells. Chemokines are classified into four subfamilies according to the first two cysteines and the amino acid residues in between at N-terminal end of the polypeptide. Based on whether the first two cysteines are adjacent, separated by one residue, or separated by three residues, a chemokine is classified into CCL, CXCL, or CX3CL family, respectively. If lacking two first cysteines, the chemokine belongs to XCL

family. Chemokine receptors are named corresponding to the subfamilies of chemokines as CCR, CXCR, CX3CR or XCR, respectively. Individual chemokines and chemokine receptors discovered to date have been reviewed elsewhere with summarized tables. [5-8] They are important both at steady state and during inflammation. Homeostatic chemokines and chemokine receptors are those important for the homing of progenitor cells and mature immune cells into the primary/secondary immune tissues for the development of the immune system and into peripheral non-immune tissues for the tissue-specific functions and immune surveillance. [5, 9] Examples are CCR7 that is expressed on naïve lymphocytes and dendritic cells for recruitment into lymph nodes by CCL19 and CCL21 as part of the normal immune system development; CXCL12 that is important for the retention of CXCR4<sup>+</sup> hematopoietic stem cells (HSCs) in HSC niches in the bone marrow; CCL2 that is critical for CCR2<sup>+</sup> monocytes emigration from the bone marrow; and CX3CL1 that is essential for CX3CR1<sup>high</sup> monocytes patrolling along the blood vessels. On the other hand, when there is an infection or injury-induced inflammation, activated immune cells will up-regulate some chemokine receptors and migrate into inflamed immune and non-immune tissues by recognizing correspondingly increased inflammatory chemokines. For example, upon the stimulation of pathogen-associated molecular pattern molecules (PAMP) or danger-associated molecular pattern molecules (DAMP), resident mast cells, macrophages and dendritic cells will release cytokine signals to induce the up-regulation of several inflammatory chemokines expressed by activated endothelial and epithelial cells, such as CCL2, CCL3, CCL4, CCL5, CXCL1, CXCL2, CXCL3, CXCL5 and CXCL8. Consequently, circulating immune cells such as neutrophils, monocytes and effector T cells will migrate into inflamed tissues using related chemokine receptors such as CCR2, CCR1, and CXCR2.



Chemokines, unlike adhesion molecules broadly shared by different types of immune cells, are selectively used by specific cell populations and have been found to be involved in the migration of leukocytes to nephritic kidney of both SLE patients and lupus-prone mice. Studies have shown that several immune cell populations are accumulated in the kidney in LN, including various T cell subsets, B cells, plasma cells, NK cells, monocytes/macrophages, dendritic cells (DC) and neutrophils. [10, 11] In this review, we summarize recent studies on the chemokines that are increased in the kidney as LN progresses, and the corresponding chemokine receptors used by renal-infiltrating leukocytes in response to the chemokines. We focus on those highlighted by multiple studies, including the chemokines CXCL13, CXCL12, CXCL9, CXCL10, CXCL11, CCL2, CCL3, CCL4, CCL5, CX3CL1, and chemokine receptors CXCR5, CXCR4, CXCR3, CCR1, CCR2, CCR5 and CX3CR1. Inflammatory factors inducing the expression of chemokines, as well as chemokines that may be used as biomarkers for the diagnosis of LN, are also discussed.

### **Key chemokines and chemokine receptors in LN**

Some chemokines are more commonly related to LN than others based on both studies of lupus-prone mouse models and SLE patients, which have been summarized in Table 1 and Table 2, respectively. To interact with specific chemokine receptors expressed on particular cell populations, these chemokines have diverse biological effects by influencing the migration of different cell populations in both healthy and disease situations. In LN disease, animal model studies suggest that these chemokines contribute to systemic autoimmune responses in immune tissues—thus indirectly promoting LN—and are involved in local renal inflammation with direct effects. Evidence from human studies, on the other hand, suggests the clinical involvement of

these chemokines in the development of LN. In the following sections we will discuss each of these LN-related chemokines regarding their biological effects, roles in mouse models of LN, and clinical evidence from studies of lupus nephritic patients.

### **CXCL13 and CXCR5**

CXCL13, also known as B cell-attracting chemokine 1 (BCA1) or B lymphocyte chemoattractant (BLC), is the chemokine recognized by CXCR5. CXCL13 is important in directing the trafficking of CXCR5<sup>+</sup> cells, including B cells, follicular helper CD4 T cells (T<sub>FH</sub>), CXCR5<sup>+</sup>CD8<sup>+</sup> T cells and CXCR5<sup>+</sup> DC, all involved in humoral immune responses. [12-14] Both B cells and T<sub>FH</sub> critical for the formation of germinal centers (GC) depend on CXCR5 to migrate into the B cell follicles in secondary immune tissues. [12, 15-17] Circulating CXCR5<sup>+</sup> central or effector memory-like T<sub>FH</sub> have also been discovered that could migrate to and function in immune and non-immune tissues. [18-21] Besides T<sub>FH</sub>, CXCR5<sup>+</sup>CD8<sup>+</sup> T cells and CXCR5<sup>+</sup> DC are also found to facilitate B cell responses. [13, 14] Therefore, by attracting different types of CXCR5<sup>+</sup> immune cells, CXCL13 contributes to B cell responses, especially the generation of high affinity antibody-producing cells in GC.

In two commonly used lupus-prone mouse models, NZB/W F1 and MRL/lpr, transcript levels of renal CXCL13 and CXCR5 are consistently increased in aged lupus nephritic mice compared to non-lupus control mice or young mice prior to disease onset, [22-25] suggesting their involvement in the development of LN. Renal macrophages and DC in lupus-prone mice may be the source of CXCL13 in the nephritic kidney, [24, 26-29] leading to increased migration of CXCR5<sup>+</sup> B cells and T<sub>FH</sub>-like cells into the inflamed kidney towards CXCL13. [24, 26, 30]

Further studies with NZB/W F1 mice have shown that among B cell populations, B1 cells compared to B2 cells express much higher CXCR5 and migrate towards CXCL13 more efficiently *in vitro*, [22, 30] and preferentially migrate into the kidney and lung of diseased mice instead of lymphoid tissues. [30] Functionally, B1 cells isolated from NZB/W F1 mice, but not B2 cells, can activate T cells in allogeneic mixed lymphocyte reaction. CXCR5<sup>+</sup> CD4<sup>+</sup> T cells, on the other hand, have been shown to promote IgG production from B1 cells *in vitro*, suggesting potential interaction of B1 and T cells *in situ* in the nephritic kidney. [30] However, another study found most renal-infiltrating B cells to be non-class-switched B2 cells in NZB/W F1 mice, [24] leaving the role of renal CXCR5<sup>+</sup> B in LN an open question. While further studies are required, these results suggest important functions of CXCR5 and its ligand in the development of LN.

The critical roles of CXCL13 and CXCR5<sup>+</sup> cells in the pathogenesis of LN are also evidenced by studies of anti-CXCL13 neutralizing antibodies in MRL/lpr lupus-prone mice and CXCR5-deficiency in B6/lpr lupus-prone mice. [31, 32] Renal pathology, including proteinuria and serum creatinine levels, glomerular and perivascular scores, deposition of IC and complement C3, and renal IL-1 $\beta$ , IL-6, IL-33 and IL-17 protein levels, was significantly lower in the neutralizing antibody-treated mice than controls. [31] Systemic autoimmune responses such as the level of circulating anti-double stranded DNA (anti-dsDNA) antibodies and the ratio of splenic Th17/Treg were reduced as well, suggesting that the pathogenic role of CXCL13/CXCR5 may not be kidney-specific. As the administration of anti-CXCL13 neutralizing antibodies is not tissue specific, it is difficult to say if reduced renal pathology is due to the secondary effect of decreased systemic autoimmune responses or the direct effect of blocking CXCL13/CXCR5

signal in the kidney. Similar to the CXCL13 blockade study, CXCR5-knockout in B6/lpr mice also down-regulated systemic autoimmune reactions, including reduced lymphadenopathy and splenomegaly with reduced GC, B cells, plasma cells and double negative (DN) T cells in secondary lymphoid organs, as well as reduced circulating IgG. [32] Importantly, this study also showed reduced infiltration of adoptively transferred DN T cells from CXCR5-deficient B6/lpr mice compared to wild type B6/lpr mice into the kidney of Rag1<sup>-/-</sup> recipient mice, indicating direct contribution of CXCL13/CXCR5 signal to local renal inflammation in LN. Therefore, CXCL13/CXCR5 contributes to the development of LN both systemically in immune tissues and locally in the kidney of lupus-prone mice.

Evidence from the studies of SLE patients with LN further suggests the clinical involvement of CXCL13/CXCR5 in the development of LN. In SLE patients with LN, but not in healthy controls (HC), CXCL13 and CXCR5 are highly expressed in the cortex of the kidney. [33] In addition, B cells and T<sub>FH</sub>-like cells that express CXCR5 have been indicated to infiltrate the nephritic kidney of SLE patients and are co-localized with CXCL13-expressing regions. [34] Besides chemoattractant functions, CXCL13 may also contribute to LN by activating CXCR5<sup>+</sup> renal non-immune cells such as human podocytes to produce pro-inflammatory molecules. [35]

### **CXCL12 and CXCR4**

CXCL12, also known as stromal cell-derived factor 1 (SDF-1), is the ligand of chemokine receptor CXCR4. It is involved in the homing, retaining, and survival of CXCR4<sup>+</sup> hematopoietic stem cells, B cell precursors and plasma cells in the bone marrow. In addition, CXCL12

maintains the homeostasis of neutrophils, T cells and B cells in immune and non-immune tissues. [36-38]

Studies have shown possible involvement of CXCL12 and CXCR4 in LN development. In lupus-prone mouse models, CXCR4 is consistently increased in various immune cell populations including B cells, plasma cells, T cells, neutrophils and monocytes in the circulation and spleen of diseased mice, suggesting enhanced chemotaxis of these cells towards CXCL12. [39-41] Importantly, both CXCL12 and CXCR4 expressions are increased in the kidney of diseased lupus-prone mice, indicating migration of CXCR4<sup>+</sup> cells into the kidney as LN progresses. [23, 24, 40, 42] In particular, studies have shown the increased accumulation of CXCR4<sup>+</sup> cells in the kidney of diseased mice, including plasma cells, Foxp3<sup>+</sup> CD4<sup>+</sup> regulatory T (Treg) cells, Foxp3<sup>-</sup> CD4<sup>+</sup> conventional T cells, and inflammatory monocytes and neutrophils. [40, 41, 43] Besides promoting leukocyte infiltration, CXCL12/CXCR4 may also deteriorate LN by directly affecting renal tissue cells. Studies have shown that activated parietal epithelial cells (PECs) as glomerular progenitor cells are involved in proliferative glomerulonephritis. [44] Normally, PECs possess regenerative potential for the repair of injured kidney. [45, 46] However, in glomerulonephritis, CXCR4 is overexpressed on PECs upon inflammatory stimulation, whereas autoantibodies and inflammatory mediators stimulate CXCL12 production on injured podocytes. [42, 44, 47] Consequently, through the interaction between CXCL12 and CXCR4, PECs migrate into the glomerular tuft during the development of LN, where they predominately form hyperplastic lesions in proliferative glomerulonephritis and lead to glomerulosclerosis by secreting extracellular matrix. [3, 44]

Blocking the interaction of CXCL12/CXCR4 in lupus-prone mice reveals their contributions to both systemic autoimmune responses in secondary lymphoid organs and local renal inflammation. Administration of anti-CXCL12 neutralizing antibodies in NZB/W F1 mice led to increased survival rate and reduced renal inflammation including decreased proteinuria and IgG deposition. [42] This may be at least partially due to decreased systemic autoimmune reactions, since circulating anti-dsDNA IgG and B1a subset in the peritoneal cavity and the spleen were reduced, as well as reduced activated CD4<sup>+</sup> T cells in the spleen and lymph nodes. The direct role of CXCL12/CXCR4 network to recruit immune cells in the lupus nephritic kidney is demonstrated in another study with administration of a CXCR4 antagonist in B6.SleYaa lupus-prone mice. [40] Similar to anti-CXCL12 neutralizing antibodies, CXCR4 antagonist ameliorated LN with decreased renal pathological scores and proteinuria and prolonged lifespan. Early administration before severe proteinuria also led to reduced splenomegaly and circulating ANA IgG, suggesting a systemic effect. Splenic monocytes, activated T cells, and B cells in marginal zone, follicular and germinal center were similarly reduced. However, late administration after the onset of severe proteinuria did not influence systemic autoimmune responses, but led to reduced infiltration of monocytes, neutrophils and CD4<sup>+</sup> T cells into the kidney, suggesting a direct effect of CXCL12/CXCR4 in the kidney.

In patients with LN, it has been consistently demonstrated that CXCL12 expression is significantly increased in tubules and glomeruli of the kidney, [48] while most circulating CD4<sup>+</sup> T cells and B cells express CXCR4 in SLE patients. [48-51] Although it is debatable whether its level on B cells is reduced [50] or increased, [48, 51] CXCR4-expressing B cells are found to be

accumulated in the renal biopsy samples of patients with LN, [51] suggesting involvement of CXCL12/CXCR4 in the kidney of patients with LN.

### **CXCL9/10/11 and CXCR3**

CXCR3 is a chemokine receptor interacting with three interferon-inducible chemokines, CXCL9 (monokine induced by gamma-interferon, MIG), CXCL10 (interferon-induced protein of 10kDa, IP-10) and CXCL11 (interferon-inducible T-cell alpha chemoattractant, I-TAC). [52] Several immune cell populations have been reported to express CXCR3, including NK cells, plasmacytoid DC (pDC), conventional DC (cDC), B cells and activated T cells. [53] Among activated T cells, T helper 1 (Th1) cells and effector CD8<sup>+</sup> T cells preferentially express CXCR3. T helper 17 (Th17) cells have also been reported to express CXCR3, although CCR6 is the predominant chemokine receptor on their surface. Since the expression of CXCR3 is induced upon activation of immune cells, especially in effector T cell populations, activated immune cells can migrate into inflamed peripheral tissues where CXCR3 ligands are induced. The three CXCR3 ligands under different circumstances have been shown redundancy, dominance, collaboration, or antagonism to one another. [52] Therefore, CXCR3 and its ligands are mainly associated with the effector stage of immune response and are regulated in a more complex fashion than single paired chemokines/chemokine receptors.

CXCR3 and its ligands are involved in the pathogenesis of SLE. In lupus-prone mice, most commonly NZB/W F1 mice and MRL/lpr mice, CXCR3-expressing T cells and plasma cells as activated effector populations in the secondary lymphoid organs are increased during the development of LN. [41, 54-56] Importantly, studies have shown that CXCR3 and its ligands

are increased in the nephritic kidney of lupus-prone mice, suggesting migration of CXCR3-expressing effector cells from the secondary lymphoid organs into the inflamed kidney. [23, 24, 54, 56-59] Detailed studies with MRL/lpr and NZB/W F1 mice reveal that CXCR3 is expressed on different renal-infiltrating cells with varied proportions, including CD4<sup>+</sup> T cells (15-33%), CD8<sup>+</sup> T cells (10-33%), B220<sup>+</sup> cells (including both B cells and pDC, 25%), plasma cells (40%) and macrophages (5%). [23, 55, 57] All renal CXCR3<sup>+</sup> T cells are confirmed as CD44<sup>+</sup> activated T cells, while CD44<sup>-low</sup> naïve T cells are CXCR3-negative. [55] In addition, renal-infiltrating CXCR3<sup>+</sup> plasma cells can secrete IgG instead of IgM, indicating their pathogenic role in promoting LN. [54]

While both CXCR3 and its ligands are increased in the kidney of lupus-prone mice with LN, their deficiencies in lupus-prone mice have shown inconsistent or even contradictory results. CXCL10 deficiency in MRL/lpr mice showed no change of LN severity. [23] However, CXCR3- or CXCL9-deficiency in the nephrotoxic serum nephritis (NSN) model showed reduced nephritic disease with decreased IgG deposits, activated T cells and macrophages in the kidney. [23] This suggests that CXCL9 rather than CXCL10 may be critical for CXCR3-dependent cellular infiltration of the kidney in LN. Consistent with this, another study showed that CXCL9 in the kidney of diseased MRL/lpr mice was the most abundant chemokine for T cell trafficking. [57] However, circulating antigen-specific IgG was also reduced in CXCR3- or CXCL9-deficient NSN model, suggesting that CXCR3/CXCL9 interaction may influence systemic immune responses and indirectly affect kidney pathology. [23] Further studies with CXCR3-knockout MRL/lpr and NZB/W F1 mice have shown different effects on the development of LN. With CXCR3-deficiency in MRL/lpr mice, glomerular pathology score was reduced with decreased T



cells and macrophages infiltration around glomeruli, ameliorated renal lesion, and decreased proteinuria. [56] IFN $\gamma$ -producing T cells and IL-17-producing T cells were also reduced in the kidney, but not in the spleen or lymph nodes of CXCR3-knockout MRL/lpr mice. Importantly, renal IgG and C3 deposits, and circulating total IgG and anti-dsDNA IgG were not different between CXCR3-knockout and wild type MRL/lpr mice, suggesting a direct effect of CXCR3 and its ligands on the kidney by recruiting activated effector T cells and macrophages. However, in NZB/W F1 mice, CXCR3 deficiency did not change either the infiltration of plasma cells and T cells to the kidney or the course of LN. [55] Therefore, further studies are required to determine whether CXCR3 is important for LN development and which ligand(s) are critical for the infiltration of CXCR3<sup>+</sup> cells to the kidney of lupus-prone mice.

Despite controversial results from studies of lupus-prone mice, evidence from SLE patients still suggests the possible involvement of CXCR3 and its chemokine ligands in the development of LN. Patients with active SLE compared to HC or patients with inactive disease have reduced CXCR3<sup>+</sup> CD4<sup>+</sup> T cells in the circulation, suggesting infiltration of the cells into peripheral tissues. [49] In addition, several studies have shown that in SLE patients with LN, compared to HC or patients without nephritic involvement, CXCR3<sup>+</sup> cells (mostly T cells) are increased in the kidney and urine, which is correlated with increased expression of renal CXCR3 ligands. [26, 56, 60] Moreover, it has been found that CXCR3<sup>+</sup> cells are accumulated in tubulointerstitial regions and around glomeruli in the kidney of lupus nephritic patients, [26] account for 60% of total infiltrating cells, and are positively correlated with proteinuria. [60] Among the three CXCR3 ligands, CXCL10 is most increased in SLE patients and localized in the same region as CXCR3<sup>+</sup> cells in the nephritic kidney. [60] Besides CXCR3-expressing T cells, a group of pathogenic

CD19<sup>high</sup> B cells also express CXCR3 at a high level in SLE patients and migrate towards CXCL9 *in vitro*, suggesting their potential to migrate into inflamed peripheral tissues such as the kidney. [61]

### CCR1, CCR5 and CCL3/4/5

CCR1 and CCR5 share the same ligands, CCL3 (macrophage inflammatory protein 1-alpha, MIP-1 $\alpha$ ) and CCL5 (regulated upon activation, normally T-expressed, and presumably secreted, RANTES). CCR5 also responds to CCL4 (macrophage inflammatory protein 1-beta, MIP-1 $\beta$ ). CCR1 is expressed on CD34<sup>+</sup> bone marrow progenitor cells, monocytes, NK cells, T cells, and preferentially on CD45RO<sup>+</sup> activated/memory T cells. [62, 63] Murine neutrophils also express CCR1. [64] CCR5 is expressed on monocytes/macrophages and T cells (both CD4<sup>+</sup> and CD8<sup>+</sup> subsets), especially on Th1 cells. [63, 65] Interestingly, monocytes express a high level of CCR1 but low CCR5, while the expression pattern is the opposite in activated/memory T cells, suggesting selective expression of CCR1 and CCR5 in monocytes and activated T cells, respectively. [66]

CCR1 or CCR5 knockout mice have been developed to study their functions in different disease models. [63] Even though CCR1 and CCR5 share the same chemokine ligands, studies with unilateral ureteral obstruction and renal ischemia-reperfusion injury models have shown that CCR1 but not CCR5 is essential for T cells, macrophages and neutrophils infiltration in the tubulointerstitial region of the kidney. [67-69] Moreover, CCR1-deficient mice have enhanced macrophage and T cell infiltration to the glomerular region of the kidney in a nephrotoxic nephritis model, suggesting that such infiltration is CCR1-independent. [70] CCR1-deficient

mice also exhibit increased circulating antigen-specific, Th1-biased, pathogenic IgG2a response, indicating that CCR1 is also involved in Th1-dependent systemic humoral immune response.

[70] However, in a host versus graft disease (HVGD) model, CCR1-deficiency shows a protective effect by inhibiting chronic cardiac allograft rejection, [71] which makes the role of CCR1 complicated in different diseases possibly depending on whether humoral immune responses are involved and/or which tissues and immune cell populations are involved.

Regarding CCR5 deficiency, macrophages from CCR5-knockout mice have reduced ability to produce inflammatory cytokines including IL-6, IL-1 $\beta$  and TNF $\alpha$ , rendering defective bacteria clearance in a *Listeria monocytogenes* infection model. [63] CCR5-deficient T cells, on the other hand, have elevated production of IFN $\gamma$ , granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 with enhanced delayed-type hypersensitivity reaction and humoral immune responses following antigen challenge in CCR5-deficient mice. [63] CCR5 also contributes to the recruitment of Treg in lymphoid and non-lymphoid tissues, which is important in suppressing effector responses in graft versus host disease (GVHD)-targeted organs. [63] Therefore, CCR5 deficiency in different diseases leads to different outcomes depending on which cell types are critical and whether initial the immune response (in lymphoid organs) or the effector phase (in non-immune tissues) is involved. [63]

In lupus-prone mice, CCR1, CCR5 and their ligands are increased in the kidney during LN development. [24, 25, 28, 57-59, 63, 72-74] Studies have shown that in nephritic NZB/W F1 mice, both renal T cells and monocytes/macrophages have elevated CCR1 expression on the surface. [63] In MRL/lpr mice, the administration of a CCR1 antagonist at late stage improved LN with reduced interstitial lesions including decreased infiltration of T cells and

monocytes/macrophages, reduced inflammation-induced proliferating and apoptotic cells, and reduction of tubular atrophy and interstitial fibrosis. [26] However, glomerular IgG deposits and different isotypes of circulating anti-dsDNA IgG reflecting systemic humoral autoimmune response did not change, suggesting a direct effect of CCR1 antagonism on preventing renal infiltration of T cells and macrophages. This was confirmed by reduced renal infiltration of adoptively transferred T cells and macrophages pre-treated with the CCR1 antagonist. The role of CCR1 in LN was limited in interstitial region, as glomerular injury and proteinuria were not improved by CCR1-antagonist administration in MRL/lpr mice. [26] In NZB/W F1 mice, the effect of CCR1 antagonist administration at late stage has also been studied. Besides the similar effects of CCR1 blocking T cells and macrophage infiltration, the study with NZB/W F1 mice also showed prolonged lifespan and improved glomerular injury including reduced proteinuria. [63]

In MRL/lpr mice, the extent of CCR5 expression is debated, as over 50% of renal T cells express CCR5 in one study, whereas only 1% of T cells are shown to express CCR5 in another study. [57, 74] Renal-infiltrating macrophages, on the other hand, are CCR5-positive in MRL/lpr mice. [74] Contrary to the effects of CCR1 blocking, CCR5 knockout in MRL/lpr mice deteriorated LN with increased proteinuria and tubulointerstitial infiltration of total CD3<sup>+</sup> T cells and F4/80<sup>+</sup> macrophages in the kidney. [75] Foxp3<sup>+</sup> Tregs were also increased in the kidney of CCR5-knockout MRL/lpr mice, but LN progression was not reversed by the increase of Treg cells. Systemic humoral immune responses were not affected by CCR5 deficiency, as the circulating anti-dsDNA IgG and renal IgG/C3 deposits were not different between CCR5-knockout and wild type MRL/lpr mice. However, CCR5-knockout MRL/lpr mice exhibited increased splenomegaly

and elevated circulating/renal CCL3, suggesting that renal-infiltrating immune cells may use alternative chemokine receptors responding to CCL3 such as CCR1 to migrate into the kidney and promote LN. This study reveals possible roles of CCR5 in negatively regulating LN progression by modulating CCL3 production and controlling lymphoproliferation in the spleen. [75] Therefore, it appears that CCR1 and CCR5 may respectively promote or attenuate the development of LN in lupus-prone mice.

In SLE patients, CCR1, CCR5 and their ligands are also increased in the kidney during the development of LN. [76-79] Evidence from SLE patients further shows that most CCR1<sup>+</sup> cells infiltrating in the kidney are CD68<sup>+</sup> macrophages, [49, 79] while CCR5, on the other hand, is expressed on both circulating and renal-infiltrating T cells in SLE patients, particularly interstitial infiltrating T cells. [77]

### **CCL2 and CCR2**

CCR2 is expressed on a fraction of monocytes, dendritic cells, NK cells and T cells, and one of its ligand is CCL2 (monocyte chemoattractant protein-1, MCP-1). [63, 80] CCR2 expression on monocytes is important for both their egression from bone marrow and extravasation into inflamed tissues. [63] Besides chemoattractant function for monocytes, CCR2 and CCL2 are also involved in the regulation of T cell activation and differentiation. T cells from CCR2-deficient mice produce less IFN $\gamma$  upon stimulation, [63] while CCL2 is associated with Th2 cell polarization and enhances IL-4 production by T cells. [80]

In lupus-prone mice, CCR2 and CCL2 are increased in the kidney during the development of LN, suggesting the recruitment of CCR2<sup>+</sup> leukocytes into the inflamed kidney by CCL2. [24, 25, 73, 81-83] Using MRL/lpr mice, studies have shown that most renal-infiltrating CCR2<sup>+</sup> cells are macrophages and not T cells. [73, 74] In addition, CCL2 is mainly expressed in the tubulointerstitial regions of the kidney in lupus-prone mice. [81, 82]

By blocking the interaction between CCR2 and CCL2 in MRL/lpr lupus-prone mice, studies have shown that CCL2/CCR2 network contributes to LN development through both systemic and local mechanisms. In both CCL2/CCR2 antagonist experiments and CCL2/CCR2-knockout models, animal lifespan was consistently prolonged with reduced LN including less glomerular and tubulointerstitial infiltration of T cells and macrophages, although severe proteinuria in old mice was not improved. [82-86] In addition, the pathology and inflammation in the lung and skin of CCL2/CCR2-deficient MRL/lpr mice were reduced, suggesting the systemic involvement of CCL2/CCR2 in multi-peripheral tissues. By further comparing the differences between antagonist and knockout models, it was evident that CCL2/CCR2 antagonists did not improve splenomegaly, lymphadenopathy and circulating total/auto-antibodies, suggesting the local involvement of CCL2/CCR2 in autoimmune target tissues, such the kidney. [83, 85, 86] In contrast, CCL2/CCR2-knockout MRL/lpr mice exhibited reduced circulating anti-dsDNA IgG, diminished lymphadenopathy and decreased percentage of circulating CD8<sup>+</sup> T cells, suggesting CCL2/CCR2 network also contributes to systemic autoimmune reactions in the immune tissues, through which indirectly promoted LN progression. [82, 84] Interestingly, anti-CCL2 spiegelmer, a CCL2 antagonist, blocked the emigration of monocytes from the bone marrow of MRL/lpr mice, which suggested an additional mechanism of how CCL2/CCR2 may promote LN

by facilitating monocytes migration from the bone marrow into the kidney. [86] Together, these results suggest the importance of CCR2 and CCL2 in promoting LN.

In SLE patients, CCR2 and CCL2 expression is also increased in the kidney during the development of LN. [62] Same as shown in lupus-prone mice, CCL2 is mainly expressed in the tubulointerstitial region of the kidney in SLE patients. [62] Renal endothelial cells, epithelial cells and infiltrating leukocytes could be the source of CCL2. [62] In patients with active SLE, a small proportion of T cells (both CD4<sup>+</sup> and CD8<sup>+</sup>) express CCR2 and are reduced in the blood circulation, suggesting their migration from the blood to inflamed peripheral tissues such as the kidney. [49]

### **CX3CL1 and CX3CR1**

CX3CL1, also known as fractalkine, is the only chemokine with CX3C-motif discovered to date that interacts with its unique chemokine receptor, CX3CR1. CX3CR1 is expressed on a fraction of monocytes/macrophages, dendritic cells, NK cells, T cells, and particularly CD8<sup>+</sup> cytotoxic T cells. [87-90] Different from other chemokines, CX3CL1 possesses a soluble form and a transmembrane form, which function to induce chemotaxis and adhesion of CX3CR1<sup>+</sup> leukocytes, respectively. CX3CR1/CX3CL1 interaction also provides anti-apoptotic signals to sustain the survival of CX3CR1<sup>+</sup> leukocytes. [63, 91, 92]

Studies have shown possible involvement of CX3CL1 and CX3CR1<sup>+</sup> leukocytes in the development of LN. In MRL/lpr mice, CD16<sup>+</sup> cells in glomeruli are increased with lupus

development, [93] with increased protein level of CX3CL1 detectable in glomeruli, interstitial microvasculature and arterial regions. [94] Unlike MRL/lpr mice, CX3CR1 and CX3CL1 expression in the kidney of NZB/W F1 mice do not change with lupus progression, suggesting differences between various lupus-prone mouse models. [23, 24, 94, 95]

Administration of NH<sub>2</sub>-terminally truncated CX3CL1 analogs blocked CX3CL1/CX3CR1 interaction and significantly ameliorates glomerular and vascular lesions in MRL/lpr mice, reducing the infiltration of macrophages and CX3CR1<sup>+</sup> cells to the glomerular, interstitial and perivascular regions. [94] T cells, however, were only reduced in the interstitial regions. With CX3CR1 blockade, the transcription level of renal IFN $\gamma$  and IL-2 was reduced as well. The levels of circulating anti-dsDNA IgG and IgG-containing IC were otherwise not affected, which, together with unchanged splenomegaly and lymphadenopathy, suggested a direct function of CX3CL1/CX3CR1 in the kidney that promotes LN progression in MRL/lpr mice. [94]

In SLE patients, CX3CL1 expression is significantly increased in the glomeruli in class IV glomerulonephritis compared to other classes. [96] In addition, glomerular CX3CL1 expression is positively correlated with the infiltration of glomerular CD16<sup>+</sup> cells that express CX3CR1, which deteriorates lupus disease, suggesting the clinical involvement of CX3CL1/CX3CR1 in LN development.

#### **Other T helper cell-associated chemokine receptors/chemokines**



Aside from the T cell-related chemokine receptors discussed above, CXCR6, CCR4 and CCR6 that are associated with the recruitment of Th1, Th2 and Th17/Treg, respectively, have been also studied in SLE. [16, 97, 98]

CXCR6 and its ligand CXCL16 have been shown to be involved in autoimmune diseases such as rheumatoid arthritis. [99] Blockade of CXCL16 in mice also attenuates glomerulonephritis induced by anti-glomerular basement membrane antibodies. [100] In both MRL/lpr and NZB/W F1 mice, the expression of CXCR6 and CXCL16 is increased during the development of LN. [24, 58] While the lack of available blocking antibodies has hindered the investigation of the role of CXCR6/CXCL16 in LN, CXCR6 has been shown to facilitate the infiltration of activated CD8<sup>+</sup> T cells to the inflamed liver. [101] It is thus possible that CXCR6<sup>+</sup> CD8<sup>+</sup> T cells may be recruited into the inflamed kidney in LN through a CXCL16-dependent mechanism. Moreover, it has been shown that the level of soluble CXCL16 (sCXCL16) in the serum of SLE patients is significantly higher compared to HC, and is positively correlated with SLE disease activity index (SLEDAI) of patients. [102] In addition, the concentration of sCXCL16 drops with disease remission. Therefore, CXCL16 may involve in LN development by recruiting CXCR6<sup>+</sup> T cells into the nephritic kidney.

CCR4 has two chemokine ligands, CCL17 (thymus and activation-regulated chemokine, TARC) and CCL22 (macrophage-derived chemokine). The expression of renal CCR4 and its two ligands is increased in MRL/lpr mice as LN progresses. [23] Interestingly, blockade of CCL22, but not CCL17, in MRL/lpr mice led to reduced proteinuria and serum creatinine with improved renal function. [83, 103] Moreover, the number of CCR4<sup>+</sup> T cells is reduced in the peripheral blood of

SLE patients compared to HC, suggesting increased migration of these cells into inflamed tissues. [78] Accordingly, CCR4<sup>+</sup> cells are found in the kidney of SLE patients that co-localize with CD4<sup>+</sup> cells. Thus, CCR4<sup>+</sup> T cells may selectively use CCL22 to migrate into the lupus nephritic kidney.

CCR6 is the chemokine receptor for CCL20 (liver and activation-regulated chemokine, LARC or macrophage inflammatory protein 3 $\alpha$ , MIP-3 $\alpha$ ). CCR6 is expressed on T cells, preferentially on Th17 and Treg cells. [40, 104-110] The interaction of CCR6 and CCL20 can recruit Treg and Th17 cells into the kidney in murine nephrotoxic nephritis. [98, 111] Whether this interaction can recruit Th17 cells into the kidney to promote LN, or recruit Treg cells to attenuate LN, remains to be explored. In NZB/W F1 mice, the expression of CCR6 and CCL20 is increased in the kidney with lupus development. [24] Renal expression of CCL20 is also increased in diseased MRL/lpr mice. [23] These results suggest that CCR6 and CCL20 may function to regulate LN by recruiting Th17 and Treg cells.

### **Mechanisms of chemokine induction in lupus nephritic kidney**

As summarized above, in the kidney of both SLE patients and lupus-prone mice, many chemokines rarely expressed at steady state are induced or significantly increased with LN progression, suggesting that local and/or systemic inflammatory factors may trigger the upregulation of these chemokines. As summarized in Table 3, targeting both renal parenchymal cells and renal-infiltrating immune cells, nucleic acid-containing antigens and autoantibodies are considered to be the major inflammatory stimulators initiating and/or accelerating chemokine release in the lupus nephritic kidney.

Mesangial cells and other intrinsic renal cells like glomerular capillary endothelial and proximal tubular epithelial cells that express several toll-like receptors (TLRs) have the potential to be activated by different antigens to produce inflammatory factors including chemokines. PolyI:C RNA that mimics viral dsRNA can induce mesangial cells from MRL/lpr mice to produce CCL2, whereas mesangial cells from humans can be activated by polyI:C to produce CXCL1 through the TLR3 signaling pathway. [112, 113] Mesangial cells from lupus-prone mice, compared to non-lupus mice, were more sensitive to lipopolysaccharide (LPS) stimulation as shown by the higher TLR4, MyD88 and NFkB expression and higher CCL2 production, suggesting a mechanism of how bacterial infections accelerate lupus disease. [114]

Besides exogenous factors, primary mesangial cells isolated from NZB/W F1 mice, upon self-nucleosome or nucleosome-containing IC stimulation *in vitro*, have been shown to produce several chemokines including CCL2, CCL7, CCL20, CXCL2 and CXCL5, suggesting self-antigen and autoantibody-mediated mesangial activation. [115] Regarding autoantibody-induced mesangial activation, it has been shown that pathogenic anti-dsDNA IgG can upregulate CXCL1 and CX3CL1 transcripts and the secretion of CXCL1 from mesangial cells isolated from MRL/lpr mice through both Fc receptor (FcR)-dependent and -independent pathways. [95] Further studies have shown that FcR-independent pathway is TLR2/4- and the receptor for advanced glycation endproducts (RAGE)-dependent but DNA/TLR9-independent, as pathogenic anti-dsDNA IgG clone 1A3F can bind high mobility group box 1, an endogenous ligand for TLR2/4 and RAGE, through which 1A3F activates TLR2/RAGE-MyD88-NFkB pathway in mesangial cells, leading to the production of several chemokines including CXCL1, CXCL2,

CXCL5, CXCL16, CCL7 and CCL20. [95, 116] Autoantibodies can also indirectly activate intrinsic renal cells. When incubated with immobilized IgG mimicking IC deposition in the kidney, lymphocytes isolated from human PBMC can be activated in a Fc $\gamma$ R-dependent way to produce IL-1 $\beta$  that in turn stimulates human mesangial cells, glomerular capillary endothelial cells, and proximal tubular epithelial cells to further produce CCL2. [117] Moreover, a transcription factor, Fli1 has been shown to directly bind the promoter region of CCL2 and CCL5 genes to promote their expression in primary endothelial cells of the kidney in NZM2410 lupus-prone mice. [118, 119]

In addition to the activation of renal parenchymal cells, renal-infiltrating macrophages and dendritic cells have been shown to produce several chemokines upon stimulation by TLR2/4, TLR3, TLR7 and TLR9 ligands. Biglycan, an endogenous stimulator of TLR2/4, is increased in the serum and kidney of both SLE patients and MRL/lpr mice. [120] An *in vitro* study further shows that macrophages and dendritic cells produce CXCL13 upon biglycan activation of TLR2/4-ROS signaling pathway that is independent of inflammasome. PolyI:C RNA mimicking viral dsRNA and a TLR7 agonist mimicking viral ssRNA can induce macrophages and dendritic cells isolated from MRL/lpr mice to produce CCL2 through the TLR3 and TLR7 signaling pathways, respectively. [112, 121] In addition, it has been shown that TLR7 and TLR9 are mostly detected in renal-infiltrating macrophages and dendritic cells rather than intrinsic renal cells of MRL/lpr mice. [26, 121] CpG, mimicking bacterial DNA or self dsDNA, can induce CCL2, CCL5 and CCR5 in the kidney through the TLR9 pathway when injected into MRL/lpr mice. [26] Detailed studies have shown that exogenous CpG or bacterial DNA particularly bind to infiltrating macrophages and dendritic cells in the glomerular and tubulointerstitial regions of

the kidney in MRL/lpr mice. [26] Chloroquine-blocked TLR9 pathway abolishes CCL5 induction in spleen monocytes, further demonstrating TLR9-dependent chemokine induction in renal-infiltrating innate immune cells upon CpG-DNA triggering. [26] Finally, miRNA-125a has been shown to indirectly down-regulate CCL5 production by activated T cells through targeting kruppel-like factor 13 (KLF13). [122] It has been demonstrated that miRNA-125a is down-regulated while KLF13/CCL5 are up-regulated in PBMC of SLE patients compared to healthy controls, suggesting that dysregulation of CCL5 in SLE patients is dependent on miRNA-125a.

### **Chemokines as biomarkers for LN**

To date, renal biopsy is still the gold standard for accurate diagnosis and classification of LN and for the prognosis of LN activity and chronicity in patients upon treatment. However, chemokines in the urine of lupus nephritic patients have been studied according to established diagnosis of LN classes that suggest their potential use as non-invasive biomarkers for LN activity monitoring, treatment responses and remission/flare prediction after the biopsy diagnosis.

Urine chemokines can be used to supplement renal biopsy diagnosis of LN. In both adult and juvenile SLE patients, urinary CCL2 (uCCL2) concentration is significantly higher in nephritic patients than non-nephritic patients and healthy controls. [123, 124] Moreover, both protein and mRNA levels of uCCL2 are significantly higher in SLE patients with active LN compared to those with inactive LN. [62, 125-130] Further studies have shown that uCCL2 alone or combined with other factors can distinguish different classes of LN, as uCCL2 concentration is positively correlated to progressive LN classes and significantly increased in diffuse proliferative group compared to focal proliferative and mesangioproliferative groups. [123, 131, 132] In addition, as

interstitial lesions are always associated with end-stage LN, it is important to monitor uCCL2 whose level is high during interstitial inflammation in moderate-severe SLE patients. [133] Besides uCCL2, urinary CXCL10 (uCXCL10) concentration is also significantly higher in nephritic patients than non-nephritic SLE patients. [134] A cut-off value 93 pg/dL of uCXCL10 has been proved to be a good prediction of nephritis with high sensitivity and specificity. Also, uCXCL10 concentration is positively correlated with renal activity score and renal biopsy grade. In addition, uCXCL10 and CXCR3 mRNA levels from class IV nephritic patients are increased compared to other classes. [135] Similarly, the urinary CXCL16 level can also distinguish inactive and active LN in SLE patients. [114]

During the treatment of LN, urinary chemokines can be useful for monitoring treatment responses. uCCL2 has been shown to be a good biomarker to predict juvenile LN improvement. [136] In this study, the cutoff uCCL2 concentration is 343 pg/mL, with a value lower than that predicting an improved renal disease activity. Two other studies have also shown that uCCL2 is reduced in SLE patients with complete or partial LN remission, while its level maintains in non-remission patients, suggesting uCCL2 as a good marker for prognosis. [129, 132] Similarly, uCXCL10 is reduced in SLE patients upon remission into inactive LN in a longitudinal follow-up study, suggesting uCXCL10 is also a good biomarker for monitoring LN improvement of SLE patients following the treatment. [137] After the remission, the elevation of uCCL2 can be detected 2 to 4 months prior to another LN flare, and changes of uCCL2 concentration can distinguish different levels of LN flare severity, suggesting that uCCL2 may be a good marker for predicting recurring LN flares. [129, 132]

## Potential role of chemokine-based drugs to treat LN

As chemokines and chemokine receptors are important in the recruitment of leukocytes to the kidney in the development of LN, one would naturally think of developing new treatments for LN that target the interaction between chemokines and chemokine receptors. However, the design of such treatments should take into consideration of potential limitations (discussed below), as many commercial drugs designed to target chemokines/chemokine receptors in different diseases have unfortunately failed in clinical trials (summarized tables in references). [138, 139]

The complexity of chemokine and chemokine receptor system and possible redundancy are a challenge for the development of new drugs to block leukocyte infiltration. [139, 140] Some chemokines, such as CCL5, can recognize several chemokine receptors (CCR1, CCR3 and CCR5), whereas some chemokine receptors, such as CXCR3, can interact with different chemokines (CXCL9, CXCL10 and CXCL11). [139] Current drugs including small chemical molecules and monoclonal antibodies are designed to simply block the interactions between chemokines and chemokine receptors by neutralizing either chemokines or chemokine receptors, which is insufficient to pinpoint the specific function of each chemokine/chemokine receptor pair. Thus, detailed studies on the dynamic interactions and functions of each particular chemokine/chemokine receptor pair in the specific diseases are critical for successful drug development. In addition, the chemokine and chemokine receptor redundancy is reflected in a situation where one chemokine receptor may function in compensation of another if the other chemokine receptor is blocked. Leukocytes always express more than one type of chemokine receptors on the surface, so blocking the ligation of one chemokine receptor may not completely

or efficiently prevent the infiltration of leukocytes. For example, while both CCR5 and CXCR3 have been shown to promote organ transplantation rejection by inducing T cells infiltration in the transplanted organ, their functions seem to be redundant. CCR5 and CXCR3 double blocking compared to either single blocking makes a much greater prolonged allograft survival in a murine heterotopic heart transplantation model. [141] Hence, future studies are necessary to clarify the effect of chemokine receptor compensation.

Another challenge for LN drug development that involves chemokines and chemokine receptors is to achieve cell-specific targeting. To this end, studies of chemokine receptor expression at the single cell level may help identify the cell type of interest. For example, renal-infiltrating T cells have been shown to express CCR1, CCR4, CCR5, CXCR3 and CXCR5. [24, 60, 63, 77, 78] However, it is unknown whether the chemokine receptors are expressed on the same T cell subsets or differentially expressed on distinct T cell subsets. If we can define T cell subsets by using different combinations of chemokine receptors, we may be able to more specifically target pathogenic T cells by blocking the corresponding chemokine receptors.

An additional limitation is the use of lupus-prone mice where most of the mechanistic studies are performed to better understand the pathogenesis of LN. The differences between human patients and mouse models make it difficult to translate the results of mouse studies to successful clinical trials. Therefore, it is important to study the differences and similarities between SLE patients and lupus-prone mice regarding their use of chemokines and chemokine receptors. An example is CXCL9, which is preferentially used in mice but not in humans, versus CXCL10, which appears to be the predominant chemokine in the kidney of SLE patients. [23, 60]



Finally, how to specifically deliver chemokine-based drugs into the kidney is another important question. It is difficult to find a kidney-specific chemokine or chemokine receptor critical for the development of LN. Systemically blocking a chemokine or chemokine receptor will likely lead to many outcomes other than attenuating LN, causing negative side effects such as an increased chance of infection or cancer. Therefore, while targeting the interaction between chemokines and chemokine receptors is a promising avenue, further studies are required to dissect and better understand the mechanisms behind such interactions before a chemokine-based drug can be developed to treat LN.

Although many commercial chemokine receptor antagonists failed to reach expectations in treating different diseases, targeting chemokines/chemokine receptors may still be a promising strategy in LN. First, studies using SLE patient cells/tissue and animal models summarized in this review have demonstrated the involvement of chemokines/chemokine receptors in LN progression, suggesting the potential of targeting this system in LN treatment. Second, the failure of previously designed drugs is due to our insufficient understanding of the complicated chemokine/chemokine receptor system, which can be improved by further studies. Third, with better understanding of chemokine/chemokine receptor system, future drugs designed to more specifically targeting particular chemokine and chemokine receptor interactions will minimize the off-target effects and side effects commonly observed for immunosuppressive drugs and monoclonal antibodies, which are non-specific. Finally, we may be able to learn from pathogens that are known to specifically target chemokine/chemokine receptor pairs [139] to design better drugs with improved specificity.

## Summary

Chemokines and chemokine receptors contribute to LN development by involving in both autoimmune initiation in immune tissues and amplification of inflammation in the nephritic kidney. Various leukocyte populations migrate into the lupus nephritic kidney through the interactions of chemokines and chemokine receptors, which, together with inflammatory chemokine-activated renal parenchymal cells, lead to acute and chronic LN. Further studies of LN should be more focused on cell-specific chemokine/chemokine receptor functions through the development of cell-specific knockouts. Moreover, detailed studies of particular chemokine/chemokine receptor interactions, as well as studies of the similarities and differences between mouse models and human patients, will serve as the basis for future drug development that benefit SLE patients with LN.

## TABLES

Table 1. Target cells and roles of chemokine receptors and chemokines in lupus-prone mouse models of LN

Chemokine receptor	Chemokine(s)	Roles	Reference(s)
CXCR5	CXCL13	<b>Targets:</b> B cells, B1 cells, T <sub>FH</sub> <b>Role:</b> promote both systemic response and local renal inflammation	[24, 26, 30-32, 34]
CXCR4	CXCL12	<b>Targets:</b> B cells, CD4 <sup>+</sup> Foxp3 <sup>+</sup> T cells, CD4 <sup>+</sup> Foxp3 <sup>-</sup> T cells, plasma cells, neutrophils, monocytes, PEC <b>Role:</b> promote both systemic response and local renal inflammation	[39-42, 44]
CXCR3	CXCL9	<b>Targets:</b> CD4 <sup>+</sup> T cells, CD8 <sup>+</sup> T cells, B220 <sup>+</sup> cells, plasma cells, macrophages <b>Role:</b> promote local renal inflammation in MRL/lpr mice	[23, 55, 57]
CCR1	CCL3, 5	<b>Targets:</b> T cells, monocytes/macrophages <b>Role:</b> promote local renal inflammation	[26, 63, 72]
CCR5	CCL3, 5	<b>Targets:</b> T cells, Foxp3 <sup>+</sup> Treg cells, macrophages <b>Role:</b> negatively regulate systemic response and local renal inflammation	[72, 74, 75]
CCR2	CCL2	<b>Target:</b> macrophages <b>Role:</b> promote both systemic response and local renal inflammation	[73, 74, 82-86]
CX3CR1	CX3CL1	<b>Target:</b> CD16 <sup>+</sup> cells <b>Role:</b> promote local renal inflammation	[93, 94]

Table 2. Chemokine receptors and chemokines likely to mediate cell infiltration into the kidney in human LN

<b>Chemokine receptor</b>	<b>Chemokine(s)</b>	<b>Target cell type(s)</b>	<b>Reference(s)</b>
CXCR5	CXCL13	Podocytes, B cells, T <sub>FH</sub>	[34, 35]
CXCR4	CXCL12	B cells, CD4 <sup>+</sup> T cells,	[48-51]
CXCR3	CXCL10	CD4 <sup>+</sup> T cells, T cells, CD19 <sup>high</sup> B cells	[26, 49, 56, 60, 61]
CCR1	CCL3, 5	CD68 <sup>+</sup> macrophages	[49, 79]
CCR5	CCL3, 4, 5	T cells	[77]
CCR2	CCL2	CD4 <sup>+</sup> T cells, CD8 <sup>+</sup> T cells	[49]
CX3CR1	CX3CL1	CD16 <sup>+</sup> cells	[96]

Table 3. Mechanisms of chemokine induction in lupus nephritic kidney

<b>Stimulators</b>	<b>Target cells</b>	<b>Signaling pathways</b>	<b>Chemokines</b>
Self-nucleosome or nucleosome-containing IC	Mesangial cells	FcR, TLR2/4-RAGE, MyD88, NFkB	CCL2, CCL7, CCL20, CXCL2, CXCL5
Pathogenic anti-dsDNA IgG	Mesangial cells	FcR, TLR2/4-RAGE, MyD88, NFkB	CXCL1, CXCL2, CXCL5, CXCL16, CCL7, CCL20, CX3CL1
Lymphocyte activated by immobilized IgG	Mesangial cells, glomerular capillary endothelial, proximal tubular epithelial cells	IL-1 $\beta$	CCL2
LPS	Mesangial cells	TLR4, MyD88, NFkB	CCL2
LPS	Glomerular capillary endothelial	Fli 1 transcription factor	CCL2, CCL5
Poly I:C RNA	Mesangial cells	TLR3, IFN $\beta$ , IRF3, NFkB	CCL2, CXCL1
Poly I:C RNA	Infiltrating macrophages and DC	TLR3	CCL2
RNA40, imiquimod	Infiltrating macrophages and DC	TLR7	CCL2
CpG-ODN, bacterial DNA	Infiltrating macrophages and DC	TLR9	CCL2, CCL5
Biglycan	Infiltrating macrophages and DC	TLR2/4, ROS	CXCL13
Phytohemagglutinin	T cells	miRNA-125a, KLF13	CCL5

## Disclosure

The authors declare that there is no conflict of interest regarding the publication of this paper.

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## Chapter 4. Paradoxical effects of all-*trans*-retinoic acid on lupus-like disease in the MRL/lpr mouse model

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## Abstract

Roles of all-*trans*-retinoic acid (tRA), a metabolite of vitamin A (VA), in both tolerogenic and immunogenic responses are documented. However, how tRA affects the development of systemic autoimmunity is poorly understood. Here we demonstrate that tRA have paradoxical effects on the development of autoimmune lupus in the MRL/lpr mouse model. We administered, orally, tRA or VA mixed with 10% of tRA (referred to as VARA) to female mice starting from 6 weeks of age. At this age, the mice do not exhibit overt clinical signs of lupus. However, the immunogenic environment preceding disease onset has been established as evidenced by an increase of total IgM/IgG in the plasma and expansion of lymphocytes and dendritic cells in secondary lymphoid organs. After 8 weeks of tRA, but not VARA treatment, significantly higher pathological scores in the skin, brain and lung were observed. These were accompanied by a marked increase in B-cell responses that included autoantibody production and enhanced expression of plasma cell-promoting cytokines. Paradoxically, the number of lymphocytes in the mesenteric lymph node decreased with tRA that led to significantly reduced lymphadenopathy. In addition, tRA differentially affected renal pathology, increasing leukocyte infiltration of renal tubulointerstitium while restoring the size of glomeruli in the kidney cortex. In contrast, minimal induction of inflammation with tRA in the absence of an immunogenic environment in the control mice was observed. Altogether, our results suggest that under a predisposed immunogenic environment in autoimmune lupus, tRA may decrease inflammation in some organs while generating more severe disease in others.

## Introduction

Vitamin A plays an important role in the development of a balanced immune system [1, 2]. All-*trans*-retinoic acid (tRA), a predominant vitamin A metabolite, exerts most of the functions attributed to vitamin A [3]. Recent studies of intestinal mucosa have shown that tRA secreted by gut-specific CD103<sup>+</sup> dendritic cells can modulate the T helper (Th)17-regulatory T cell (Treg) balance [4-6]. tRA has also been shown to induce gut-tropic, IgA-producing B cells [7]. Systemically, tRA is known to regulate Th1-Th2 balance [8, 9] and increase antigen-specific antibody response by promoting the activation and the differentiation of B cells into plasma cells [10-12]. More recently, tRA has been shown to be essential for the differentiation of conventional dendritic cells [13]. These evidences imply that tRA may affect autoimmunity but whether and how tRA, or vitamin A in general, may do so is not clearly understood.

Systemic lupus erythematosus (SLE) is an autoimmune disease with persistent inflammation that damages multiple organs including kidney, skin, lung, heart, joints and brain [14]. A majority of patients are women of childbearing age [14, 15]. SLE is initiated by a breach of immunotolerance to self, which promotes the generation of high affinity autoantibodies primarily against nuclear components and phospholipids [16, 17]. The autoantibodies recognize and bind self antigens, forming immune complexes (IC) that deposit in the peripheral tissues. The complement system is subsequently activated by IC *in situ* and induces inflammation, which amplifies itself by recruiting inflammatory leukocytes [18, 19]. Commonly-used drugs for the treatment of SLE include nonsteroidal anti-inflammatory drugs, antimalarial medicine, glucocorticoids, and immunosuppressive drugs [14]. Recently, several antibody products specifically perturbing autoimmune reactions have been developed to replace the traditional, more toxic chemical agents [14, 20, 21]. However, they may compromise the normal immune

response to infections [22, 23]. Therefore, there is a need for new, more natural interventions with minimal side effects.

A beneficial effect of tRA, alone or in combination with low-dose immunosuppressive drugs, on lupus nephritis has been reported in both mouse models and SLE patients [24-28]. However, SLE is a systemic autoimmune disease involving many other organs besides the kidney. Evidence is lacking on how tRA affects other SLE-manifested organs, such as the brain and lung. We herein demonstrate the complex effects of tRA on different peripheral tissues using the classical lupus-prone MRL/lpr mouse model.

## Materials and Methods

### ***Ethics statement***

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech College of Veterinary Medicine (Animal Welfare Assurance Number: A3208-01). All animal experiments were conducted under IACUC protocol #12-062. For anesthesia and euthanasia, isoflurane and CO<sub>2</sub> were used, respectively, according to the IACUC protocol.

### ***Mice and vitamin A administration***

MRL/Mp (MRL) and MRL/Mp-*Fas*<sup>lpr</sup> (MRL/lpr) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and bred and maintained in a specific pathogen-free facility following the requirements of Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University. All-*trans*-retinoic acid (tRA) and all-*trans*-retinyl palmitate (RP) were purchased from Sigma (St. Louis, MO), and prepared and used in the dark to avoid exposure to light. Both retinoids were dissolved in canola oil (vehicle) and administered orally (directly into the mouth) to female mice from 6 till 14 weeks of age. For tRA treatment, 6 mg tRA/kg body weight (BW) was used per day. This dose was reduced from the reported dose of 10 mg tRA/kg BW [27] that led to skin lesions in MRL/lpr mice in our pilot study, which could affect our analysis of the skin. For daily VARA treatment, 11.2 mg RP/kg BW (equivalent to 6 mg retinol/kg BW) was mixed with 0.6 mg tRA/kg BW (10% of the amount of retinol) before being given to the mice. Mice were weighed twice weekly and the retinoid doses were adjusted accordingly.

### ***Leukocyte isolation and flow cytometry***

For bone marrow cells, bones from both hind limbs of each mouse were cracked gently in a mortar containing phosphate buffered saline (PBS) by using a pestle. Bone marrow was released by gentle stirring after the addition of C10 medium (RPMI 1640, 10% fetal bovine serum, 1 mM sodium pyruvate, 1% 100× MEM non-essential amino acids, 10 mM HEPES, 55 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, all from Life Technologies, Grand Island, NY). The suspension was cleared by passing through a 70-μm sterile cell strainer and carefully layered on the top of Ficoll-Paque Plus (GE Healthcare, Pittsburg, PA). After centrifugation at 1,363×g for 30 min at room temperature, mononuclear cells in the buffy coat layer were collected. Spleen and all lymph nodes in the mesenteric region (MLN) were collected and mashed in 70-μm cell strainers with C10. For splenocytes, red blood cells were lysed with RBC lysis buffer (eBioscience, San Diego, CA). For surface marker staining, cells were blocked by anti-mouse CD16/32 (eBioscience), stained with fluorochrome-conjugated antibodies, and analyzed with BD FACSAria II flow cytometer (BD Biosciences, San Jose, CA). For intracellular staining, Foxp3 Fixation/Permeabilization kit (eBioscience) was used. Anti-mouse antibodies used in this study include: B220-FITC, CD3-FITC, I-E/I-A-FITC, CD25-Alexa Fluor® 488, CD11c-PE, CD19-PerCP-Cy5.5, CD4-PerCP-Cy5.5, rat IgG2a-APC, CD40-APC, and Foxp3-PE-Cy7 (eBioscience); CD27-PE, Siglec-H-PerCP-Cy5.5, CD138-APC, CD44-APC-Cy7, and CD62L-BV510 (Biolegend, San Diego, CA); mouse IgG2a-PE, I-E<sup>k</sup>-PE, CD11c-PE-Cy7, Ly6C-PE-Cy7, CD11b-APC-Cy7, CD8a-V450, I-A/I-E-V500, and B220-V500 (BD Biosciences); CD3e-APC (Miltenyi Biotec, Auburn, CA). Flow cytometry data were analyzed with FlowJo.

### ***Enzyme-linked immunosorbent assay (ELISA)***

Blood was collected into anticoagulant-coated Capiject tubes (Terumo Medical, Somerset, NJ) and centrifuged at 15,000×g for 30 sec. Plasma was collected and stored at -80°C. For detection of anti-double-stranded DNA (dsDNA) IgG, the plate was coated with 0.1 mg/ml of calf DNA (Sigma) in 1× saline-sodium citrate (SSC) buffer at 4°C overnight, followed by washing with PBS containing 0.05% Tween-20 (PBS-T). Wells were then blocked with PBS containing 1% bovine serum albumin (BSA) for 1 h at room temperature and washed. Samples were added and incubated for 1 h at room temperature. After additional washing, HRP-conjugated goat anti-mouse IgG-Fc secondary antibody (Bethyl Laboratories, Montgomery, TX) was added and incubated for 1 h at room temperature, following by more washes with PBS-T. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Biolegend) was then added. After the reaction was stopped, the plate was read at 450 nm with SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). Total IgG and IgM concentrations were determined with mouse IgG and IgM ELISA kits according to the manufacturer's instructions (Bethyl Laboratories).

### ***Histology***

All fixed tissues were paraffin-embedded, sectioned, and stained for Hematoxylin and Eosin (H&E) or Periodic Acid-Schiff (PAS) at the Histopathology Laboratory at Virginia-Maryland Regional College of Veterinary Medicine. After immersion-fixation in 10% neutral buffered formalin, brains were sectioned in the transverse plane at levels of the following structures: olfactory bulb, head of caudate nucleus, rostral level of hippocampus, caudal level of hippocampus, midlevel of cerebellum with underlying medulla oblongata, caudal level of

cerebellum with underlying medulla oblongata. In addition, longitudinal sections of the trigeminal ganglion and adjacent nerve were also obtained. Brain slides were read with Nikon ECLIPSE Ci-L microscope and pictures were taken by using NIS-ElementsD 3.2 64-bit software under 20× objective lense (Nikon Plan 20×/0.40, OFN22 WD1.2) at room temperature. Inflammatory lesions were graded as 0 (no lesions), 1, 2 or 3 (increasing severity of lesions). All brain slides were scored by a board certified veterinary neuropathologist (Jortner) in a blinded fashion. Kidneys were fixed in formalin immediately after isolation, while lung tissues were inflated with formalin through the trachea before submerged in formalin. Lung and kidney slides were read with Olympus BX43 microscope and pictures were taken by using Olympus cellSens software. Lung lesions were scored semiquantitatively (0-4) based on the extent of peribronchial, perivascular, or interstitial lymphocytic infiltration as previously described [29]. Glomerular lesions were graded on a scale of 0-3 for increased cellularity, increased mesangial matrix, necrosis, percentage of sclerotic glomeruli, and presence of crescents [27]. Similarly, tubulointerstitial lesions were graded on a scale of 0-3 for interstitial mononuclear infiltration, tubular damage, interstitial fibrosis, and vasculitis. Slides were scored by a board certified veterinary pathologist (Cecere) in a blinded fashion.

### ***Immunohistochemistry***

Kidneys were embedded in Tissue-Tek<sup>®</sup> O.C.T.<sup>™</sup> Compound (Sakura Finetek, Torrance, CA) and rapidly frozen in a freezing bath of dry ice and 2-methylbutane. Frozen OCT samples were cryosectioned and unstained slides were stored at -80°C. Frozen slides were warmed to room temperature and let dry for 30 min, followed by fixation in -20°C cold acetone at room temperature for 10 min. After washing in PBS, slides were blocked with PBS containing 1%



BSA and anti-mouse CD16/32 for 20 min at room temperature. Slides were then incubated with fluorochrome-conjugated antibody mixture for 1 h at room temperature in a dark humid box. Slides were mounted with Prolong Gold containing DAPI (Life Technologies). The following anti-mouse antibodies were used in immunohistochemical analysis: complement C3-PE (Cedarlane, Burlington, NC); IgG-FITC (Sigma); CD11c-PE, CD3e-FITC (eBioscience); and CD138-APC (Biolegend). Slides stained with anti-complement C3 and anti-IgG were read and pictured with EVOS<sup>®</sup> FL microscope (Advanced Microscopy Group, Grand Island, NY) and a 20× objective. Slides stained with antibodies against CD11c, CD3e and CD138 were read and pictured with BX51 upright Olympus microscope (Olympus, Center Valley, PA), a 20× objective and Stereo Investigator software (MBF Bioscience, Williston, VT).

#### ***Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)***

Spleen and MLN were homogenized with Bullet Blender<sup>®</sup> homogenizer (Next Advance, Averill Park, NY) and total RNA was extracted with RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. Genomic DNA was removed by digestion with RNase-free DNase I (Qiagen). Reverse transcription was performed by using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed with iTaq<sup>™</sup> Universal SYBR Green Supermix (Bio-Rad) and ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY). Relative quantities were calculated using L32 as the housekeeping gene. Primer sequences for mouse IL-6, IL-21, IFN $\alpha$ , and L32 can be found in the Supporting Information.

#### ***Other measurements***

Proteinuria was measured weekly with Chemstrip 2GP (Roche, Indianapolis, IN). A scale of 0-4 was used that corresponded to negative, trace (5-20 mg/dL), 30 mg/dL, 100 mg/dL, and  $\geq 500$  mg/dL total protein, respectively. Dermatitis on the back of the neck and/or face of the mice was observed and recorded in a blinded fashion. Total retinol from liver samples was quantified by Ultra Performance Liquid Chromatography (UPLC) after extraction and saponification. Briefly, portions of each sample (around 0.05 g) were saponified in 5% potassium hydroxide, 1% pyrogallol and 98% ethanol, at 55°C. After extraction into hexanes and phase separation with water, an aliquot of the upper phase lipid extract was mixed with a known amount of internal standard, trimethylmethoxyphenyl-retinol (provided by M. Klaus, Hoffmann-La Roche, Basel, Switzerland). Samples were dried under nitrogen and reconstituted in methanol for UPLC analysis using a C-18 reversed-phase column and mobile phase of 92.5% methanol and 7.5% water at a flow rate of 0.6 ml/min with monitoring at 325 nm. The liver total retinol concentrations were calculated based on areas of the peaks for trimethylmethoxyphenyl-retinol (known amount) and total retinol.

### ***Statistical analysis***

For the comparison of two groups, unpaired student's *t*-test was used. For the comparison of more than two groups, one-way ANOVA and Tukey's post-test were used. Results were considered statistically significant when  $P < 0.05$ . In some experiments, Spearman correlation test and Grubbs' test for identification of outliers were used. All analyses were performed with Prism software.

## Results

### Identifying an appropriate age of female MRL/lpr mice for intervention

Depending on the immunological state, tRA can promote either immunogenic or tolerogenic immune responses [2]. It is immunosuppressive under steady state [30-32]. However, under an inflammatory environment, evidence has shown that tRA can be immunogenic and deteriorate pre-existing inflammation [4, 33, 34]. Although a beneficial effect of tRA on lupus nephritis has been reported [24-27], whether it would be of benefit or detriment, systemically, to SLE patients with early stages of lupus—where inflammation has initiated but clinical signs are minimal—is unclear. To find an appropriate experimental model to mimic these patients, we first assessed the immunological environment in young, female MRL/lpr mice that were reaching sexual maturity (i.e., around 6 weeks old [35]). We found that, unlike 9- and 17-week-old mice, 6-week-old MRL/lpr mice had a comparable level of anti-dsDNA IgG in the plasma as age-matched MRL controls (Fig. 1A). No kidney pathology was observed, suggesting minimal clinical signs of lupus at this age [27, 36, 37]. However, lymphoproliferation had already initiated as evidenced by higher levels of total IgG and IgM in the plasma of 6-week-old MRL/lpr mice than the controls (Fig. 1B) and accumulations of B cells and double-negative (DN) T in the spleen and MLN of lupus-prone mice (Fig. 1C). These two cell types can contribute to lupus pathogenesis by producing autoantibodies and the proinflammatory cytokine IL-17, respectively [38-42]. Dendritic cells, recently shown to be a strong mediator in lupus development [43, 44], were also investigated. We found that significantly more plasmacytoid dendritic cells (pDCs) were present in the bone marrow and MLN of 6-week-old MRL/lpr mice than age-matched MRL controls (Fig. 1D). In addition, although the number of pDCs in the spleen did not differ, the percentage of splenic pDCs that were MHC-II<sup>+</sup>CD40<sup>+</sup> or MHC-II<sup>high</sup> was higher in lupus-prone mice (Fig. 1E),

suggesting their activation [45, 46]. Moreover, in both spleen and MLN, accumulation of CD11b<sup>-</sup> (Fig. 1F) and CD11b<sup>+</sup> (Fig. 1G) conventional dendritic cells (cDCs) was observed for 6-week-old female MRL/lpr mice. Most accumulated CD11b<sup>+</sup> cDCs were Ly6C<sup>+</sup> (Fig. 1H), suggesting that they may have derived from monocytes [47, 48]. Like pDCs, CD11b<sup>+</sup> cDCs in MRL/lpr mice appear to be activated based on upregulated CD40 and MHC-II expression (Fig. 1I). Taken together, these results suggest that an immunogenic environment has been established in 6-week-old female MRL/lpr mice albeit the lack of overt clinical signs of lupus. Therefore, we decided to investigate the effects of tRA on these mice that mimic patients with early-stage lupus.

### **Inflammation of the skin, brain and lung with tRA**

To evaluate the effect of tRA on lupus pathogenesis, we treated 6-week-old, female MRL/lpr mice, orally and daily, with vehicle, tRA or VARA till 14 weeks of age. Compared to tRA alone, VARA contained both tRA and retinyl palmitate, the latter being a primary ingredient in vitamin A supplements and thus more clinically relevant than tRA. MRL mice treated with vehicle were used as the control. Retinol analysis showed that vitamin A accumulated in the liver of VARA-treated mice (Fig. S1A). Because tRA is a metabolite of retinol [49-51], tRA-treated mice did not have retinol accumulation in liver. Results of liver function tests (Fig. S1B) and body weight (Fig. S1C) were not different among MRL/lpr groups, suggesting that the administered doses of tRA and VARA were not toxic to the mice.

tRA of comparable doses and with similar treatment time frame was previously reported to improve renal pathology in the same mouse model [27]. However, we noted that tRA increased serum concentrations of total antibodies and autoantibodies in the reported study, and wondered if the antibodies would affect the mice at the systemic level, as SLE is a systemic

autoimmune disease. Strikingly, we found that tRA worsened lupus-like disease in tissues not investigated in the previous study, including skin, brain and lung. The percentage of mice with dermatitis (Fig. S1D), and the leukocyte infiltration scores of the brain (Fig. 2A and 2C) and lung (Fig. 2B and 2D) increased significantly with tRA treatment compared to MRL/lpr mice treated with vehicle. For the brain, lesions were most profound in the tela choroidea of the 3<sup>rd</sup> ventricle and adjacent leptomeninges, and in choroid plexus of the 4<sup>th</sup> ventricle including choroid in the lateral recesses and adjacent leptomeninges. For the lung, although normal peribronchial lymphoid aggregates were present in the MRL control mice, all three MRL/lpr groups exhibited increased severity of peribronchial lymphoid infiltration characterized by mixed small and large lymphocytes and few Mott cells. Perivascular and interstitial infiltrates of lymphoid cells were also observed in the lungs of these mice. In contrast, the effect of VARA on the brain and lung of MRL/lpr mice was comparable to the vehicle control (Fig. 2C and 2D). These results suggest that tRA can increase inflammation in lupus-affected tissues other than the kidney.

#### **Differential effects of tRA on glomeruli and tubulointerstitium of the kidney**

Nephritis is one of the most common symptoms in SLE that can cause death [52]. Although the effect of tRA on glomerular pathology had been reported in lupus-prone mice [27], its effect on tubulointerstitium of the kidney was unclear. We found a slight, albeit statistically non-significant, increase of leukocytes infiltrating the tubulointerstitial region of the kidney in tRA-treated MRL/lpr mice compared to lupus-prone mice treated with vehicle (Fig. 3A and 3B).

These interstitial lesions contained multiple coalescing perivascular and peritubular infiltrates of mononuclear cells and low numbers of Mott cells. Cellular infiltration was most severe in the medulla but extended into the cortex. In addition, immunohistochemical staining showed the

accumulation of a large number of T cells and dendritic cells, and to a lesser extent, of plasma cells, in the interstitium (Fig. 3C). In contrast, although not statistically significant, less severe glomerulonephritis was observed with tRA treatment compared to vehicle-treated MRL/lpr mice (Fig. 3D), which was accompanied by a slight decrease in proteinuria (Fig. 3E). In addition, the size of glomeruli that was enlarged in MRL/lpr mice (as compared to MRL) decreased with tRA treatment (Fig. 3F and 3G), consistent with prior reports [25, 27]. IgG and complement C3 levels in the glomerular region were comparable among the three MRL/lpr mouse groups (Fig. 3H). Taken together, these results suggest that the effect of tRA on the kidney may be region-specific. It increases leukocyte infiltration in the tubulointerstitium while restoring the size of glomeruli in the renal cortex. Treatment with VARA did not affect kidney pathology.

### **Reduced lymphadenopathy with tRA**

MRL/lpr mice had significantly larger lymph nodes in the mesenteric region than MRL mice (Fig. 4A and [53]). We found that treatment with tRA significantly decreased the size of MLN in MRL/lpr mice (Fig. 4A), consistent with a prior report [27]. A slight decrease in spleen size was also observed but not statistically significant (Fig. S2A). Closer analysis revealed that the change of MLN size was due to decreased numbers of T cells and B cells, which together represented nearly all mononuclear cells in the MLN (Fig. 4B). VARA exerted similar, but less obvious, effects. Interestingly, the proportions of different T-cell subsets, including CD4<sup>+</sup>, CD8<sup>+</sup>, DN T cells (Fig. S2B) and naïve, central memory, effector memory T cells (Fig. S2C), did not change with tRA or VARA treatment. Because tRA is critical for the generation of gut-tropic Tregs that can suppress intestinal inflammation [31], we examined whether a changed proportion of Tregs in the secondary lymphoid organs accompanied tRA-mediated reduction of lymphadenopathy. It

was found that tRA did not change the percentage of Tregs in the spleen or MLN (Fig. S2D). This could be due to the presence of transforming growth factor (TGF)  $\beta$ , a cytokine known to be at an increased level in MRL/lpr mice [54], that was recently shown to suppress tRA-mediated expansion of Tregs from peripheral blood CD4<sup>+</sup> T cells isolated from SLE patients [55]. In addition, the percentage of activated B cells in the MLN was not affected by tRA, either (see below in Fig. 5B). These results suggest that tRA might have decreased the number of lymphocytes in the MLN without changing their composition or activation. One possible explanation for this phenomenon may be increased trafficking of lymphocytes from MLN to nonlymphoid organs, such as the brain or lung. Dendritic cells, which represented <1% of total cells in the MLN of 14-week-old MRL/lpr mice, were not affected by either tRA or VARA treatment (Fig. S2E).

### **Increased circulating autoantibodies with tRA and VARA**

We next investigated the humoral immune response that is the cause of type III hypersensitivity in SLE disease [56]. Both anti-dsDNA and total IgG increased significantly in the tRA- and VARA-treated groups compared to the vehicle control in MRL/lpr mice (Fig. 5A). Interestingly, the ratio of anti-dsDNA IgG to total IgG did not change, suggesting that tRA and VARA affected antibody response in general and did not specifically target autoantibodies for expansion. In addition, while neither tRA nor VARA affected the numbers of antibody-secreting B cells in the MLN and bone marrow, the number of plasma cells in the spleen of VARA-treated mice was significantly greater than that of vehicle-treated MRL/lpr mice (Fig. 5B). Moreover, tRA and VARA respectively increase the mRNA levels of IL-21 and IL-6 in the spleen (Fig. 5C). However, another cytokine known to promote plasma cell formation, IFN $\alpha$ , was lower in

MRL/lpr than MRL mice and did not change with retinoid treatments. This was not entirely surprising because IFN $\alpha$  production had been shown to diminish in old mice with late stage of lupus disease [57]. In the MLN, tRA significantly increased IL-6 and IL-21 mRNA per unit weight but not on the tissue level (Fig. S3A) due to significantly smaller MLN with tRA treatment (Fig. 4A). These results indicate that tRA and VARA increased circulating autoantibodies in MRL/lpr mice, and they might do so through enhancing IL-6/IL-21 production and/or inducing plasma cell differentiation in lymphoid tissues.

To determine whether the increase in circulating autoantibodies correlated with increased pathological scores for the brain, lung and kidney, we performed Spearman correlation analysis (Fig. 6). In the analysis, we excluded one outlier identified by Grubbs' test (Fig. S3B). It was found that among the 3 organs, the brain pathology score had the strongest correlation with autoantibody accumulation in the blood ( $R^2 = 0.53$ ,  $P < 0.001$ ). The lung had a weaker correlation ( $R^2 = 0.42$ ,  $P < 0.01$ ), whereas no correlation was observed for the kidney. The lack of correlation between kidney pathology and circulating autoantibodies is consistent with recent recognition that pathogenic IgG in the kidney are likely produced *in situ* rather than from the circulation [58-60]. These results and analyses indicate that circulating anti-dsDNA antibodies are detrimental to the brain and lung in lupus-prone mice. It is unclear, however, why VARA increased autoantibody levels in the circulation without affecting brain or lung pathology (Fig. 2). It is possible that the retinol component of the VARA formulation exerted certain protective effects in these organs. This will be investigated in the future.

#### **Minimal induction of inflammation with tRA in the absence of an immunogenic environment**



To evaluate whether the proinflammatory effect of tRA seen in MRL/lpr mice was specific to a predisposed immunogenic environment, we tested the effects of tRA in the absence of such an environment. MRL control mice, which did not display any inflammation at 6 weeks old (Fig. 1), were treated with vehicle or tRA from 6 to 14 weeks of age. Compared to the vehicle group, tRA-treated MRL mice developed dermatitis at the first week of treatment but quickly recovered in the following week. At the end point, there was no dermatitis or leukocyte infiltration in the brain of either vehicle- or tRA-treated mice (data not shown). Minimal amounts of infiltrates were seen in the lung with tRA (Fig. 7A and 7D) and in the kidney for both treatment groups (Fig. 7B and 7E). The cellular composition of renal infiltrates was the same regardless of treatment (Fig. 7C). In addition, the level of proteinuria was not different between the two groups (Fig. 7F). Importantly, while tRA significantly increased the levels of circulating anti-dsDNA and total IgG in MRL/lpr mice (Fig. 5A), it did not do so in MRL mice (Fig. 7G). This was consistent with unchanged or reduced numbers of plasma cells and plasmablasts with tRA in the spleen and MLN (Fig. S4A). Neither did tRA activate T cells in these mice (Fig. S4B). These results suggest that tRA is not a strong inducer of inflammation in the absence of an immunogenic environment.

Taken together, we found in this study that, under a predisposed immunogenic environment in autoimmune lupus, tRA could reduce glomerular injury and lymphadenopathy while generating more severe disease in other lupus-affected organs such as the brain and lung. Administration of this retinoid as a supplement for patients with early-stage lupus should therefore be approached with caution.

## Discussion

A beneficial effect of tRA on lupus nephritis has been reported [24-27]. In lupus-prone MRL/lpr mice, we observed similar decreases in glomerulonephritis (Fig. 3D) and proteinuria (Fig. 3E) with tRA in this study. However, our study extended the investigation to other lupus-affected tissues, as the development of SLE involves multiple organs besides the kidney. Our results showed that although tRA could ameliorate glomerular inflammation, it exerted an opposite effect and worsened inflammation in the skin (Fig. S1D), brain and lung (Fig. 2). In addition, treatment with tRA led to the accumulation of more leukocytes in the renal tubulointerstitium (Fig. 3A). Interestingly, the number of lymphocytes in the MLN decreased with tRA that led to significantly reduced MLN weight (Fig. 4). We hypothesize that this may be due to increased trafficking of lymphocytes from MLN to nonlymphoid organs such as the brain, lung, and tubulointerstitial region of the kidney. Furthermore, we showed that tRA significantly increased the level of circulating autoantibodies (Fig. 5A), which strongly correlated with exacerbated pathology of the brain, and to a lesser extent, that of the lung (Fig. 6). Our results suggest that tRA exerted paradoxical effects on peripheral tissue inflammation in the MRL/lpr mouse model. Table 1 summarizes the changes of all the variables measured in this study.

tRA is essential for the induction of immune tolerance in steady-state conditions but it can also promote an immunogenic response under inflammatory state [2]. Several lines of evidence in our results support the notion that tRA acts like an adjuvant and facilitates tissue inflammation under an immunogenic environment. First, tRA increased B-cell responses that included more antibody production (Fig. 5A), increased number of plasma cells in the spleen (Fig. 5B), and heightened production of B cell-activating cytokines in MRL/lpr mice (Fig. 5C and Fig. S3A). Second, tRA increased the accumulation of leukocytes in the brain (Fig. 2A),

lung (Fig. 2B), and tubulointerstitium region of the kidney in the same lupus-prone mice (Fig. 3A-C), potentially by facilitating leukocyte trafficking into these tissues and promoting local inflammation. Importantly, tRA failed to induce such inflammation in the absence of a predisposed immunogenic state in MRL control mice (Fig. 7 and Fig. S4). In addition, we found that retinol storage in the liver was significantly lower in MRL/lpr than MRL mice (Fig. S1A). Although the level of retinol in lupus-affected organs was not determined, we speculate that more retinol may have been depleted from the liver in order to facilitate immune response in other organs, thereby promoting lupus-like disease in MRL/lpr mice. Together, these results suggest that although tRA is able to improve glomerular pathology and reduce the size of lymph nodes in the mesenteric area, it has a generally deteriorating effect on peripheral inflammation when given after the initiation of disease in the MRL/lpr mouse model.

We observed a ~50% reduction in the size of MLN in MRL/lpr mice (Fig. 4A), consistent with a previous report [27]. However, the proportion of B- and T-cell subsets did not change (Fig. 5B, S2B and S2C), suggesting normal differentiation and activation of lymphocytes with tRA treatment. Since tRA has been shown to induce apoptosis of cancer cells in the treatment of acute promyelocytic leukemia [61], the diminished lymphocyte numbers in MLN might be due to tRA-induced apoptosis. Another possibility is that tRA increased the trafficking of immune cells from MLN to peripheral tissues [62, 63]. The observation that tRA increased leukocyte infiltration in the brain, lung and kidney interstitium supported this hypothesis. Whether or not tRA regulated chemokine receptors CCR6, CCR4, and CX3CR1, which respectively regulate leukocyte migration to these tissues [64-66], required further investigation.

tRA is critical for the generation of Tregs under steady state [31]. Methods to enhance Tregs have been considered for several autoimmune diseases [67-70]. However, as shown in this

study (Fig. S2D), tRA failed to induce peripheral Tregs under an immunogenic environment established early in lupus pathogenesis. In fact, when combined with proinflammatory cytokines such as IL-6, which was shown to be induced in tRA-treated MRL/lpr mice (Fig. S3A), tRA may facilitate the differentiation and activation of Th1 or Th17 cells instead of Tregs [2, 4]. The lack of Treg response upon tRA treatment may explain why tRA failed to induce systemic immunosuppression in MRL/lpr mice.

Two formulations were used in our study to administer vitamin A. One was a high dose of tRA alone, and the other was a low dose of tRA combined with retinyl palmitate (VARA). VARA was formulated with 10% of tRA, previously shown to enhance the esterification and storage of retinyl palmitate in the lung, and to a lesser extent, in the liver [49]. By comparing tRA to VARA treatment, we could potentially find out how different vitamin A metabolites affect autoimmunity. Because the concentration of tRA in the plasma and peripheral tissues is tightly regulated [71], even high doses of tRA can only sustain a high plasma level for a few hours, which is followed by dramatic decrease to the normal, physiological level [71-73]. However, tRA as a direct dose may be able to exert cellular functions faster than VARA, which requires time to generate tRA from retinyl palmitate. Indeed, our results showed that direct administration of tRA led to distinct changes in MRL/lpr mice that were not shared by VARA treatment (Table 1). In particular, while correlation analysis suggests that circulating autoantibodies detriment brain and lung pathology (Fig. 6), VARA increased blood levels of total and autoantibodies without exacerbating lupus-like disease in the brain and lung, indicating a possibly protective effect from this formulation. Together, these results suggest that different formulations of vitamin A may affect the immune system differently, and thus should be carefully considered if supplementation were to be used. It is worth mentioning that, for both

tRA and VARA used in our study, the total amount of vitamin A was about 6 mg/kg body weight per day, which is 500× of Recommended Dietary Allowance for vitamin A and clearly a pharmacological dose, though liver toxicity was not observed (Fig. S1B).

It is worth mentioning that in human SLE, it has been well established that autoantibodies and the amount of IC deposits in the glomeruli are correlated with disease activity and in particular with lupus nephritis activity [74]. In our study of MRL/lpr mice, we observed increased anti-dsDNA IgG levels with tRA or VARA treatment, but that did not directly correlate with renal damages. This phenomenon might be due to the short lifespan of the mice and the fact that no single mouse model can mimic the complexity of human SLE [75]. Indeed, while another study in MRL/lpr mice observed a similar increase of autoantibodies with tRA treatment [27], in the NZBWF1 mouse model, tRA did not increase anti-dsDNA IgG titers [26, 28].

Together, the results of this study suggest that tRA may play paradoxical roles in SLE if it is given after an immunogenic environment has been established early in lupus pathogenesis. We showed that while treatment with tRA improved glomerular pathology, it also caused severe inflammation in other peripheral organs. The increased tissue inflammation may be associated with tRA-mediated augmentation of B-cell responses. Immunosuppressive Tregs, on the other hand, were not induced by tRA treatment in MRL/lpr mice. Paradoxically, tRA was able to shrink the lymph nodes in the mesenteric area by decreasing the number of lymphocytes, likely through promoting the trafficking of lymphocytes to the brain, lung, or kidney interstitium. In SLE patients, tRA combined with glucocorticoids has been shown to decrease the level of autoantibodies [24-27]. However, the effect of tRA alone on autoantibody production was not investigated. Nor was the effect of tRA on skin, lung, and brain of lupus patients. Our study in

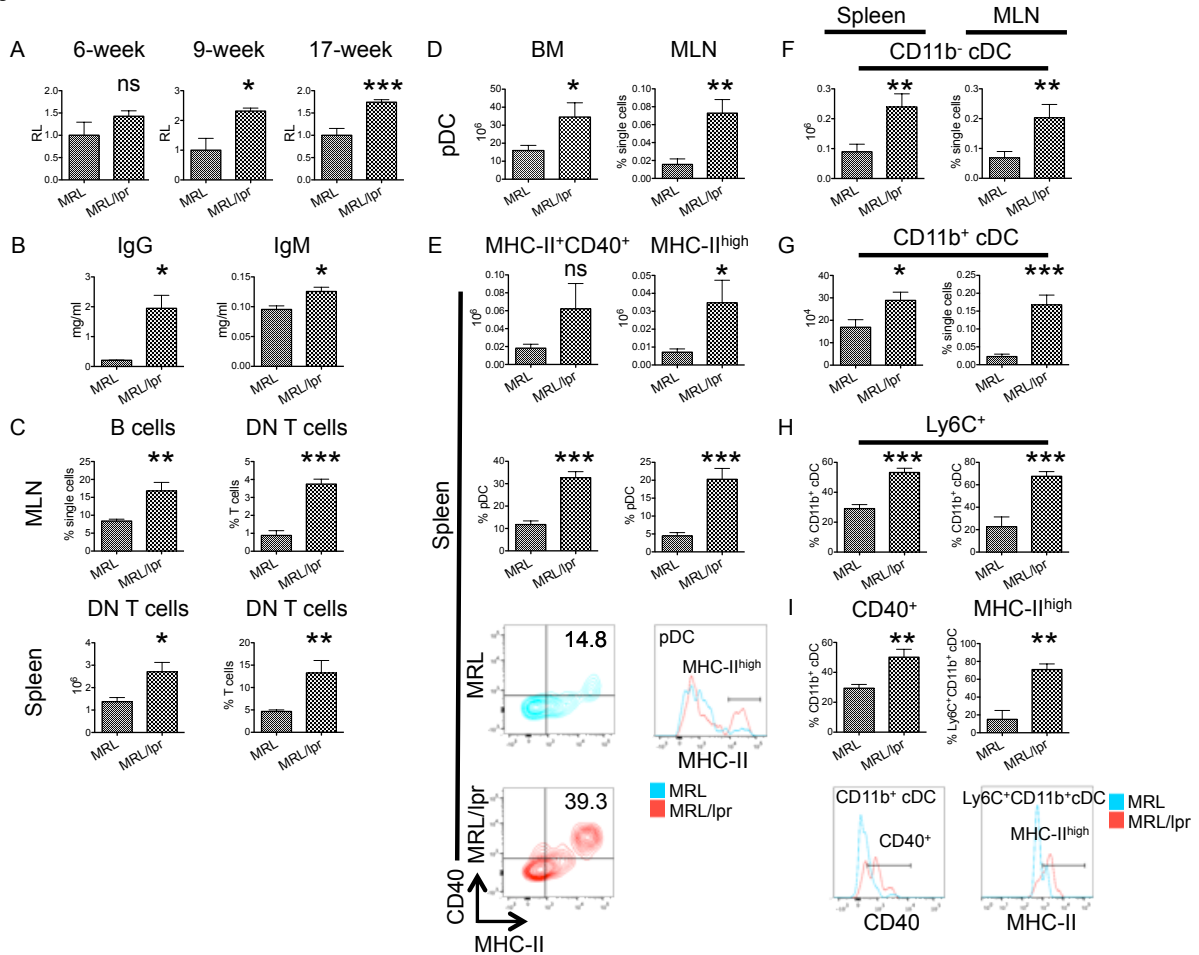
the MRL/lpr mice provides evidence that tRA may be harmful for lupus patients when administered alone. Although we analyzed younger mice without established clinical symptoms, we hypothesize that the effects of tRA on older mice with symptoms would be similar to those on younger ones, since the immunogenic microenvironment has already been established in younger mice and would be sustained when they get older. This hypothesis will be tested in the future. Nevertheless, our current observations suggest that further studies are necessary before a recommendation on the use of retinoid supplements can be made for SLE patients.

## Acknowledgments

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## FIGURES

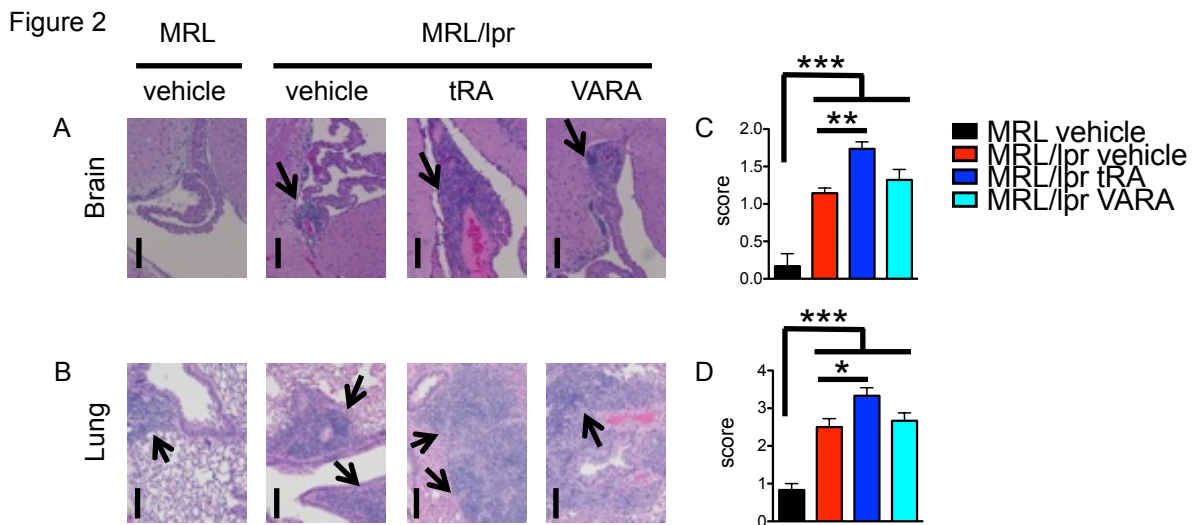
Figure 1



**Figure 1. Immunogenic environment in 6-week-old MRL/lpr mice.** (A) Anti-dsDNA IgG in the plasma of 6-week-, 9-week- and 17-week-old MRL and MRL/lpr mice as detected by ELISA. Data shown as relative levels (RL) were normalized to the average absorbance value of MRL mice at the same age, which was defined as 1. (B) T total IgG and IgM concentrations in the plasma of 6-week-old mice as detected by ELISA. (C-I) The percentages and absolute numbers of T, B, and dendritic cells in the spleen, mesenteric lymph node (MLN), and bone marrow (BM) of 6-week-old mice as determined by flow cytometry. (C, upper plots) The



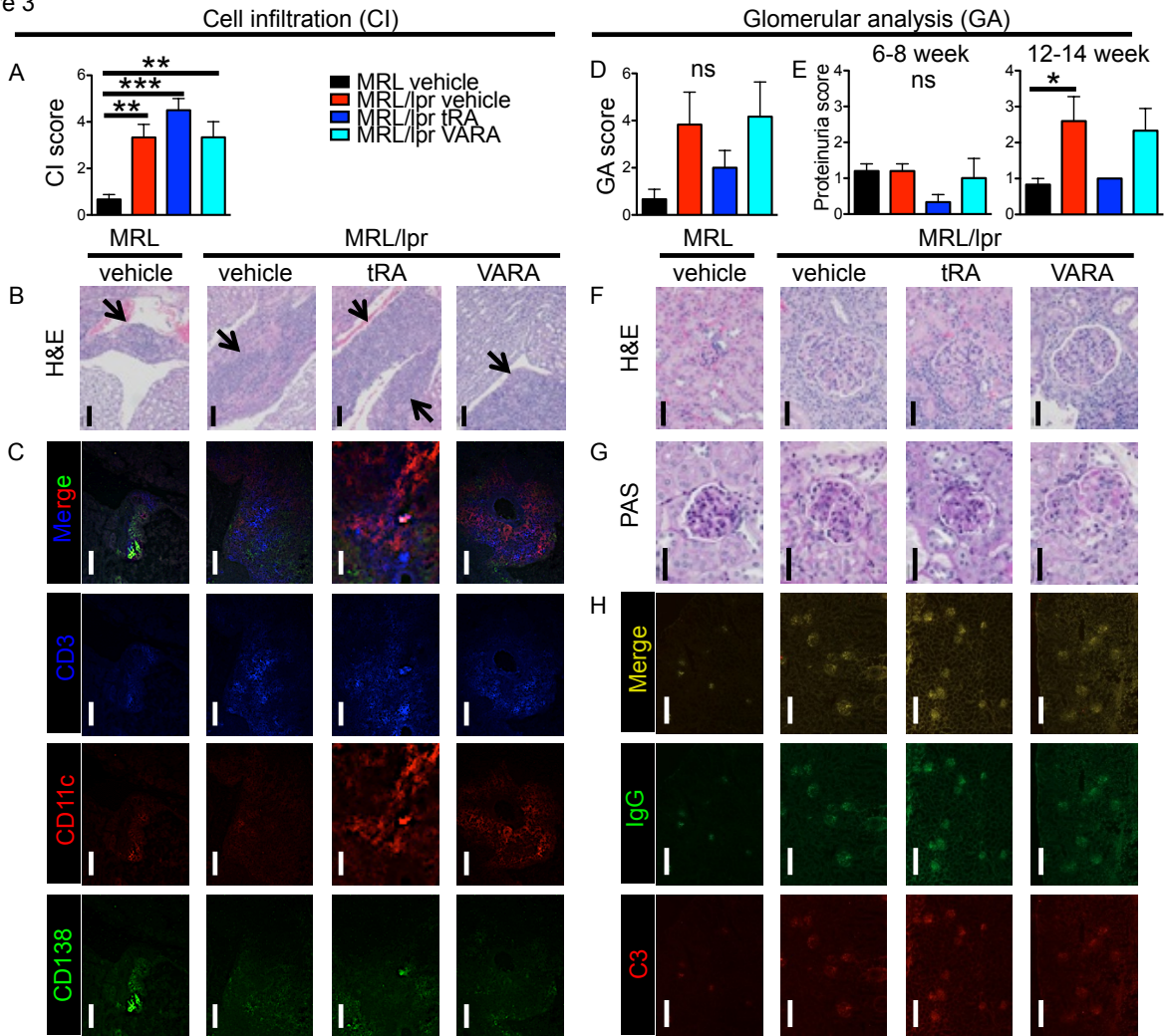
percentages of B cells (CD19<sup>+</sup>) and CD4<sup>+</sup>CD8<sup>-</sup> T cells (DN T cells, pre-gated on CD3<sup>+</sup>) in the MLN. (C, lower plots) The absolute cell numbers and percentages of DN T cells in the spleen. (D) pDCs (defined as CD11c<sup>+</sup>CD11b<sup>-</sup>B220<sup>+</sup>Siglec-H<sup>+</sup>) in the BM and MLN. (E) The absolute numbers and percentages of activated pDCs (MHC-II<sup>+</sup>CD40<sup>+</sup> or MHC-II<sup>high</sup> pDCs) in the spleen. Representative flow cytometry plots of MRL and MRL/lpr are shown. (F and G) The absolute cell numbers and percentages of CD11b<sup>-</sup> cDCs (CD11c<sup>+</sup>CD11b<sup>-</sup>B220<sup>-</sup>Siglec-H<sup>-</sup>MHC-II<sup>+</sup>) and CD11b<sup>+</sup> cDCs (CD11c<sup>+</sup>CD11b<sup>+</sup>B220<sup>-</sup>MHC-II<sup>+</sup>) in the spleen and MLN. (H) Percentages of Ly6C<sup>+</sup> cells in CD11b<sup>+</sup> cDCs in the spleen and MLN. (I) The percentage of CD40<sup>+</sup> cells in CD11b<sup>+</sup> cDCs in the spleen and the percentage of MHC-II<sup>high</sup> cells in Ly6C<sup>+</sup>CD11b<sup>+</sup> cDCs in the MLN. Representative flow cytometry plots of MRL and MRL/lpr are shown. ns: not significant, \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, student's *t*-test. Data are shown as mean + standard error of the mean (SEM), n=3 mice in each group.



**Figure 2. tRA-induced pathology in the brain and lung.** Starting from 6 weeks of age, MRL and MRL/lpr mice were given, orally and daily, vehicle (canola oil), tRA (6 mg/kg BW), or

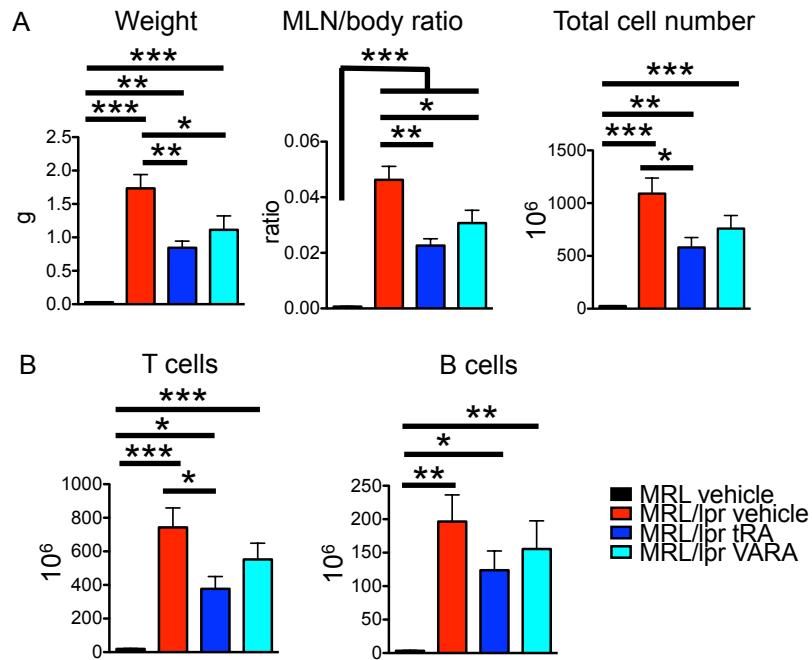
VARA (6 mg retinol and 0.6 mg tRA per kg BW) till 14 weeks old when tissues were collected. n=6 mice in each group. (A) H&E stains of the brain. Representative micrographs are shown. Bar equals 100  $\mu$ m. (B) H&E stains of the lung. Representative micrographs are shown. Bar equals 50  $\mu$ m. Arrows indicate areas of infiltration. (C-D) Leukocyte infiltration scores of the brain (C) and lung (D) according to H&E stains. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA. Data are shown as mean + SEM.

Figure 3



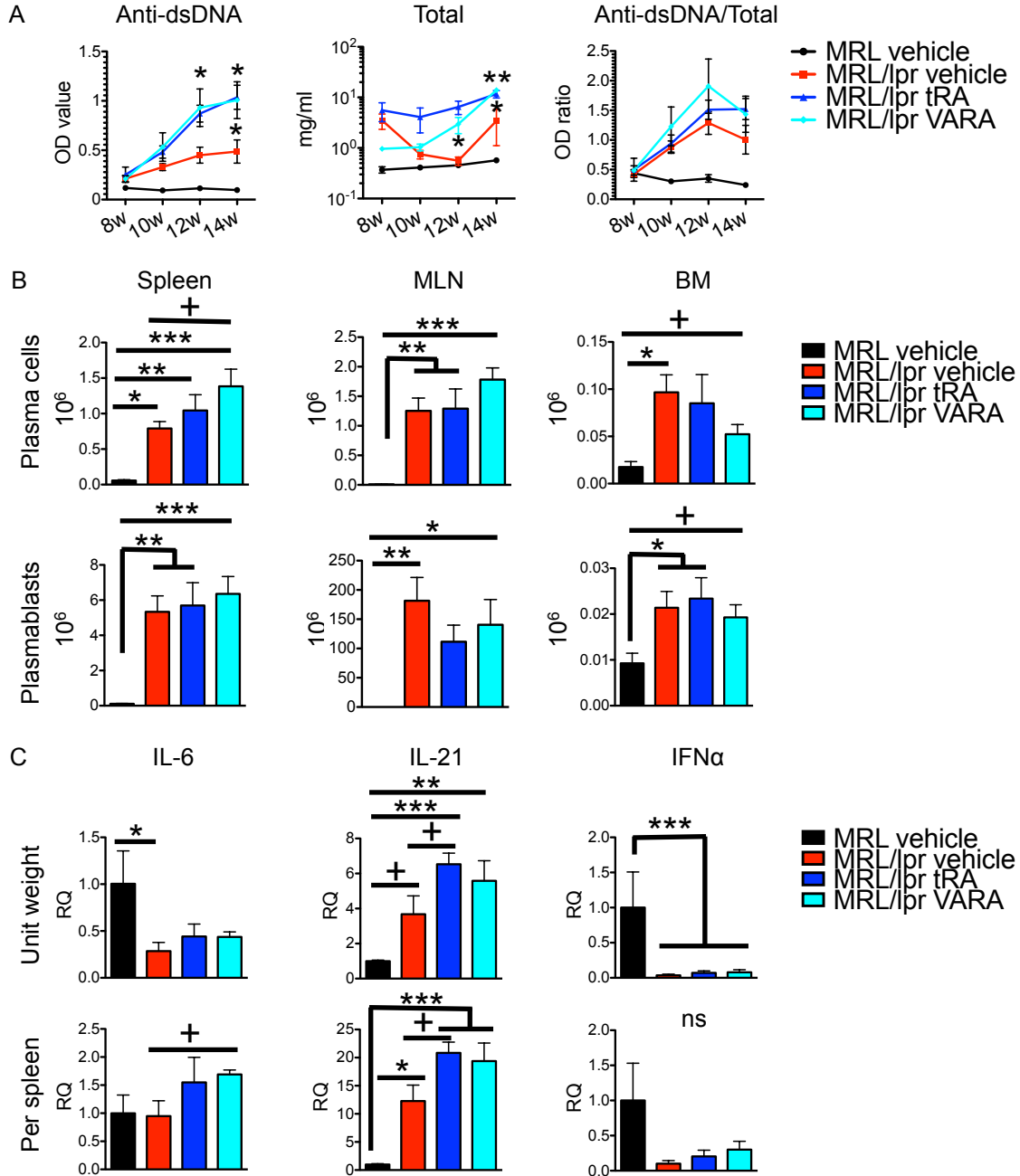
**Figure 3. tRA-mediated modulation of kidney pathology.** (A-C) Leukocyte infiltration of the tubulointerstitial region. (A) Cell infiltration (CI) scores according to H&E stains. (B) Representative micrographs of H&E stains of the tubulointerstitial region. Bar equals 100  $\mu$ m. Areas of infiltration are indicated by arrows. (C) Immunohistochemical stains of T cells (CD3-blue), dendritic cells (CD11c-red), and plasma cells (CD138-green). Representative images are shown. Bar equals 100  $\mu$ m. (D-H) Glomerular analysis (GA). (D) Average GA scores of hypercellularity, mesangial matrix expansion, necrosis, percentage of sclerotic glomeruli, and glomerular crescents. (E) Analysis of proteinuria. The level of total protein in the urine was measured weekly with Chemstrip 2GP. The data of 6- to 8-week-old time points were combined as the early stage, and those of 12- to 14-week-old time points were combined as the late stage. (F) Representative H&E stains showing kidney glomeruli. Bar equals 60  $\mu$ m. (G) PAS stains showing kidney glomeruli. Bar equals 40  $\mu$ m. (H) Immunohistochemical stains of IgG (green) and complement C3 (red) deposition in the kidney cortex. Bar equals 200  $\mu$ m. ns: not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , one-way ANOVA. Data are shown as mean + SEM (n=6 mice in each group).

Figure 4



**Figure 4. tRA-mediated decrease of lymphocyte accumulation in the MLN.** (A) MLN weight, MLN/body weight ratio, and total number of cells in MLN. (B) Absolute numbers of T and B cells in the MLN. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA. Data are shown as mean + SEM (n=6 mice in each group).

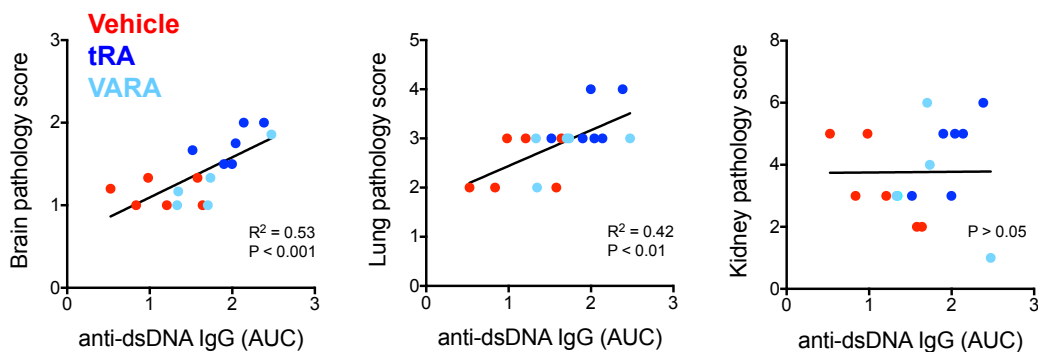
Figure 5



**Figure 5. Vitamin A-mediated increase of B-cell responses.** (A) Anti-dsDNA IgG, total IgG, and their ratios in the plasma of 8-, 10-, 12- and 14-week-old mice as determined by respective ELISA. One-way ANOVA at each time point was performed but only comparisons between a vitamin A group (either tRA or VARA) and the MRL/lpr vehicle group are labeled in the graphs

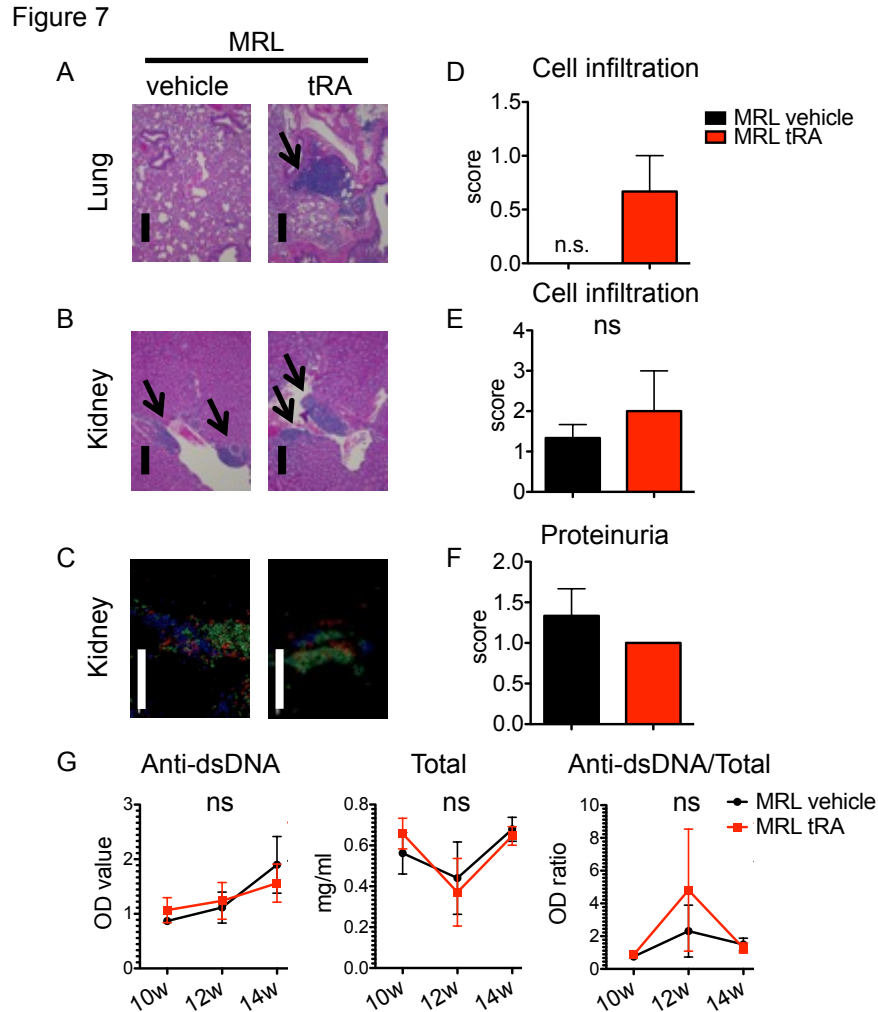
for simplicity. Although not labeled here, the MRL vehicle group is statistically different from all other groups. Data are shown as mean  $\pm$  SEM (n=6). (B) The absolute cell numbers of plasma cells (CD19<sup>+</sup>CD27<sup>-</sup>CD138<sup>+</sup>CD44<sup>+</sup>) and plasmablasts (CD19<sup>+/low</sup>CD27<sup>+/low</sup>CD138<sup>+</sup>CD44<sup>+</sup>) in the spleen, MLN, and BM at 14 weeks old as measured by flow cytometry. (C) mRNA levels of IL-6, IL-21, and IFN $\alpha$  in the spleen at 14 weeks old as determined by RT-qPCR. Relative quantities (RQ) of cytokine mRNA were normalized to that of L32. The average RQ value of MRL vehicle group was defined as 1. ns: not significant, \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA; +: P<0.05, student's *t*-test. Except for (A), data are shown as mean + SEM (n=6 mice in each group).

Figure 6



**Figure 6. Correlation analysis between blood autoantibody levels and pathological scores.**

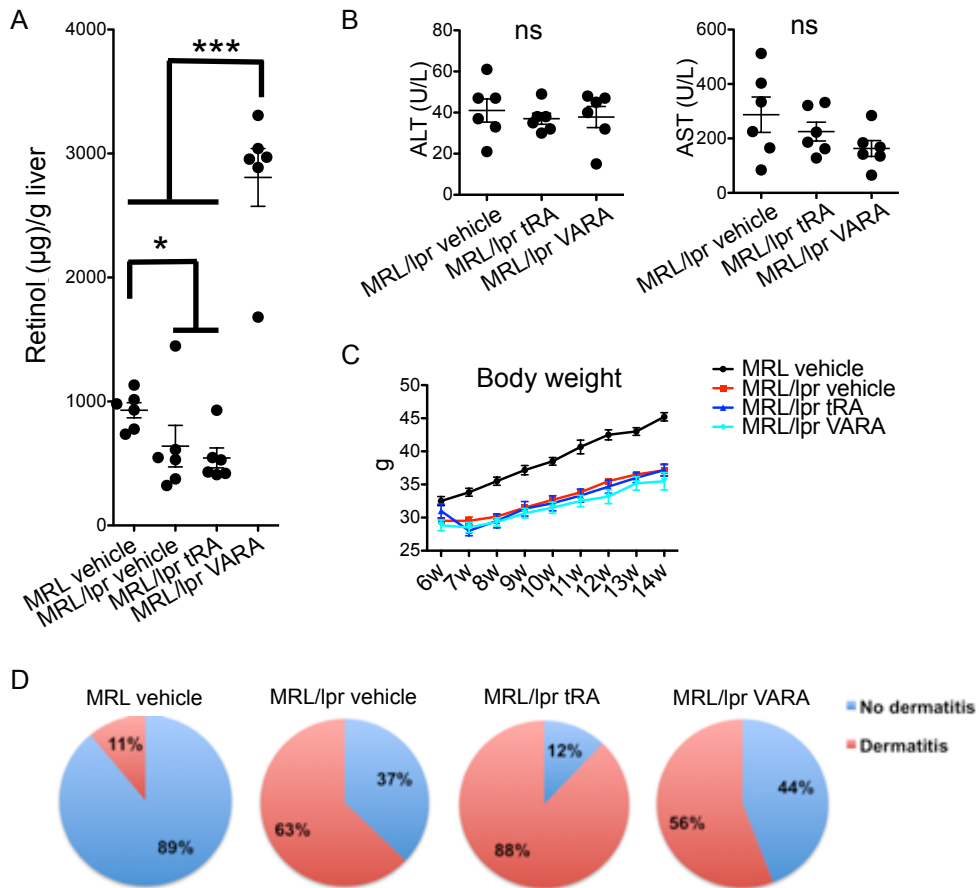
The area under the curve (AUC) was calculated for the level of anti-dsDNA IgG in the circulation and plotted against pathological scores of the brain, lung and kidney. Spearman correlation tests were performed.



**Figure 7. Minimal induction of inflammation with tRA in the absence of an immunogenic environment.** MRL mice were given, orally and daily, vehicle (canola oil) or tRA (6 mg/kg BW) from 6 to 14 weeks of age when tissues were collected. n=3 mice in each group. (A) H&E stains of the lung. Representative micrographs are shown. Bar equals 400  $\mu$ m. Areas of infiltration are indicated by arrows. (B) Representative micrographs of H&E stains of the kidney. Bar equals 400  $\mu$ m. (C) Immunohistochemical stains of T cells (CD3-green), dendritic cells (CD11c-red), and plasma cells (CD138-blue). Representative images are shown. Bar equals 200  $\mu$ m. (D-E) Leukocyte infiltration scores of the lung (D) and kidney (E) according to H&E stains.

(F) Analysis of proteinuria. The level of total protein in the urine of 14-week-old mice was measured with Chemstrip 2GP. (G) Anti-dsDNA IgG, total IgG, and their ratios in the plasma of 10-, 12- and 14-week-old mice as determined by respective ELISA. ns: not significant, student's *t*-test. Data are shown as mean + SEM or mean  $\pm$  SEM.

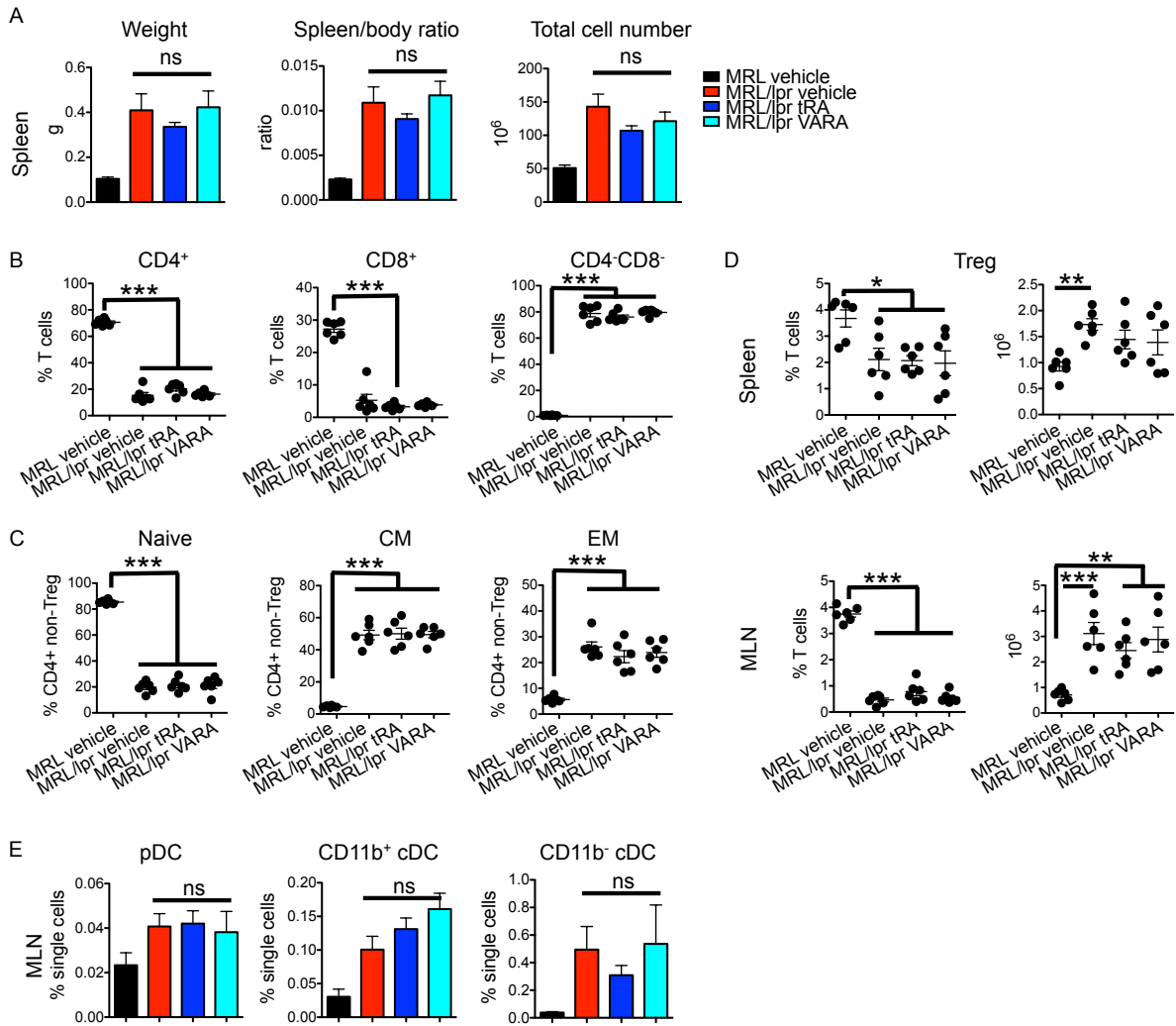
Figure S1



**Figure S1.** (A) Total retinol in the liver. (B) Liver function tests. Concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the plasma of 14-week-old mice are shown. (C) Body weight. ns: not significant. Data are shown as mean  $\pm$  SEM (n=6 mice in each group). (D) The percentage of mice with (red) or without (blue) dermatitis on the back of the neck and/or face.



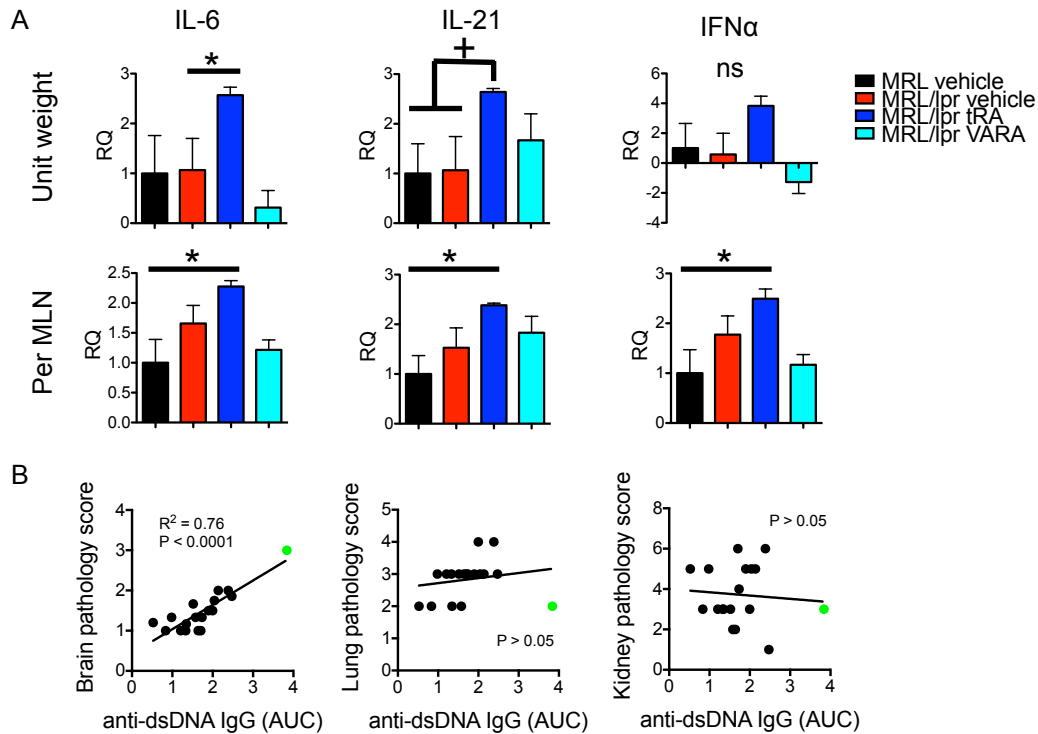
Figure S2



**Figure S2.** (A) Spleen weight, spleen/body weight ratio, and total number of cells in the spleen. ns: not significant, one-way ANOVA. (B) Percentages of CD4<sup>+</sup>CD8<sup>-</sup>, CD4<sup>-</sup>CD8<sup>+</sup> and CD4<sup>-</sup>CD8<sup>-</sup> in CD3<sup>+</sup> T cells in the MLN as determined by flow cytometry. (C) Percentages of naïve T cells (CD62L<sup>+</sup>CD44<sup>-</sup>), central memory (CM) T cells (CD62L<sup>+</sup>CD44<sup>+</sup>) and effector memory (EM) T cells (CD62L<sup>-</sup>CD44<sup>+</sup>) in CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>-</sup>CD25<sup>-</sup> (non-Treg CD4<sup>+</sup>) T cells in the MLN. (D) Percentages and absolute numbers of Tregs (CD3<sup>+</sup>CD4<sup>+</sup>Foxp3<sup>+</sup>CD25<sup>+</sup>) in the spleen and MLN. (E) Percentages of pDC (CD11b<sup>-</sup>CD11c<sup>+</sup>Siglec-H<sup>+</sup>B220<sup>+</sup>), CD11b<sup>+</sup> cDC (B220<sup>-</sup>

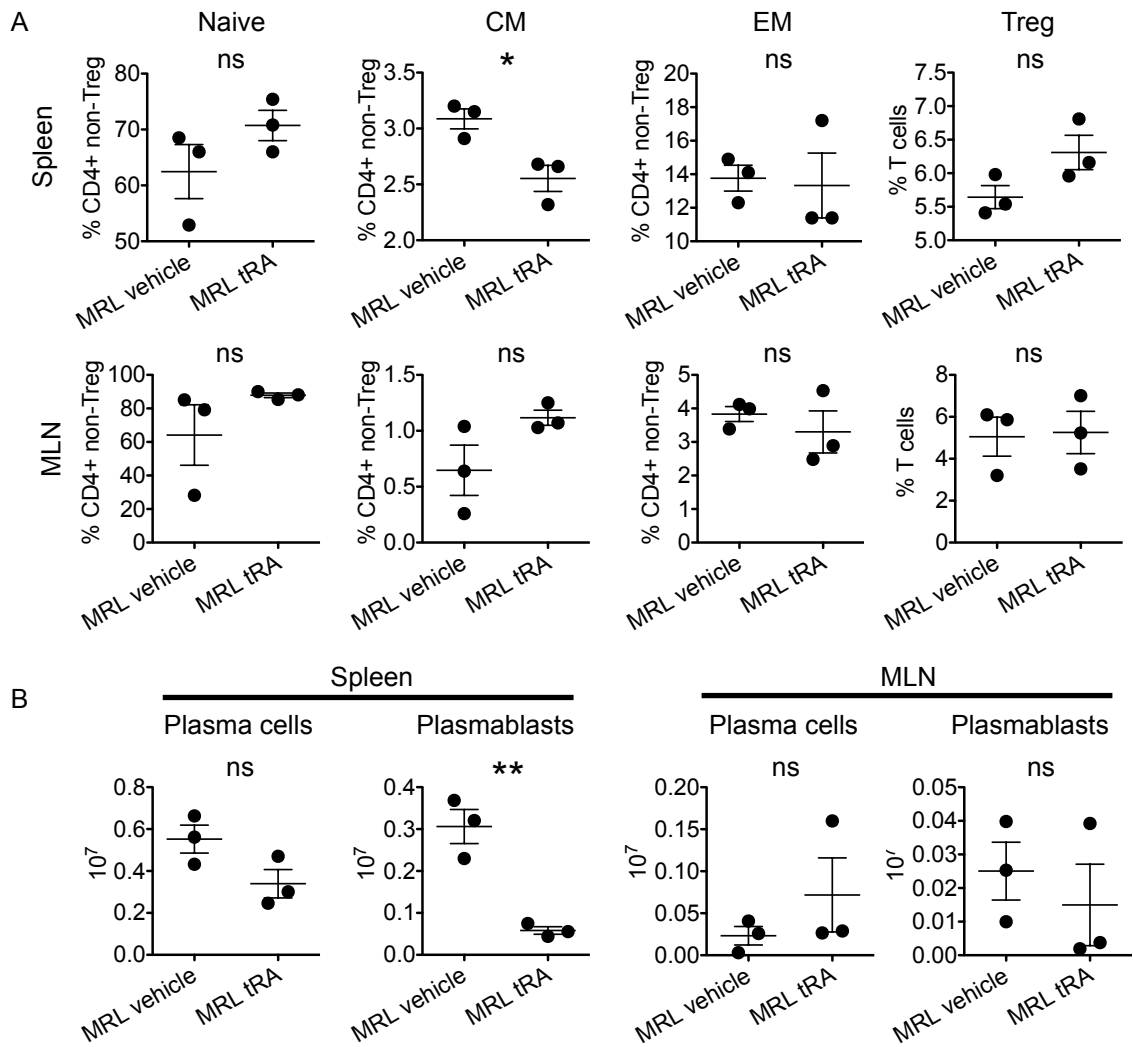
CD11c<sup>+</sup>CD11b<sup>+</sup>MHC-II<sup>+</sup>), and CD11b<sup>-</sup>cDC (CD11b<sup>-</sup>Siglec-H<sup>-</sup>B220<sup>-</sup>CD11c<sup>+</sup>MHC-II<sup>+</sup>) in the MLN. ns: not significant, \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA; +: P<0.05, student's *t*-test. Data are shown as mean + SEM or mean ± SEM (n=6 mice in each group).

Figure S3



**Figure S3.** (A) mRNA levels of IL-6, IL-21 and IFN $\alpha$  in the MLN at 14 weeks old as determined by RT-qPCR. Relative quantities (RQ, log scale) of cytokine mRNA were normalized to that of L32. The average log RQ value of MRL vehicle group was defined as 1. (B) Correlation analysis between blood autoantibody levels and pathological scores with the outlier included (shown in green).

Figure S4



**Figure S4.** (A) The absolute cell numbers of plasma cells ( $CD19^+CD27^+CD138^+CD44^+$ ) and plasmablasts ( $CD19^{+/low}CD27^{+/low}CD138^+CD44^+$ ) in the spleen and MLN at 14 weeks old as measured by flow cytometry. (B) Percentages of naïve T cells ( $CD62L^+CD44^-$ ), central memory (CM) T cells ( $CD62L^+CD44^+$ ) and effector memory (EM) T cells ( $CD62L^-CD44^+$ ) in  $CD3^+CD4^+Foxp3^-CD25^-$  (non-Treg  $CD4^+$ ) T cells, and percentages of Tregs ( $CD3^+CD4^+Foxp3^+CD25^+$ ) in  $CD3^+$  T cells in the spleen and MLN at 14 weeks old as measured

by flow cytometry. ns: not significant, \*  $P < 0.05$ , \*\*  $P < 0.01$ , student's  $t$ -test. Data are shown as mean  $\pm$  SEM (n=3 mice in each group).

## TABLES

**Table 1. Effects of tRA and VARA on MRL/lpr mice.**

Variables			Compared to vehicle		
			tRA	VARA	
Peripheral tissue pathology	Dermatitis		↑ #	– <sup>c</sup>	
	Brain pathology		↑ ***	–	
	Lung pathology		↑ *	–	
	Kidney pathology	Glomerular score		– (↓)	–
		Leukocyte infiltration (total)		– (↑)	–
		T-cell and DC infiltration		↑ #	–
		Complement/IgG deposition		↑ #	–
Proteinuria		– (↓)	–		
Lymphoid tissues	Spleen	Tissue weight	– (↓)	–	
		T cells <sup>a</sup>	–	–	
		B cells	–	–	
		Plasmablasts	–	– (↑)	
		Plasma cells	–	↑ *	
		Inflammatory cytokines <sup>b</sup>	↑ *	↑ *	
	MLN	Tissue weight	↓ **	↓ *	
		T cells	↓ *	–	
		B cells	– (↓)	–	
		Plasmablasts	–	–	
		Plasma cells	–	–	
		Inflammatory cytokines	↑ *	–	
Plasma	Anti-dsDNA IgG		↑ **	↑ *	
	Total IgG		↑ *	↑ **	

<sup>a</sup>Changes in the percentage or number of T cells, B cells, plasmablasts, and plasma cells.

<sup>b</sup>Transcript levels of IL-21 or IL-6.

<sup>c</sup>–: no change, ↑: increase, ↓: decrease; (↑) and (↓): changes that were not statistically significant; \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, student's *t*-test. # Statistical tests not performed.

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## Chapter 5. *Cutting Edge*: Plasmacytoid Dendritic Cells in Late-Stage Lupus Mice Defective in Producing IFN $\alpha$

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## ABSTRACT

Plasmacytoid dendritic cells (pDCs) are professional type I IFN producers believed to promote lupus. However, questions exist on whether they function at the same level throughout the course of lupus disease. We analyzed high purity pDCs sorted from lupus mice. Producing a large amount of IFN $\alpha$  during the initiation stage of lupus, pDCs sorted from late-stage lupus mice were found to be defective in producing IFN $\alpha$ . These pDCs expressed an increased level of MHC, suggesting a functional drift to antigen presentation. We examined the potential mechanism behind the defect and identified a novel transcriptional factor, Foxj2, which repressed the expression of several genes in pDCs. Dysregulation in pDCs appears to be predisposed, as pDCs exhibited an altered transcriptional profile before the onset of clinical signs. Our results suggest that pDCs do not function the same throughout the disease course, and lose the ability to produce IFN $\alpha$  in late-stage lupus mice.

## INTRODUCTION

It has been long recognized that type I IFNs, including IFN $\alpha$ , facilitate the progression of systemic lupus erythematosus (SLE). Based on large amounts of supporting evidence obtained with human patient samples [1-6] and murine models [7-11], 4 antibodies targeting IFN $\alpha$  or type I IFN receptor have been tested in human clinical trials for SLE [12-16]. A couple of them have shown promising efficacies (*AstraZeneca* press releases). Beneficial effect was observed in SLE patients with a “low” type I IFN signature—i.e., those with lower expression of IFN-responsive genes—where IFN $\alpha$  blockade partially inhibited such expression [13-15]. This suggests that early intervention (when the IFN signature is still low) may be required for IFN $\alpha$ -targeted therapies for SLE. Interestingly, IFN signatures returned to pre-treatment levels in all patients by 6 months after the last dose of anti-IFN $\alpha$  antibodies [17], suggesting that type I IFNs are constantly produced and pathogenic throughout disease progression. As virtually all cells can produce IFN $\alpha$  upon stimulation, identifying which types of cells play a predominant role in driving early- and late-stage SLE has become an interesting but challenging question.

Plasmacytoid dendritic cells (pDCs) were discovered as professional IFN-producing cells [18, 19]. They produce a large amount of IFN $\alpha$  in response to TLR7/9 ligation [20]. Studies of SLE patient samples have revealed that immune complexes in the patient sera are also capable of inducing IFN $\alpha$  in pDCs [21, 22], a process where neutrophil extracellular traps are involved [23, 24]. Recently, two groups of researchers have independently shown that depletion of pDCs from mouse models of SLE ameliorates lupus-like disease [25, 26]. Deletion of pDCs in pre-disease lupus-prone mice significantly reduced lymphadenopathy and improved kidney pathology later in life. Strikingly, the benefit was sustained even after the pDC population recovered [26]. This

suggests that pDCs contribute to disease early in SLE pathogenesis, and that the recovered pDCs present in late-stage lupus mice might be less pathogenic.

In this study, we have used a classical mouse model of SLE, MRL/Mp-*Fas*<sup>*lpr*</sup> (*lpr*), as well as its parent strain MRL, to explore pDC functions in lupus mice. Female *lpr* mice exhibit systemic autoimmunity and glomerulonephritis, and die at an average age of 18 weeks. Like SLE patients [27], diseased *lpr* mice show an elevated level of IFN $\alpha$  in the circulation [10, 28], but the source of this cytokine remains unclear.

## Materials and Methods

***Mice and cell sorting.*** Mice were purchased from Jackson Laboratory and bred in-house. All mice used were female. PDCs were sorted as CD11c<sup>+</sup>CD11b<sup>-</sup>PDCA1<sup>+</sup>B220<sup>+</sup>. Siglec-H inhibits IFN $\alpha$  induction and was not used for sorting. Anti-mouse antibodies were from eBioscience. RNA-seq was performed and data are available at BioProject #PRJNA284002.

***Cell culture and analyses.*** CpG (ODN1585, 5 $\mu$ M) was used to stimulate sorted pDCs or total bone marrow cells. For gene knockdown, mouse Foxj2 siRNA and negative control were purchased from Qiagen. For quantitative PCR of cDNA, iTaq<sup>TM</sup> Universal SYBR Green Supermix and ABI 7500 Fast System were used, with ribosomal protein L32 as the housekeeping gene. For FACS, cells were Fc-blocked, stained and analyzed with BD FACSAria II. Mouse IFN $\alpha$  levels were measured with PBL ELISA kit.

***Statistical analysis.*** Analysis of non-Seq data was performed with student's *t*-test, one-way ANOVA and Tukey's post-test. Results were considered statistically significant when  $P < 0.05$ .

## Results and Discussion

### IFN signatures in lupus pDCs

Early depletion of pDCs from lupus mice ameliorates disease [26]. However, whether pDCs are the primary source of IFN $\alpha$  for both early- and late-stage lupus remains unclear. We sorted pDCs (**Fig. 1A**) from the bone marrow of 6- and 16-week-old female *lpr* mice—representing pre-disease and late-stage lupus disease, respectively—and analyzed the gene expression profile. As controls, pDCs were sorted from the bone marrow of age-matched female MRL mice. MRL mice carry wild-type *Fas* and display lupus much later than *lpr*. They appear normal at 6 weeks and are considered to be at pre-disease stage at 16 weeks [29]. Therefore, our mouse groups included 16-week-old *lpr* (late-stage lupus), 6-week-old *lpr* and 16-week-old MRL (pre-disease), and 6-week-old MRL mice (control). While mouse pDCs are most enriched in the bone marrow than other organs, they are still a minor population in the bone marrow [29]. Rigorous quality control measures were taken to ensure accurate processing of samples. Measurement of pDC-specific transcription factor *E2-2* showed high purity of our sorted cells (**Fig. 1B**). As B-cell progenitors in the bone marrow share surface markers with pDCs, we measured the expression levels of B-cell genes *Cd20*, *Ebf1*, *Vpreb3* and *Igha*. Less than 5% of our sorted pDCs might have been contaminated with B-cell progenitors (data not shown). With high purity pDCs, we found that many IFN-responsive genes were upregulated comparing 16-week-old *lpr* mice to age-matched MRL mice (**Fig. 1D**, highlighted with yellow bars). This suggests that bone marrow pDCs in late-stage *lpr* mice exhibit both type I and II IFN signatures indicating previous exposure to IFNs.



### PDCs from late-disease lupus mice unable to produce IFN $\alpha$ upon CpG stimulation

We next identified signaling pathways based on the downstream transcript signatures. Among 5 top canonical pathways identified, 4 were found in the comparison between 16-week-old *lpr* mice and age-matched MRL mice (**Fig. 2A**). A common feature of the 4 pathways was upregulation of MHC molecules (data not shown). Detection of MHC-II on the surface of pDCs confirmed its upregulation in *lpr* mice with late-stage disease (**Fig. 2B**). This suggests that pDCs from late-stage lupus mice might excel at antigen presentation, a phenomenon similar to human SLE where patient pDCs have been shown to be better antigen-presenting cells than normal pDCs [30].

We next questioned whether pDCs isolated from older *lpr* mice, expressing a higher level of MHC-II, were still capable of producing IFN $\alpha$  upon stimulation. A previous study showed some defect in IFN $\alpha$  production from a mixed population of bone marrow dendritic cells from late-stage lupus mice [31]. With sorted pDCs of high purity, we showed almost complete loss of CpG-mediated induction of IFN $\alpha$  from late-stage lupus pDCs (**Fig. 2C**). Interestingly, pDCs from 16-week-old MRL mice (pre-disease) showed enhanced IFN $\alpha$  production, consistent with a significant lower level of MHC-II on the surface of these cells (Fig. 2B). This suggests that pDCs produce a large amount of IFN $\alpha$  to initiate the disease. While the bone marrow has the highest percentage of pDCs [29], *lpr* mice with late-stage disease possess very large lymph nodes increasing the absolute numbers of pDCs. Indeed, we found that pDCs were mainly found in lymph nodes in 16-week-old *lpr* mice (**Fig. 2D**). However, lymph node pDCs were also found to be defective in producing IFN $\alpha$  (**Fig. 2E**). This suggests that the high level of IFN $\alpha$  in late-stage *lpr* mice [10, 28] may not have come from pDCs. Additionally, we measured the transcript level of *Atg7*, a gene required for IFN $\alpha$  induction by immune complexes [32], and found it be

significantly lower in pDCs from 16-week-old *lpr* mice than age-matched MRL mice (**Fig. 2F**). This suggests that immune complex-mediated IFN $\alpha$  production from pDCs may be defective as well. Interestingly, we found a significantly higher level of *Sca1* in late-stage lupus pDCs; in contrast, the expression of this gene was negligible in pDCs from 16-week-old MRL mice (**Fig. 2G**). This observation is consistent with a previous report that *Sca1* expression on pDCs was negatively correlated with their ability to produce IFN $\alpha$  [33]. Lastly, we confirmed that the defect of pDCs to produce IFN $\alpha$  in late-stage lupus mice was not exclusive for the *lpr* model. In another classical SLE mouse model NZB/W F1, pDCs also exhibited reduced capability to produce IFN $\alpha$  during late stage compared to early disease (**Fig. 2H**). These results suggest that pDCs from late-disease lupus mice lose their ability to produce IFN $\alpha$ . Instead, they express more MHC molecules and may become better antigen-presenting cells.

### Defect of IFN $\alpha$ production in pDCs independent of the transcriptional repressor Foxj2

We next investigated the mechanism behind the functional loss of pDCs to produce IFN $\alpha$ . Upstream receptors leading to IFN $\alpha$  induction in pDCs, including TLR9 for DNA antigens and Fc receptors for immune complexes, were expressed in pDCs isolated from late-stage lupus mice (**Fig. 3A** and Supplemental Fig. 1A). We also measured the transcript level of another potential sensor TLR7 and found it to be overexpressed in these pDCs (Supplemental Fig. 1B). This suggests that the defect of IFN $\alpha$  production was not due to a lack of upstream receptors. However, the problem seemed to be related to transcription or RNA stability, as pDCs from late-stage lupus mice expressed little IFN $\alpha$  mRNA upon CpG stimulation (**Fig. 3B**). Thus, we explored a novel strategy of using RNA-seq data to identify differentially expressed genes that

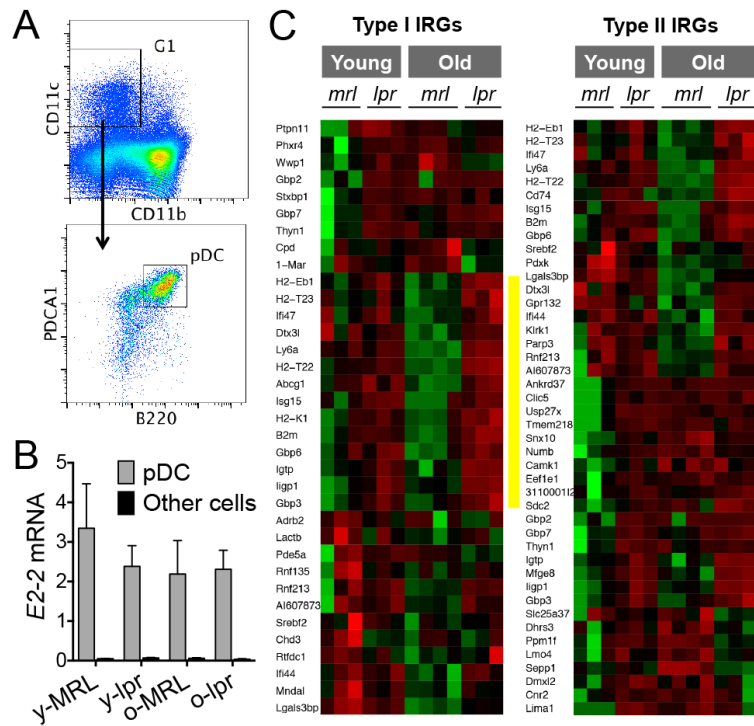
followed the same or opposite pattern of CpG-induced IFN $\alpha$ . We reasoned that common machineries might modulate IFN $\alpha$  transcripts together with other gene transcripts. By studying regulatory elements of the other genes, we might be able to identify the mechanism behind the defect of IFN $\alpha$  production. To identify genes with the same expression pattern as CpG-induced IFN $\alpha$  (Fig. 2C), we set the following criteria: (1) expression in pDCs of older MRL mice had to be  $\geq 2$ -fold higher than that of younger MRL mice; (2) expression in pDCs of older *lpr* mice had to be  $\geq 2$ -fold lower than that of older MRL mice. Similar criteria were applied to identify genes of the opposite pattern. Using this strategy we identified a total of 7 genes (**Fig. 3C**). We searched for shared microRNAs using DIANA-microT but found no matches. However, promoter analysis revealed two shared *cis*-regulatory elements that were present in the promoter regions of all genes except one (Supplemental Fig. 1C). One of the two motifs, TGTTT, can be bound by 3 members of the Fox family of transcription factors (Supplemental Fig. 1D). Among them, only Foxj2 was expressed in pDCs. In *lpr* mice, Foxj2 was expressed significantly higher in pDCs than other bone marrow cells (**Fig. 3D**). The other shared *cis*-regulatory element was an Rreb1-binding site, but Rreb1 was not preferentially expressed in pDCs (Supplemental Fig. 1E).

We hypothesized that Foxj2 overexpression in pDCs repressed the transcription of both IFN $\alpha$  and genes with the same pattern (*Afap1*, *Numb*, *Tmem201*). To test this hypothesis, we used siRNA to knockdown Foxj2 in pDCs from late-stage lupus mice (**Fig. 3E**). Unexpectedly, such knockdown did not restore IFN $\alpha$  production in pDCs from *lpr* mice (**Fig. 3F**), suggesting that the defect was independent of Foxj2. In contrast, knockdown of Foxj2 did increase the transcript levels of *Afap1*, *Numb*, and *Tmem201* (**Fig. 3G**). Although Foxj2 does not seem to directly repress IFN $\alpha$  transcription, our results suggest that Foxj2 is a functional transcriptional repressor involved in shaping the gene expression profile of pDCs in late-stage lupus mice.

Additional comparisons revealed that 207 genes were differentially expressed when comparing pDCs in older vs. younger MRL mice (Supplemental Fig. 2A, green). In contrast, only 25 genes were differentially expressed when comparing pDCs in older vs. younger *lpr* mice (blue). This suggests that there might be little changes in transcript abundance in pDCs as lupus progressed from pre-disease stage to late stage. We thus hypothesized that an altered gene expression profile, i.e., a “lupus-prone” profile, might be already present in pDCs prior to disease onset. To identify “lupus-prone” genes independent of *Fas*<sup>*lpr*</sup> mutation—as few SLE patients carry mutations of the *Fas* gene—we investigated whether pDCs from older MRL mice at pre-disease stage of lupus would share differentially expressed genes with pDCs from younger *lpr*, which represents pre-disease stage for the *lpr* mouse strain. We identified 54 such genes, including 44 protein-coding genes (Supplemental Fig. 2B/C) and 10 long unannotated transcripts (Supplemental Fig. 2D/E). The significance of the “lupus-prone” profile in pDCs remains to be explored. However, our findings raise an important question of whether pDCs in SLE patients or lupus mice were “born” with the predisposed, dysregulated gene expression profile. In future investigations, we will study pDCs from even younger mice to determine potential early defects, and whether triggers later in life might manifest the defects.

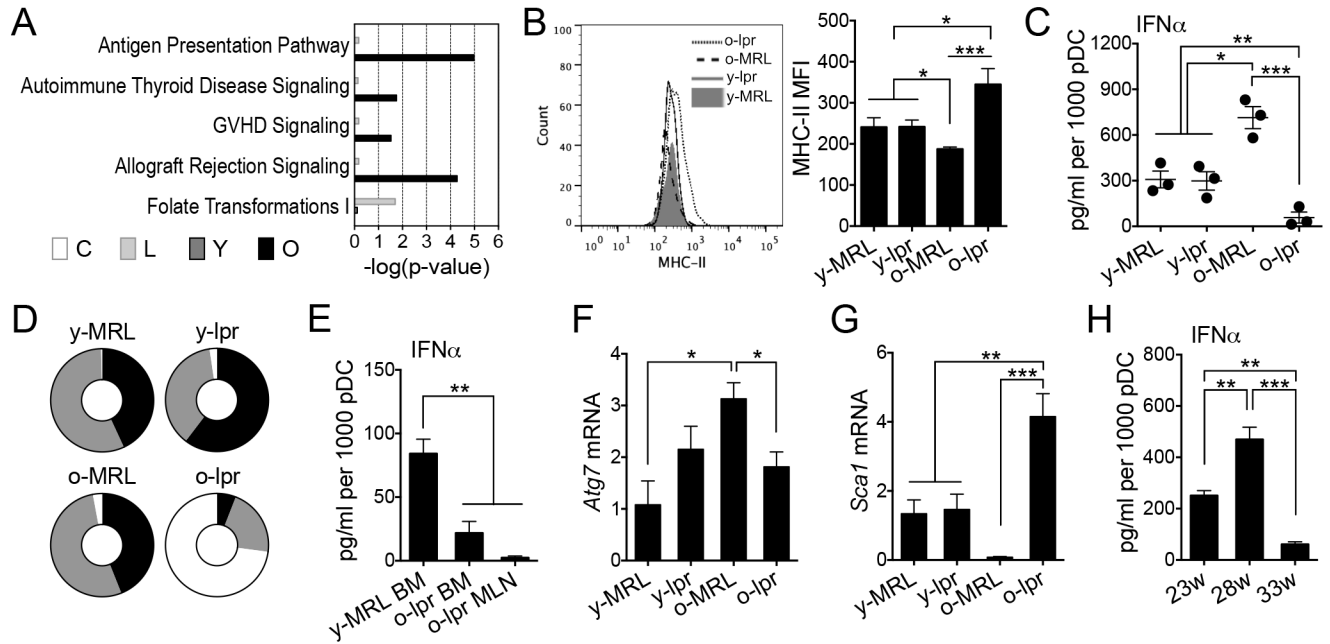
## FIGURES

**Fig 1.**



**Figure 1. IFN signatures in pDCs sorted from lupus mice. (A)** Sorting strategy. **(B)** Transcript level of pDC-specific gene *E2-2* in sorted pDCs as compared to pDC-depleted bone marrow cells (other cells). **(C)** Heatmaps of type I & II IFN-responsive genes (IRGs). Red, upregulation. Green, downregulation. Yellow bars highlight the genes that were upregulated in older *lpr* vs. older MRL mice.

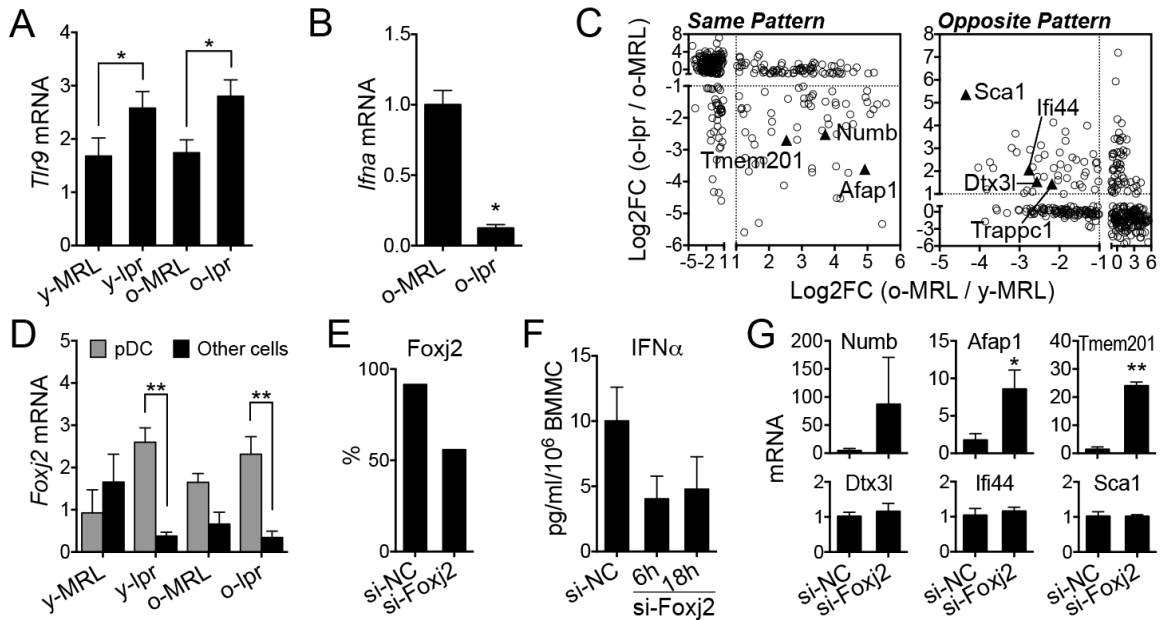
Fig 2.



**Figure 2. PDCs from late-stage lupus mice unable to produce IFN $\alpha$  upon CpG stimulation.**

**(A)** Ingenuity Pathway Analysis showing the top canonical pathways. C, older vs. younger MRL; L, older vs. younger *lpr*; Y, younger *lpr* vs. younger MRL; O, older *lpr* vs. older MRL. **(B)** MHC-II expression on bone marrow pDCs. MFI, mean fluorescence intensity. y, younger; o, older. **(C)** Production of IFN $\alpha$  in sorted bone marrow pDCs upon CpG stimulation for 6 h. **(D)** Distribution of pDCs among bone marrow (black), spleen (gray) and mesenteric lymph node (white). **(E)** Production of IFN $\alpha$  from sorted pDCs upon CpG stimulation for 6 h. BM, bone marrow. MLN, mesenteric lymph node. **(F)** Transcript level of *Atg7* in sorted pDCs. **(G)** Transcript level of *Sca1* in sorted pDCs. **(H)** Production of IFN $\alpha$  from bone marrow pDCs from 23-, 28- and 33-week-old NZB/W F1 female mice upon CpG stimulation for 6 h. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, ANOVA,  $n \geq 3$  mice per group. Error bars equal standard errors.

**Fig.3**



**Figure 3. Defect of IFN $\alpha$  production in pDCs independent of the transcriptional repressor**

**Foxj2.** (A) Transcript level of *Tlr9* in sorted pDCs. (B) Transcript level of *IFN $\alpha$*  in pDCs from older mice stimulated with CpG for 6 h. (C) Differentially expressed genes following the same or opposite pattern of CpG-induced IFN $\alpha$ . Log<sub>2</sub>FC, log<sub>2</sub> fold changes. (D) Transcript level of *Foxj2* in sorted pDCs and pDC-depleted bone marrow cells (other cells). (E) Transcript level of *Foxj2* with negative control siRNA (si-NC) or *Foxj2*-specific siRNAs (si-*Foxj2*) in bone marrow cells from *lpr* mice. Percentages from the untransfected control are shown. (F) Production of IFN $\alpha$  in total bone marrow cells (BMMC) isolated from 16-week-old *lpr* mice, transfected with siRNAs, and stimulated with CpG for 6 or 18 h. (G) Transcript levels of other genes after siRNA transfection. Fold differences are shown. \* $P < 0.05$ ,

\*\* $P < 0.01$ , student's *t*-test,  $n \geq 3$  mice per group.

Fig S1.

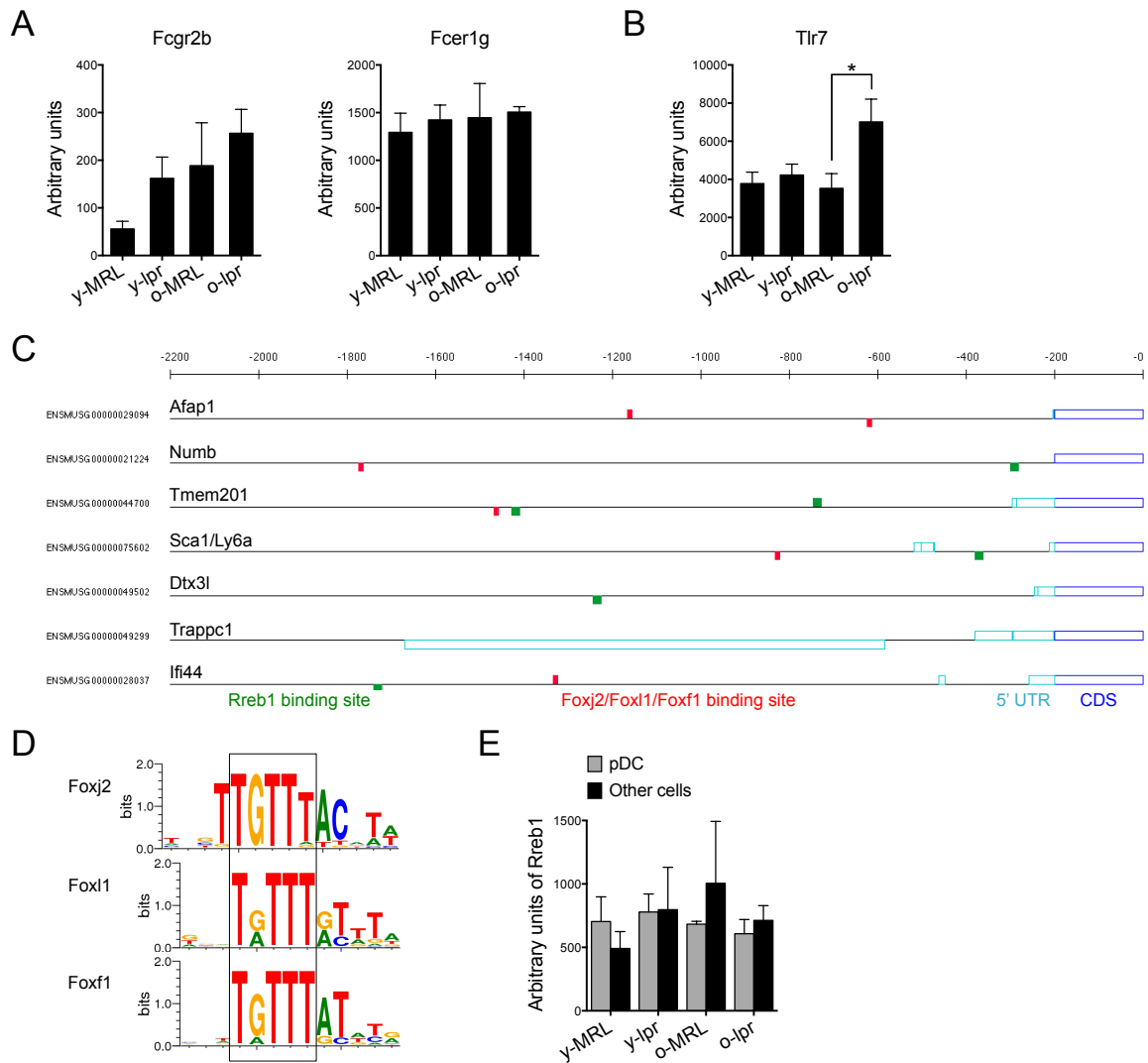


Figure S1. **PDCs unable to produce IFN $\alpha$  in late-stage lupus mice.** (A) Transcript levels of immune complex receptors Fcgr2b and Fcer1g. PDCs from these mice did not express Fcgr1 or Fcgr3. (B) Transcript levels of Tlr7. \*P<0.05, Student's t-test, n=3 per group. (C) Promoter analysis by using TOUCAN



(<https://gbiomed.kuleuven.be/english/research/50000622/lcb/tools/toucan>). (D) Shared binding motif of Foxj2, Foxl1 and Foxf1. Maps were provided by MotifMap (<http://motifmap.ics.uci.edu/>). Only Foxj2 was expressed in the pDCs. (E) Transcript levels of Rreb1 in pDCs and pDC-depleted cells (other cells).

Fig S2.

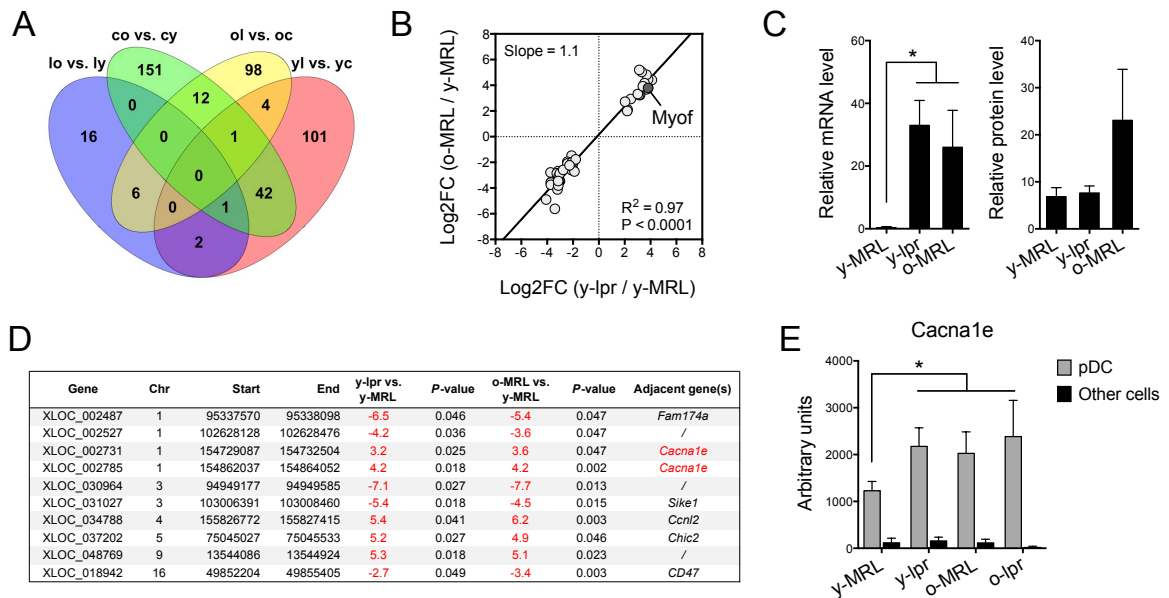


Figure S2. **Predisposed dysregulation of pDCs in lupus mice.** (A) Venn diagram showing the number of differentially expressed genes in 4 comparisons (FDR<0.1). Blue: older vs. younger in MRL/lpr mice. Green: older vs. younger in MRL mice. Yellow: MRL/lpr vs. MRL in older mice. Red: MRL/lpr vs. MRL in younger mice. (B) Log2 fold changes (FC) of 44 overlapped, differentially expressed genes in both Green and Red. (C) Transcript levels of Myof in sorted pDCs and protein levels of Myof in bone marrow pDCs. For protein levels, percentage and mean fluorescence intensity of Myof+ pDCs were measured by using flow cytometry and the numbers were multiplied to represent the total expression level. \*P<0.05, student's t-test. Data are shown

as mean + SEM (n=3 mice per group). (D) List of predicted lncRNAs that were overlapped. (E) Transcript level of Cacna1e, a gene next to predicted lncRNAs XLOC\_002731 and XLOC\_2785 on Chromosome 1. This gene appeared to be pDC-specific, and was upregulated in lupus mice with either early or late disease. The two predicted lncRNAs were downregulated during lupus progression. \*P<0.05, Student's t-test, n=3 per group.

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## **Chapter 6. Renal-infiltrating CD11c<sup>+</sup> cells are pathogenic in murine lupus nephritis through promoting CD4<sup>+</sup> T cell responses**

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## SUMMARY

Lupus nephritis (LN) is a major manifestation of systemic lupus erythematosus (SLE) causing morbidity and mortality in 40-60% of SLE patients. The pathogenic mechanisms of LN are not completely understood. Recent studies have demonstrated the presence of various immune cell populations in lupus nephritic kidneys of both SLE patients and lupus-prone mice. These cells may play important pathogenic or regulatory roles *in situ* to promote or sustain LN. Here, using lupus-prone mouse models, we showed the pathogenic role of a kidney-infiltrating CD11c<sup>+</sup> myeloid cell population in LN. These CD11c<sup>+</sup> cells accumulated in the kidneys of lupus-prone mice as LN progressed. Surface markers of this population suggest their dendritic cell identity and differentiation from Ly6C<sup>low</sup> mature monocytes. The cytokine/chemokine profile of these renal-infiltrating CD11c<sup>+</sup> cells suggests their roles in promoting LN, which was further confirmed in a loss-of-function *in vivo* study by using an antibody-drug conjugate (ADC) strategy targeting CX<sub>3</sub>CR1, a chemokine receptor highly expressed on these CD11c<sup>+</sup> cells. However, CX<sub>3</sub>CR1 was dispensable for the homing of CD11c<sup>+</sup> cells into lupus nephritic kidneys. Finally, we found that these CD11c<sup>+</sup> cells co-localized with infiltrating T cells in the kidney. Using an *ex vivo* co-culture system, we showed that renal-infiltrating CD11c<sup>+</sup> cells promoted the survival, proliferation and IFN $\gamma$  production of renal-infiltrating CD4<sup>+</sup> T cells, suggesting a T cell-dependent mechanism by which these CD11c<sup>+</sup> cells promote LN. Together, our results identify a pathogenic kidney-infiltrating CD11c<sup>+</sup> cell population promoting LN progression, which could be a new therapeutic target for the treatment of LN.

## Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease induced by the breach of systemic immunotolerance to self antigens that are typically nuclear components and phospholipids [1, 2]. It manifests as persistent inflammation in multiple organs including kidney, lung, skin, heart, joints and brain [3]. Among different manifestations, lupus nephritis (LN), which is the inflammation of the kidney, occurs in about 50% of SLE patients and is a major cause of morbidity and mortality in SLE patients [4]. Commonly used anti-inflammatory and immunosuppressive chemical drugs have significantly prolonged the survival of patients with LN [3], but systemic side effects are a major cause of concern. Recently, several antibody-based products aimed to perturb specific immune reactions have been developed and evaluated in clinical trials to reduce or replace the use of chemical drugs [3, 5, 6]. However, these treatments are not specific for autoimmune reactions that they can compromise the normal immune response to infections [7, 8]. In addition, some LN patients are resistant to current standard of care treatments or experience relapses of symptoms [9]. Therefore, it is urgent and necessary to continue investigating the kidney-specific immune mechanisms behind LN, which may lead to the development of more effective drugs with fewer side effects.

LN is known to be initiated by renal deposition of high-affinity autoantibodies generated by activated autoreactive B cells with the help from activated autoreactive T cells [3, 10]. However, the downstream immune mechanisms causing the progression of LN are not well understood. Studies have shown that different types of leukocytes including various T cell subsets, B cells, plasma cells, natural killer cells, monocytes/macrophages, dendritic cells (DCs) and neutrophils are accumulated in the kidneys of both patients and lupus-prone mice with active LN [11, 12].

Using lupus-prone mice, one study further demonstrated that LN could still develop in the absence of humoral immune responses, and such development was associated with leukocyte infiltrations in the kidney [13]. This highlights the critical roles of cellular immune responses in LN progression.

Among different renal-infiltrating cell types, DCs are of interest because there are many subtypes of DCs and that each of them has specific and diverse immune functions through interacting with other immune cells [14]. CD11c as a general surface marker for DCs has been utilized in lupus-prone mice to demonstrate the pathogenic roles of CD11c<sup>+</sup> cells in SLE, particularly in the development of LN [15-20]. However, as CD11c is also expressed on some macrophages [14], it is unclear whether the pathogenic CD11c<sup>+</sup> cells belong to DCs; and if they do, which subpopulation(s) of DCs are more important for LN progression. Plasmacytoid DCs (pDCs), a subpopulation of DCs, have been demonstrated to be critical in initiating autoimmune responses in SLE by producing IFN $\alpha$  [17, 18]. Unlike pDCs, the role of conventional DCs (cDCs) subsets in LN remains unclear. The aim of this study was to reveal the pathogenic roles of cDCs in the development of LN. Here, using lupus-prone mice, we show that a subpopulation of CD11c<sup>+</sup> cells with surface markers representing mature monocyte-derived cDCs accumulates in lupus nephritic kidneys, and has a pathogenic role in promoting LN at least partially by enhancing kidney-infiltrating T cell responses.



## Materials and methods

### *Mice*

MRL/Mp (MRL), MRL/Mp-*Fas*<sup>lpr</sup> (MRL/lpr), NZW/Lac (NZW), NZBWF1 (NZB/W) and B6-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility following the requirements of Institutional Animal Care and Use Committee (IACUC) at Virginia Polytechnic Institute and State University. MRL and MRL/lpr are classical mouse models of LN. Mice with MRL background possess multiple SLE susceptibility loci and exhibit autoimmune disorders similar to SLE-associated manifestations in humans. With the spontaneous mutation *Fas*<sup>lpr</sup>, MRL/lpr mice show lymphadenopathy and glomerulonephritis early in life. Female MRL/lpr mice die at an average age of 16 weeks. These mice develop severe kidney disease, with proteinuria detectable starting at 8 weeks of age. In contrast, the parent and control strain (MRL) does not show any sign of kidney disease before 18 weeks of age. Nevertheless, MRL mice are another lupus-prone mouse model that develops kidney disease at around 9 months of age. Like MRL and MRL/lpr mice, NZB/W mice display high levels of antinuclear antibodies, proteinuria, and glomerulonephritis. The time course of LN in NZB/W mice is similar to that in MRL mice.

### *Leukocyte isolation*

For kidney leukocytes, kidneys were cut into 1-2 mm<sup>3</sup> pieces and digested in 5 ml digestion buffer [10% fetal bovine serum (FBS), 10 mM HEPES, 1 mg/ml collagenase D (Sigma-Aldrich, St. Louis, MO) and 0.2 mg/ml DNase I (Sigma-Aldrich) in RPMI 1640 medium] for 30 min at 37°C with gentle shaking. Ice-cold 1× phosphate buffered saline (PBS, 10 ml) containing 10 mM Ethylenediaminetetraacetic acid (EDTA) was added, followed by another 5 min of incubation on

ice. After being vortexed several times, tissue pieces were filtered, smashed and washed twice with wash buffer [0.1% bovine serum albumin (BSA), 5 mM EDTA and 10 mM Hepes in 1× Hank's balanced salt solution (HBSS) without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , all from Life Technologies, Grand Island, NY] through 70- $\mu\text{m}$  cell strainers. The flow-through solution containing cells was then centrifuged. The cell pellet was resuspended in 10 ml 37% Stock Isotonic Percoll (SIP) solution (100% SIP was made by mixing 9 volume Percoll with 1 volume 10× PBS; then the 100% SIP was diluted with wash buffer to make 70% SIP and 37% SIP) and carefully layered on the top of 5 ml 70% SIP. After centrifugation at 1000×g without break for 30 min at room temperature, enriched leukocytes were collected from the layer between 37% and 70% SIP. For bone marrow mononuclear cells, bones from both hind limbs of each mouse were cracked gently in a mortar containing PBS by using a pestle. Bone marrow was released by gentle stirring after the addition of C10 medium (RPMI 1640, 10% FBS, 1 mM sodium pyruvate, 1% 100× MEM non-essential amino acids, 10 mM Hepes, 55  $\mu\text{M}$  2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, all from Life Technologies). The suspension was cleared by passing through a 70- $\mu\text{m}$  sterile cell strainer and carefully layered on the top of Ficoll-Paque Plus (GE Healthcare, Pittsburg, PA). After centrifugation at 1,363×g without break for 30 min at room temperature, mononuclear cells in the buffy coat layer were collected. For direct flow cytometry detection, spleens and all lymph nodes in the mesenteric region (MLN) were collected and mashed in 70- $\mu\text{m}$  cell strainers with C10. For purification of splenic  $\text{CD8}^+$  cDCs, spleens were injected with 500  $\mu\text{l}$  digestion buffer (as used in kidney digestion), cut into 1-2  $\text{mm}^3$  pieces, and digested in 5 ml digestion buffer for 30 min at 37°C with gentle shaking. Ice-cold 1× PBS containing 10 mM EDTA was added, followed by another 5 min of incubation on ice. After being pipetted several times, cell suspensions were filtered through a 70- $\mu\text{m}$  cell strainer. The remaining tissue pieces

in the cell strainer were smashed and washed. For total splenocytes or purification of CD8<sup>+</sup> DCs from the spleen, red blood cells were lysed with RBC lysis buffer (eBioscience, San Diego, CA).

#### *In vivo antibody-drug conjugate (ADC) treatment*

Anti-mouse CX<sub>3</sub>CR1-biotin (clone SA011F11, Biolegend, San Diego, CA) and streptavidin-saporin (Advanced Targeting Systems, San Diego, CA) were mixed at 1:1 molar ratio to make ADC according to the manufacturer's instructions. ADC was aliquoted and stored at -20°C. Six-week-old MRL/lpr females were randomly divided into two groups (ADC group and control group) with 4 mice in each group. ADC (6 µg per dose) or 1× PBS was intravenously injected into each mouse, weekly from 8-week-old to 15-week-old. Body weight and proteinuria score were monitored weekly. Mice were euthanized at 15-week-old to collect plasma, splenocytes, kidney leukocytes and kidney tissues for further study.

#### *Ex vivo T cell stimulation in a co-culture system*

Isolated leukocytes from the kidney of 4-month-old MRL/lpr females were blocked with anti-mouse CD16/32 (clone 93, eBioscience) and then stained with anti-mouse CD45-PE (clone 30-F11, eBioscience), CD11c-PerCP-Cy5.5 (clone HL3, BD Biosciences, San Jose, CA), CD11b-APC-Cy7 (clone M1/70, BD Biosciences), CD4-APC (clone RM4-5, eBioscience), and CD8-PE-Cy7 (clone 53-6.7, BD Biosciences). CD45<sup>+</sup> leukocytes were further enriched by anti-PE microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Enriched leukocytes were resuspended in sorting buffer (2% FBS, 10 mM HEPES, 2 mM EDTA in 1× PBS) containing 2 µM 4',6-diamidino-2-phenylindole (DAPI; Life Technologies) and sorted with BD FACSAria II flow cytometer (BD Biosciences). CD11c<sup>+</sup> cells were sorted as

DAPI<sup>-</sup>CD45<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>, CD4<sup>+</sup> T cells were sorted as DAPI<sup>-</sup>CD45<sup>+</sup>CD11c<sup>-</sup>CD11b<sup>-</sup>CD4<sup>+</sup>CD8<sup>-</sup>, CD8<sup>+</sup> T cells were sorted as DAPI<sup>-</sup>CD45<sup>+</sup>CD11c<sup>-</sup>CD11b<sup>-</sup>CD4<sup>-</sup>CD8<sup>+</sup>, and double negative (DN) T cells were sorted as DAPI<sup>-</sup>CD45<sup>+</sup>CD11c<sup>-</sup>CD11b<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup>. For T cell stimulation,  $1 \times 10^5$  CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and DN T cells were respectively resuspended in 200  $\mu$ l C10 containing 2  $\mu$ g/ml anti-mouse CD28 (clone 37.51, BD Biosciences) and cultured in a 96-well flat-bottom plate (Costar 3595, tissue culture treated nonpyrogenic polystyrene) coated with 10  $\mu$ g/ml anti-mouse CD3 (clone 145-2C11, BD Biosciences) for 3 days. Supernatants were harvested and stored at -80 °C. For co-culture experiments, sorted CD4<sup>+</sup> T cells were stained with 5  $\mu$ M carboxyfluorescein succinimidyl ester (CFSE). CD4<sup>+</sup> T cells ( $1 \times 10^5$ /well), either alone or mixed with  $5 \times 10^4$  CD11c<sup>+</sup> cells, were resuspended in 200  $\mu$ l C10 containing 2  $\mu$ g/ml anti-mouse CD28, 50 ng/ml mouse macrophage colony-stimulating factor (M-CSF; Sigma-Aldrich) and 5  $\mu$ M ODN1585 CpG (InvivoGen, San Diego, CA) or 5  $\mu$ g/ml Imiquimod (InvivoGen), and cultured in a 96-well flat-bottom plate coated with 10  $\mu$ g/ml anti-mouse CD3 for 3 days. Cells were collected for flow cytometry and supernatants were harvested and stored at -80°C.

#### *Immunohistochemistry (IHC)*

Kidneys were embedded in Tissue-Tek<sup>®</sup> O.C.T.<sup>™</sup> Compound (Sakura Finetek, Torrance, CA) and rapidly frozen in a freezing bath of dry ice and 2-methylbutane. Frozen OCT samples were cryosectioned and unstained slides were stored at -80°C. Frozen slides were warmed to room temperature and let dry for 30 min, followed by fixation in -20°C cold acetone at room temperature for 10 min. After washing in PBS, slides were blocked with PBS containing 1% BSA and anti-mouse CD16/32 for 20 min at room temperature. Slides were then incubated with

fluorochrome-conjugated antibody mixture for 1 h at room temperature in a dark humid box. Slides were mounted with Prolong Gold containing DAPI (Life Technologies). The following anti-mouse antibodies were used in immunohistochemical analysis: CD11c-PE (clone N418, eBioscience), CD3-FITC (clone 145-2C11, eBioscience). Slides were read and pictured with EVOS<sup>®</sup> FL microscope (Advanced Microscopy Group, Grand Island, NY) and a 20× objective (LPlanFL PH2 20x/0.4, ∞/1.2). All infiltrating areas of CD11c<sup>+</sup> cells and CD3<sup>+</sup> cells were captured and analyzed with ImageJ software (version 1.47v).

### *Flow cytometry*

For surface marker staining, cells were blocked with anti-mouse CD16/32 (eBioscience), stained with fluorochrome-conjugated antibodies, and analyzed with BD FACSAria II (BD Biosciences) or Attune NxT (Thermo Fisher Scientific, Waltham, MA) flow cytometer. Anti-mouse antibodies used in this study include eBioscience: CD3-biotin (clone 145-2C11), CD19-biotin (clone 1D3), CD11c-biotin (clone N418), CD11c-PE, CD11b-PerCP-Cy5.5 (clone M1/70), CD45-FITC (clone, 30-F11), CD45-PE, CD80-PE (clone RMMP-1), CD103-APC (clone 2E7) and F4/80-eFluor 450 (clone BM8); Biolegend: CD16-2 (FcγR IV)-APC (clone 9E9), CD40-APC (clone 3.23), CD44-APC-Cy7 (clone IM7), CD64 (FcγR I)-PE (clone X54-5/7.1), CD138-APC (clone 281-2), CD49b-biotin (clone DX5), PD-L1-APC (clone 10F.9G2), ICOSL-PE (clone HK5.3), IgG-APC (clone poly4053), IgG2a-PE (clone RMG2a-62), CCR2-PE (clone SA203G11), CX<sub>3</sub>CR1-biotin, CX<sub>3</sub>CR1-PE (clone SA011F11) and CX<sub>3</sub>CR1-APC (clone SA011F11); BD Biosciences: B220-V500 (clone RA3-6B2), CD4-PE-Cy7 (clone RM4-5), CD8a-V450 (clone 53-6.7), CD11b-APC-Cy7, CD11c-PerCP-Cy5.5, CD11c-BV510 (clone HL3), CD45-APC-Cy7 (clone 30-F11), CD86-PE (clone GL1), Ly6C-PE-Cy7 (clone AL-21), I-E/I-A-BV421 (clone

M5/114), I-E/I-A-V500 (clone M5/114), OX40L-BV421 (clone RM134L) and PD-L2-BV510 (clone TY25); Miltenyi Biotec: CD3-APC (clone 145-2C11), CD115-PE (clone AFS98), anti-biotin-FITC, anti-biotin-APC and Ly6G-biotin (clone 1A8). Flow cytometry data were analyzed with FlowJo (version 10.2).

*Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

Bone marrow neutrophils were sorted as DAPI<sup>-</sup>Ly6G<sup>+</sup>CD11b<sup>+</sup> cells. Bone marrow monocytes were sorted as DAPI<sup>-</sup>CD11c<sup>-</sup>CD11b<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>high</sup> cells. Spleen CD8<sup>+</sup> cDCs were pre-enriched by staining with anti-mouse CD11c-biotin and anti-biotin microbeads (Miltenyi Biotec), then further sorted as DAPI<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>+</sup>CD8<sup>+</sup>MHC-II<sup>+</sup> cells. Kidney neutrophils were sorted as DAPI<sup>-</sup>CD45<sup>+</sup>Lin (CD3, CD19 and CD49b)<sup>-</sup>CD11c<sup>-</sup>CD11b<sup>+</sup>SSC-H<sup>high</sup> cells. Kidney monocytes were sorted as DAPI<sup>-</sup>CD45<sup>+</sup>Lin<sup>-</sup>CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup> cells. Kidney CD11c<sup>+</sup> cells were sorted as DAPI<sup>-</sup>CD45<sup>+</sup>Lin<sup>-</sup>CD11c<sup>+</sup>CD11b<sup>+</sup> cells. Total RNA from sorted cell populations was extracted with RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. Reverse transcription was performed by using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed with iTaq<sup>™</sup> Universal SYBR Green Supermix (Bio-Rad) and ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY). Relative quantities were calculated using L32 as the housekeeping gene. Primer sequences for RT-qPCR can be found in the supplemental materials (Table S1).

*Speed congenic backcrossing to generate MRL/lpr-CX<sub>3</sub>CR1-knockout mice*

B6-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> mice were bred with MRL/lpr mice to generate F1 mice. The F1 generation was intercrossed to generate Fas<sup>lpr/lpr</sup>CX<sub>3</sub>CR1<sup>gfp/gfp</sup> F2 mice determined by the genotyping of *Fas* and *Cx3cr1* genes. Starting from the selected F2 mice, each subsequent generation was backcrossed with MRL/lpr mice and selected by speed congenic strategy [21] plus *Cx3cr1* gene to obtain CX<sub>3</sub>CR1<sup>gfp/+</sup> mice with maximal MRL/lpr genetic background. Tail tips of the mice were used to extract gDNA by the Quick DNA purification protocol from The Jackson laboratory. Genotyping of *Fas* gene (wild type and Fas<sup>lpr</sup> mutation) and *Cx3cr1* gene (wild type and CX<sub>3</sub>CR1<sup>gfp</sup> replacement) was also performed following the protocols from The Jackson laboratory. Speed congenic PCR was performed by using the following steps: (1) 94°C, 3 min; (2) [94°C, 20 sec; 57°C, 15 sec, -1°C/cycle; 68 °C, 1 min] ×10 cycles; (3) [94°C, 15 sec; 47°C, 15 sec; 72°C, 1 min] ×28 cycles; (4) 72°C, 2 min. KAPA2G Robust HotStart ReadyMix PCR Kit with dye (KAPA Biosystems, Wilmington, MA) was used for the PCR. Primer sequences can be found in the supplemental materials (Table S2). Tail tips of three 5<sup>th</sup> generation CX<sub>3</sub>CR1<sup>gfp/+</sup> mice were sent to The Jackson laboratory for single-nucleotide polymorphism screen to determine the purity of the MRL background.

#### *Enzyme-linked immunosorbent assay (ELISA)*

Blood was collected into anticoagulant (Potassium EDTA)-coated Capiject tubes (Terumo Medical, Somerset, NJ) and centrifuged at 15,000×g for 30 sec. Plasma was collected and stored at -80°C. Detection of anti-double-stranded DNA (dsDNA) IgG was described previously [22]. Total IgG concentrations were determined with mouse IgG ELISA kits according to the manufacturer's instructions (Bethyl Laboratories, city, state).

### *Proteinuria score and kidney histopathology*

Proteinuria was measured weekly with Chemstrip 2GP (Roche, Indianapolis, IN). A scale of 0-3 was used that corresponded to negative-trace (0-20 mg/dL), 30 mg/dL, 100 mg/dL, and  $\geq 500$  mg/dL total protein, respectively. Kidneys were fixed in 10% neutral buffered formalin immediately after isolation, paraffin-embedded, sectioned, and stained for Periodic Acid-Schiff (PAS) at the Histopathology Laboratory at Virginia-Maryland College of Veterinary Medicine. Kidney slides were read with Olympus BX43 microscope. Glomerular lesions were graded on a scale of 0-3 for increased cellularity, increased mesangial matrix, necrosis, percentage of sclerotic glomeruli, and presence of crescents [23]. Similarly, tubulointerstitial lesions were graded on a scale of 0-3 for interstitial mononuclear infiltration, tubular damage, interstitial fibrosis, and vasculitis. Slides were scored by a board certified veterinary pathologist (Cecere) in a blinded fashion.

### *Statistical analysis*

For the comparison of two groups, unpaired student's *t*-test was used. For the comparison of more than two groups, one-way ANOVA and Tukey's post-test were used. Results were considered statistically significant when  $P < 0.05$ . In some experiments, linear regression analysis and Grubbs' test for identification of outliers were used. All analyses were performed with Prism Graphpad software (version 5.0b).



## Results

### Renal accumulation of CD11c<sup>+</sup> myeloid cells as LN progresses

To study the possible influence of CD11c<sup>+</sup> cells on LN, we first investigated their presence in the kidney of lupus-prone mice. IHC staining of CD11c on the kidney sections of 4-month-old MRL control mice and MRL/lpr lupus-prone mice showed that CD11c<sup>+</sup> cells specifically accumulated in the kidney medulla of MRL/lpr but not MRL mice (Fig. 1A). As active LN has been established in 4-month-old MRL/lpr mice but not MRL mice [24], this result suggests that the renal accumulation of CD11c<sup>+</sup> cells may be associated with LN progression. To further quantify these renal-infiltrating CD11c<sup>+</sup> cells, we performed flow cytometry on isolated renal leukocytes. Most renal-infiltrating CD11c<sup>+</sup> cells showed a CD45<sup>+</sup>Lin<sup>-</sup>(CD3, CD19 and CD49b)<sup>-</sup>CD11b<sup>+</sup>CD11c<sup>+</sup> phenotype (Fig. 1B), suggesting that they might be of myeloid lineage. The co-expression of CD11c and CD11b was confirmed by IHC staining, with the co-localization of CD11c and CD11b signals in the kidney medulla of 4-month-old MRL/lpr mice (Fig. 1C). Quantification of renal-infiltrating CD11c<sup>+</sup> cells in the kidney of MRL/lpr mice showed a significant increase of the cells from 6 weeks to 15 weeks of age (Fig. 1D). Using the same gating strategy shown in Fig. 1B, we also found that both the percentage of CD11c<sup>+</sup> cells in Lin<sup>-</sup> and their absolute cell number were much higher in the kidney of another lupus-prone mouse model, NZB/W, compared to NZW controls, when NZB/W mice developed active LN at 35 weeks of age (Fig. 1E). MRL mice, while disease-free at 4 months of age, do develop LN later in life (Fig. S1A) and is another classical model of LN. We found that in MRL mice, the percentage and absolute cell number of renal-infiltrating CD11c<sup>+</sup> cells were increased at the age with active LN (37-week-old) compared to younger ages (Fig. 1F). Taken together, these results

demonstrated that the accumulation of CD11c<sup>+</sup> cells in the nephritic kidney was a common phenotype shared by different lupus-prone mouse models.

### **Renal-infiltrating CD11c<sup>+</sup> cells exhibiting a mature monocyte-derived dendritic cell phenotype**

To further study the possible origin of renal-infiltrating CD11c<sup>+</sup> cells, we included additional surface markers and compared their expression with neutrophils, monocytes and CD11b<sup>-</sup> cDC (see Fig. S1B for the gating strategy) from the same kidney using 4-month-old MRL/lpr mice as our model. MHC-II expression on renal-infiltrating CD11c<sup>+</sup> cells was not uniform, with a majority of the population expressing a lower level of MHC-II, which however was still higher than that of neutrophils and monocytes (Fig. 2A). The rest of the CD11c<sup>+</sup> population expressed a high level of MHC-II that was comparable to that of mature CD11b<sup>-</sup> cDCs. In addition, renal-infiltrating CD11c<sup>+</sup> cells expressed a low level of F4/80 (Fig. 2B), suggesting that these cells may be DCs instead of F4/80<sup>high</sup> macrophages. Moreover, renal-infiltrating CD11c<sup>+</sup> cells were CD103<sup>-</sup>CD115<sup>high</sup> and different from CD103<sup>+</sup>CD115<sup>-</sup> CD11b<sup>-</sup> cDCs (Fig. 2C and 2D), suggesting that they may be derived from monocytes rather than cDC precursors. The Ly6C<sup>low</sup>CCR2<sup>-</sup> phenotype of renal-infiltrating CD11c<sup>+</sup> cells, which was distinct from Ly6C<sup>high</sup>CCR2<sup>+</sup> immature monocytes, further suggests that they may be specifically derived from Ly6C<sup>low</sup>CCR2<sup>-</sup> mature monocytes (Fig. 2E and 2F). Collectively, these results suggest that renal-infiltrating CD11c<sup>+</sup> cells possessed a phenotype of mature monocyte-derived DCs.

As immature monocytes usually differentiate into inflammatory DCs while mature monocytes regularly patrolling blood vessels are responsible for tissue repairs with anti-inflammatory effects [25, 26], it is possible that these renal-infiltrating CD11c<sup>+</sup> cells have anti-inflammatory

functions. Therefore, we sorted renal-infiltrating CD11c<sup>+</sup> cells from the kidney of 4-month-old MRL/lpr mice and compared the transcript levels of several cytokines and chemokines with sorted bone marrow monocytes, bone marrow neutrophils, and splenic CD8<sup>+</sup> cDC from the same mice. To our surprise, these renal-infiltrating CD11c<sup>+</sup> cells showed a much more complicated cytokine/chemokine profile with the expression of both pro- and anti-inflammatory molecules (Fig. 2G). Compared to bone marrow monocytes and splenic CD8<sup>+</sup> cDC, renal-infiltrating CD11c<sup>+</sup> cells expressed higher levels of pro-inflammatory cytokines IL-1 $\beta$ , IL-18 and TNF. At the same time, they also expressed high levels of anti-inflammatory cytokines IL-10 and TGF $\beta$ . In addition, the expression of IL-10, together with that of IL-21, can promote B cell responses; whereas the expression of TGF $\beta$  and IL-6 suggests their potential to promote Th17 responses. Moreover, renal-infiltrating CD11c<sup>+</sup> cells highly expressed a set of chemokines including CCL2, CCL3, CCL9, CXCL13 and IL-18 that could attract the homing of monocytes, DC, T cells, B cells and pDCs into the kidney. Altogether, the cytokine and chemokine profile suggests that these renal-infiltrating CD11c<sup>+</sup> cells might be pathogenic and could potentially promote LN.

#### **High expression of CX<sub>3</sub>CR1 on kidney-infiltrating CD11c<sup>+</sup> cells that is dispensable for renal homing**

We next sought to determine the pathogenic role of renal-infiltrating CD11c<sup>+</sup> cells *in vivo* by blocking their infiltration into the kidney of MRL/lpr mice. The migration of leukocytes into specific tissues requires the expression of certain chemokine receptors [27]. As the phenotype of renal-infiltrating CD11c<sup>+</sup> cells suggested that they might be derived from mature monocytes, and that mature monocytes should express a high level of CX<sub>3</sub>CR1 on their surface [26], we measured the expression level of CX<sub>3</sub>CR1 on renal-infiltrating CD11c<sup>+</sup> cells. As expected, these

cells highly expressed CX<sub>3</sub>CR1 at both the transcriptional (Fig. 3A) and protein levels (Fig. 3B). To knock out CX<sub>3</sub>CR1, we performed speed congenic backcrossing of B6-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> mice onto the MRL/lpr background by genotyping *Fas*<sup>lpr</sup> (Fig. S1C), *Cx3cr1* (Fig. S1D) and other SLE susceptibility loci, and generated MRL/lpr-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> (CX<sub>3</sub>CR1<sup>gfp/gfp</sup>) and MRL/lpr-CX<sub>3</sub>CR1<sup>+/+</sup> (CX<sub>3</sub>CR1<sup>+/+</sup>) littermates after 5 generations of backcrossing that achieved >90% of MRL/lpr genetic background (data not shown). The replacement of CX<sub>3</sub>CR1 with green fluorescent protein (GFP) in the 5<sup>th</sup> generation was further confirmed by flow cytometric analysis (Fig. 3C). Unexpectedly, at the age of 15-week-old, the absolute cell number of renal-infiltrating CD11c<sup>+</sup> cells in MRL/lpr-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> mice was not significantly different from that in MRL/lpr-CX<sub>3</sub>CR1<sup>+/+</sup> mice, although both of them were higher than that in age-matched MRL control mice (Fig. 3D). Consistently, the proteinuria scores were not different between MRL/lpr-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> and MRL/lpr-CX<sub>3</sub>CR1<sup>+/+</sup> mice, either; and both were higher than that of MRL controls (Fig. 3E). Together, these results suggest that CX<sub>3</sub>CR1 may be dispensable for renal infiltration of CD11c<sup>+</sup> cells and not critical for the development of LN in MRL/lpr mice. Further studies on other chemotactic receptors revealed that renal-infiltrating CD11c<sup>+</sup> cells also expressed CXCR4, CCR1, CCR10 and chemR23 (Fig. 3F). CD11c<sup>+</sup> cells may be able to use one or more of these receptors to infiltrate the nephritic kidneys of MRL/lpr mice.

### **The pathogenic role of renal-infiltrating CD11c<sup>+</sup> cells in vivo**

Although CX<sub>3</sub>CR1 is not critical for renal infiltration of CD11c<sup>+</sup> cells, its high expression on these cells still suggests CX<sub>3</sub>CR1 as a good target for the removal or functional disruption of renal-infiltrating CD11c<sup>+</sup> cells. We thus utilized an antibody-drug conjugate (ADC) method that has been mainly used in cancer therapies [28] to remove or disable renal-infiltrating CD11c<sup>+</sup>

cells *in vivo*. To exclude possible off-target effects, we screened the expression of CX<sub>3</sub>CR1 on as many types of leukocytes as possible in the kidney, MLN and spleen of 4-month-old MRL/lpr mice, and found that renal-infiltrating CD11c<sup>+</sup> cells had the highest percentage of CX<sub>3</sub>CR1<sup>+</sup> cells (Fig. 4A). We next compared the mean fluorescent intensity (MFI) of CX<sub>3</sub>CR1 among leukocyte populations with >5% of CX<sub>3</sub>CR1<sup>+</sup> cells, and found that except for renal neutrophils and splenic CD11c<sup>+</sup>CD11b<sup>+</sup> cells, the intensity of CX<sub>3</sub>CR1 on renal-infiltrating CD11c<sup>+</sup> cells was significantly higher than that on all other cell types (Fig. 4B). Taking both the percentage and MFI of CX<sub>3</sub>CR1<sup>+</sup> cells into consideration, we calculated the CX<sub>3</sub>CR1 expression index by multiplying the two, and showed that renal-infiltrating CD11c<sup>+</sup> cells expressed a significantly higher level of CX<sub>3</sub>CR1 than all other cell types not only in the kidney but also in the secondary immune tissues (Fig. 4C). This suggests that targeting CX<sub>3</sub>CR1 would specifically remove or disable renal-infiltrating CD11c<sup>+</sup> cells with very limited off-target effects. Nevertheless, splenic CD11c<sup>+</sup>CD11b<sup>+</sup> cells expressed the second highest level of CX<sub>3</sub>CR1 after renal-infiltrating CD11c<sup>+</sup> cells (Fig. 4C) and could be a target of ADC. Another requirement for successful application of ADC is that target cells should efficiently internalize ADC after antibody binding to the target cells. To study the internalization of CX<sub>3</sub>CR1 after ligation with the anti-CX<sub>3</sub>CR1 antibody, we stained renal-infiltrating CD11c<sup>+</sup> cells with anti-CX<sub>3</sub>CR1-biotin and incubated cells at 37°C for different periods of time (0, 30, 60 or 90 min), allowing for the internalization of surface CX<sub>3</sub>CR1-anti-CX<sub>3</sub>CR1-biotin complexes. The cells were then stained with anti-biotin-APC to detect the remaining surface CX<sub>3</sub>CR1 by flow cytometry. The result showed a time-dependent decrease of APC signal on renal-infiltrating CD11c<sup>+</sup> cells (Fig. 4D), suggesting efficient internalization of CX<sub>3</sub>CR1 upon antibody ligation. With renal-infiltrating CD11c<sup>+</sup> cells expressing the highest level of CX<sub>3</sub>CR1, and that CX<sub>3</sub>CR1 can be effectively internalized upon

ligation with an anti-CX<sub>3</sub>CR1 antibody, we concluded that CX<sub>3</sub>CR1 was a good target for investigating the role of renal-infiltrating CD11c<sup>+</sup> cells in LN by the ADC method.

The ADC we used *in vivo* was a monoclonal anti-mouse CX<sub>3</sub>CR1-saporin conjugate. In our study, we injected 6 µg ADC intravenously into each MRL/lpr mouse once a week starting from 8-week-old till 15-week-old. The same volume of PBS was injected into control MRL/lpr mice. Weekly monitoring of mouse body weight and proteinuria scores revealed that the ADC-treated group had higher body weight and significantly lower proteinuria scores at 15 weeks of age (Fig. 4E). In addition, linear regression analysis showed that the development of proteinuria was significantly slower in the ADC group than the PBS-treated control group (Fig. 4E). The histopathological scores (glomerular score and tubulointerstitial score) were lower in the ADC group but the difference was not statistically significant (Fig. S2A). To exclude the possible influence by the change of systemic autoimmune response, we measured the activation of T cells in the spleen (Fig. S2B) and antibody levels in the plasma (Fig. S2C). No difference was found between the ADC and PBS groups, suggesting that ADC did not affect the systemic autoimmune response, and that its effects were kidney-specific. Collectively, these results indicate that ADC administration ameliorated LN in MRL/lpr mice without influencing systemic autoimmune response. As our ADC specifically targeted CX<sub>3</sub>CR1-expressing renal-infiltrating CD11c<sup>+</sup> cells, these results suggest the *in vivo* pathogenic role of renal-infiltrating CD11c<sup>+</sup> cells in the development of LN.

### **Promotion of renal-infiltrating CD4<sup>+</sup> T cell response by syngeneic renal-infiltrating CD11c<sup>+</sup> cells**

To study the mechanism by which renal-infiltrating CD11c<sup>+</sup> cells promote LN, we determined their interactions with renal-infiltrating T cells that are known to be pathogenic in LN [29]. Although many renal-infiltrating innate immune cell types including CD11b<sup>-</sup> cDCs, monocytes and neutrophils can interact with T cells, we showed that renal-infiltrating CD11c<sup>+</sup> cells significantly outnumbered the other innate immune cell populations in the kidney of MRL/lpr mice with active LN (15-week-old) (Fig. 5A). This suggests that renal-infiltrating CD11c<sup>+</sup> cells may be the predominant innate immune cell type interacting with T cells. Co-staining of the kidney sections of MRL/lpr mice with CD11c and CD3 showed that CD11c<sup>+</sup> cells and T cells localized in the same regions adjacent to each other. In addition, the time courses of renal infiltration of CD11c<sup>+</sup> cells and T cells were very similar (Fig. 5B), suggesting that they might interact or facilitate each other's infiltration into the kidney. As shown earlier, the renal-infiltrating CD11c<sup>+</sup> cells possessed the phenotype of DCs (Fig. 2A and 2B) that could interact with T cells as typical antigen-presenting cells. We found that these CD11c<sup>+</sup> cells highly expressed Fc-gamma-receptor type IV (FcγR IV) and were coated with IgG, in particular pathogenic IgG2a on their surface (Fig. 5C), suggesting that they may be able to capture self-antigen in the immune complexes. Moreover, the renal-infiltrating CD11c<sup>+</sup> cells expressed a high level of MHC-II as well as a high ratio of CD86/CD80 (Fig. S2D and S2E, Fig. 5D), suggesting their potential ability to present self-antigen to and stimulate autoreactive T cells. Furthermore, we found that these CD11c<sup>+</sup> cells expressed co-stimulatory molecules such as CD40, ICOSL and OX40L (Fig. S2F), which could provide additional activation signals for T cells. Interestingly, the renal-infiltrating CD11c<sup>+</sup> cells also expressed higher levels of co-suppressive molecules PD-L1 and PD-L2 (Fig. 5D).

We next performed co-culture experiments between renal-infiltrating CD11c<sup>+</sup> cells and syngeneic renal-infiltrating T cells to study their interactions *ex vivo*. Three major T cell subpopulations including CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and CD4<sup>-</sup>CD8<sup>-</sup>B220<sup>+</sup> (DN) T cells infiltrated in the nephritic kidney of MRL/lpr mice. The number of CD4<sup>+</sup> T cells was the highest among the 3 subpopulations (Fig. 5E). In addition, upon *ex vivo* stimulation with anti-CD3/CD28, CD4<sup>+</sup> T cells were able to produce higher levels of two pathogenic cytokines, IFN $\gamma$  and IL-17a, than CD8<sup>+</sup> and DN T cells (Fig. 5F). We thus focused our attention on the interaction between CD11c<sup>+</sup> cells and CD4<sup>+</sup> T cells. In the co-culture system, in addition to the presence of CD4<sup>+</sup> T cells, we provided M-CSF as a survival signal to renal-infiltrating CD11c<sup>+</sup> cells, and stimulated CD11c<sup>+</sup> cells with either Toll-like receptor (TLR) 7 agonist, Imiquimod or TLR 9 agonist, ODN1585 CpG to imitate self-RNA or self-DNA, respectively. CD4<sup>+</sup> T cells, on the other hand, were stained with CFSE and stimulated with anti-CD3/CD28. After three days of co-culturing, we found that renal-infiltrating CD11c<sup>+</sup> cells promoted both the survival (DAPI) and proliferation (CFSE<sup>low</sup>) of CD4<sup>+</sup> T cells compared to CD4<sup>+</sup> T cells alone (Fig. 5H and 5I, with representative flow cytometry plots of DAPI and CFSE staining shown in Fig. 5G). Furthermore, when stimulated with a TLR9 agonist, renal-infiltrating CD11c<sup>+</sup> cells also enhanced IFN $\gamma$  production from CD4<sup>+</sup> T cells (Fig. 5J). Taken together, our results suggest that renal-infiltrating CD11c<sup>+</sup> cells were able to promote the activation of renal-infiltrating CD4<sup>+</sup> T cells, which may be one of the mechanisms by which these CD11c<sup>+</sup> cells deteriorated LN in MRL/lpr mice.

## Discussion



CD11c<sup>+</sup> cells have been demonstrated to play pathogenic roles in the development of LN in lupus-prone mice [15, 17-19]. In addition, the accumulation of CD11c<sup>+</sup> cells has been found in the kidney of both SLE patients and lupus-prone mice with active LN [20, 30-34]. However, as CD11c<sup>+</sup> cells are very heterogeneous, the spatial and temporal roles of different CD11c<sup>+</sup> subsets in LN development have not been well investigated. In this study, we identified a population of DC-like CD11c<sup>+</sup> cells, which accumulated in the kidney of different types of lupus-prone mice with active LN. We demonstrated that they were pathogenic in promoting proteinuria through enhancing renal-infiltrating T helper cell responses in the MRL/lpr mouse model. The similar accumulation of CD11c<sup>+</sup> cells in the nephritic kidney of both MRL and MRL/lpr mice suggests that the increase of renal-infiltrating CD11c<sup>+</sup> cells and their pathogenic functions should be due to the multiple SLE susceptibility loci present in the MRL mouse background rather than the *Fas*<sup>lpr</sup> gene mutation in MRL/lpr mice. While the accumulation and subpopulations of renal-infiltrating CD11c<sup>+</sup> cells, especially CD11c<sup>+</sup>CD11b<sup>+</sup> dendritic cells, have also been studied in some other lupus-prone mouse models by other groups [34, 35], the renal-infiltrating CD11c<sup>+</sup> cells we identified in MRL/lpr mice have shown both similarities and differences compared to them. Regarding F4/80 and MHC-II expression, unlike the three subpopulations (F4/80<sup>+</sup>, F4/80<sup>-</sup>MHC-II<sup>-</sup>, and F4/80<sup>-</sup>MHC-II<sup>+</sup>) of renal-infiltrating CD11c<sup>+</sup>CD11b<sup>+</sup> cells in Sle1Tg7 mice [35], those in the kidneys of MRL/lpr mice are all F4/80<sup>low</sup>MHC-II<sup>+</sup> and similar to the renal-infiltrating CD11c<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>low</sup> cells identified in NZB/W F1 mice that express MHC-II from low to high levels [34], suggesting that these cells belong to the same population with different activating status. Furthermore, combined with additional markers, these renal-infiltrating CD11c<sup>+</sup> cells possessed a surface phenotype of mature monocytes, especially with a high expression of CX<sub>3</sub>CR1, which is consistent with studies in SLE patients where CX<sub>3</sub>CR1<sup>+</sup>

cells and CD16<sup>+</sup> cells are found in the kidney biopsies of patients with active LN [36]. The similar cell population found in NZB/W F1 mice, however, is negative for CX<sub>3</sub>CR1 expression [34]. This suggests that different lupus-prone mouse models have their unique characteristics and our findings in MRL/lpr mice are clinically relevant, as the same population of renal-infiltrating CD11c<sup>+</sup> cells are found in both MRL/lpr mice and SLE patients.

Notably, different from well accepted concepts that immature monocytes enhance the inflammation whereas mature monocytes downregulate inflammation [25, 26], in SLE patients, mature monocytes have been suggested to possess a pathogenic role to promote lupus disease by enhancing pathogenic T cell responses [37]. The results of the present study support this notion, as our cell population of interest, which is derived from mature monocytes, appears to be pro-inflammatory and contributes to disease pathogenesis in LN. Therefore, the functions of a particular cell type may change depending on the microenvironment.

In our study of MRL/lpr mice, renal-infiltrating CD11c<sup>+</sup> cells expressed higher levels of MHC-II and CD86/CD80 co-stimulatory molecules than renal-infiltrating monocytes and neutrophils, suggesting their activated state and ability to activate T helper cells, which was then confirmed by the *ex vivo* co-culture experiments. Additionally, other co-stimulatory molecules, ICOSL and OX40L, in particular, also expressed by these renal-infiltrating CD11c<sup>+</sup> cells, have been demonstrated to be pathogenic through activating autoreactive T cells in both lupus-prone mice and SLE patients [15, 16]. However, renal-infiltrating CD11c<sup>+</sup> cells also expressed high levels of co-suppressive molecules PD-L1 and PD-L2. As PD-L1 and PD-L2 are IFN $\gamma$ -inducible genes that can be upregulated on IFN $\gamma$ -activated APCs [38], this result is consistent with the activated

state of renal-infiltrating CD11c<sup>+</sup> cells and suggests their possible interaction with renal-infiltrating T helper cells, in particular IFN $\gamma$ -producing pathogenic T helper cells. In addition, we found that renal-infiltrating CD4<sup>+</sup> T cells only expressed a low level of PD-1 (data not shown), suggesting a limited suppressive effect of renal-infiltrating CD11c<sup>+</sup> cells on renal-infiltrating CD4<sup>+</sup> T cells. Indeed, the results of our *ex vivo* co-culture experiments suggest that renal-infiltrating CD11c<sup>+</sup> cells actually promoted the syngeneic renal-infiltrating CD4<sup>+</sup> T cell response.

To investigate the potentially pathogenic role of renal-infiltrating CD11c<sup>+</sup> cells *in vivo*, we tried two different methods, CX<sub>3</sub>CR1-knockout and ADC. Studies have shown that the interaction between CX<sub>3</sub>CR1 and its ligand CX<sub>3</sub>CL1 can promote LN in MRL/lpr mice [39] and that the interaction is responsible for the infiltration of pathogenic DCs into the kidney in a nephrotoxic nephritis mouse model [40]. We thus hypothesized that by knocking out CX<sub>3</sub>CR1 from MRL/lpr mice, these renal-infiltrating CD11c<sup>+</sup> cells would be unable to infiltrate into the kidney, and LN would be ameliorated. However, these two strategies resulted in different outcomes with no disease change in CX<sub>3</sub>CR1-knockout mice but amelioration of LN in ADC-treated mice. This suggests that renal-infiltrating CD11c<sup>+</sup> cells are pathogenic but their effects may be independent of CX<sub>3</sub>CR1. As the knockout of CX<sub>3</sub>CR1 failed to prevent their renal accumulation, the infiltration of renal-infiltrating CD11c<sup>+</sup> cells appeared to be CX<sub>3</sub>CR1-independent. This partially explains the unfavorable phenotype in MRL/lpr-CX<sub>3</sub>CR1<sup>-/-</sup> mice. To exclude the influence of the remaining B6 genetic background (less than 10%) in the 5<sup>th</sup> generation of MRL/lpr-CX<sub>3</sub>CR1<sup>-/-</sup> mice, we further backcrossed the mice onto MRL/lpr background for a total of 10 generations that achieved >99% MRL/lpr genetic purity. Unfortunately, the severity and course of kidney disease were still unaffected (data not shown). The ADC method, on the other hand, targets

renal-infiltrating CD11c<sup>+</sup> cells by directly depleting them or disabling their functions, thus a better way to demonstrate the *in vivo* pathogenic role of renal-infiltrating CD11c<sup>+</sup> cells in LN. The ADC used in this study was optimally designed to maximize its effects. The anti-mouse CX<sub>3</sub>CR1 antibody was of mouse IgG2a origin to reduce host immune responses to ADC. Saporin is a very stable cytotoxic drug that functions by preventing protein synthesis in the cell. It has been used to induce apoptosis of tumor cells in cancer therapies [41]. The potential off-target effect of ADC treatment was a concern, as splenic CD11c<sup>+</sup>CD11b<sup>+</sup> cells expressed the second highest level of CX<sub>3</sub>CR1, but we found that the activated T cells in the spleen and the levels of circulating antibodies including anti-dsDNA antibodies were not different between ADC-treated and PBS-treated groups. This suggests that ADC treatment did not affect systemic immune responses, and that the pathogenic role of renal-infiltrating CD11c<sup>+</sup> cells might be kidney-specific.

The ADC strategy to target renal-infiltrating CD11c<sup>+</sup> cells appears to be efficacious in MRL/lpr mice. This can be highly translatable, where a similar ADC-based drug may be used to target a similar population in LN patients with high efficiency and low side effects. However, further studies are required to characterize these renal-infiltrating CD11c<sup>+</sup> cells in the kidney of LN patients. For example, although the surface markers suggest their mature monocyte origin, this should be confirmed *in vivo*. In addition, it is worthy to further investigate the molecular mechanisms by which renal-infiltrating CD11c<sup>+</sup> cells promote CD4<sup>+</sup> T cell responses *in vivo*. Moreover, based on their cytokine/chemokine profile, it appears that renal-infiltrating CD11c<sup>+</sup> cells may be able to influence renal-infiltrating monocytes [42], pDCs [43] and B cells [44, 45] in addition to CD4<sup>+</sup> T cells. This would be also interesting to explore.

In conclusion, we identified a renal-infiltrating CD11c<sup>+</sup> cell population associated with the progression of LN in different lupus-prone mouse models. They had a phenotype of mature monocyte-derived DCs with a complicated cytokine/chemokine profile. These renal-infiltrating CD11c<sup>+</sup> cells have shown a pathogenic role in promoting LN in MRL/lpr mice partially by enhancing syngeneic renal-infiltrating CD4<sup>+</sup> T cell responses. Moreover, their preferential surface expression of CX<sub>3</sub>CR1 makes them a potential therapeutic target. Our results could be highly translatable if a similar population of CD11c<sup>+</sup> cells exists in the nephritic kidneys of SLE patients.

### **Acknowledgments**

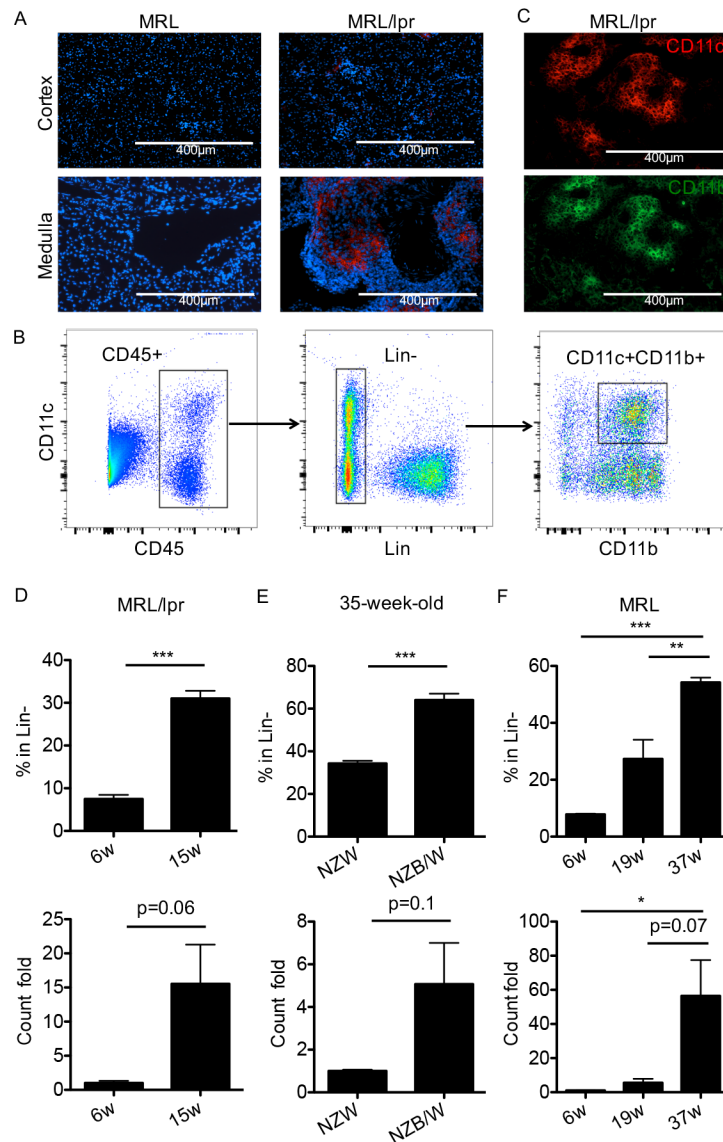
We thank Melissa Makris and Dr. Caroline Leeth for the use of flow cytometry core facility at Virginia Tech. This work was supported by various internal grants awarded to XML. XL is a Stamps Fellow in the Biomedical and Veterinary Sciences graduate program.

### **Conflict of interest**

The authors declare no conflict of interest.

## FIGURES

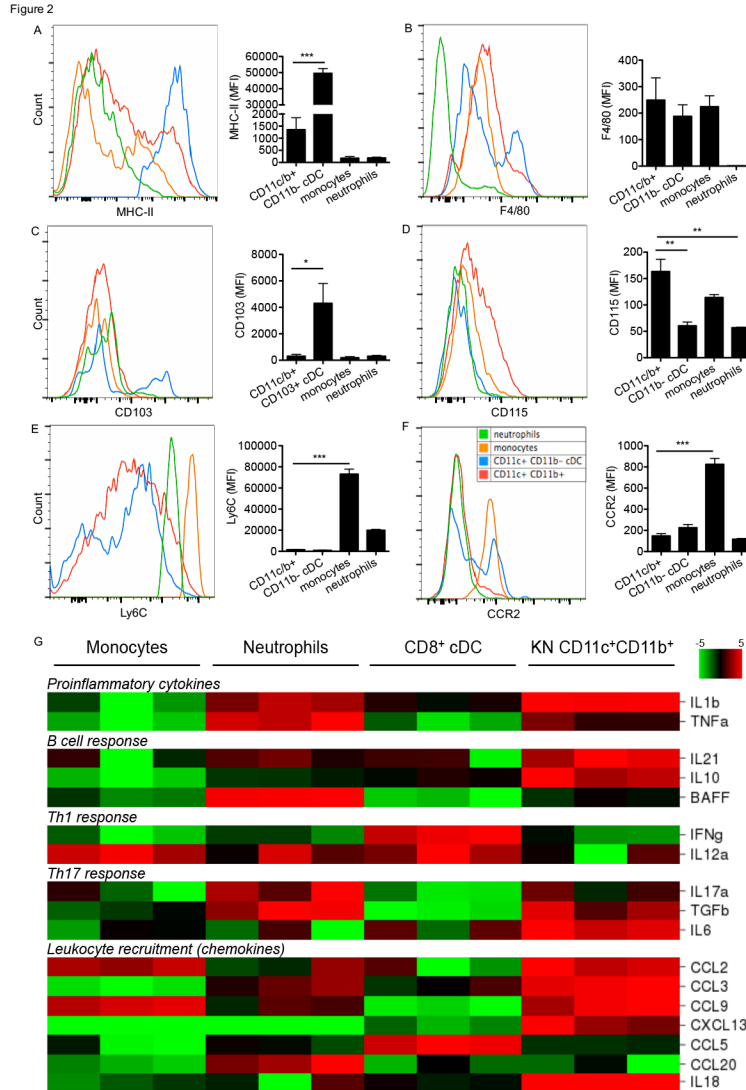
Figure 1



**Figure 1. Accumulation of a CD11c<sup>+</sup> cell population in the kidney of lupus-prone mice.** (A) IHC stains of CD11c<sup>+</sup> cells (red) on the kidney sections of 4-month-old MRL and MRL/lpr mice. Representative images are shown. Blue, DAPI. (B) Step-wise gating of CD11c<sup>+</sup> cells by flow cytometry as CD11c<sup>+</sup>CD45<sup>+</sup>Lin(CD3, CD19 and CD49b)<sup>-</sup>CD11b<sup>+</sup> cells from isolated kidney mononuclear cells from MRL/lpr mice. Representative flow cytometry plots are shown. (C) IHC

stains of CD11c<sup>+</sup> cells (red) and CD11b<sup>+</sup> cells (green) on the kidney sections of 4-month-old MRL/lpr mice. Representative images of the medulla region are shown. (D-F) The percentages of renal-infiltrating CD11c<sup>+</sup> cells in Lin<sup>-</sup> population (top row) as gated in (B) and the relative cell count changes of renal-infiltrating CD11c<sup>+</sup> cells (bottom row) in (D) 6-week- and 15-week-old MRL/lpr mice, (E) 35-week-old NZW mice and NZB/W mice, and (F) 6-week-, 19-week- and 37-week-old MRL mice. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, student's *t*-test for (D and E) and one-way analysis of variance (ANOVA) for (F). Data are shown as mean + standard error of the mean (SEM), n=3 mice in each group.

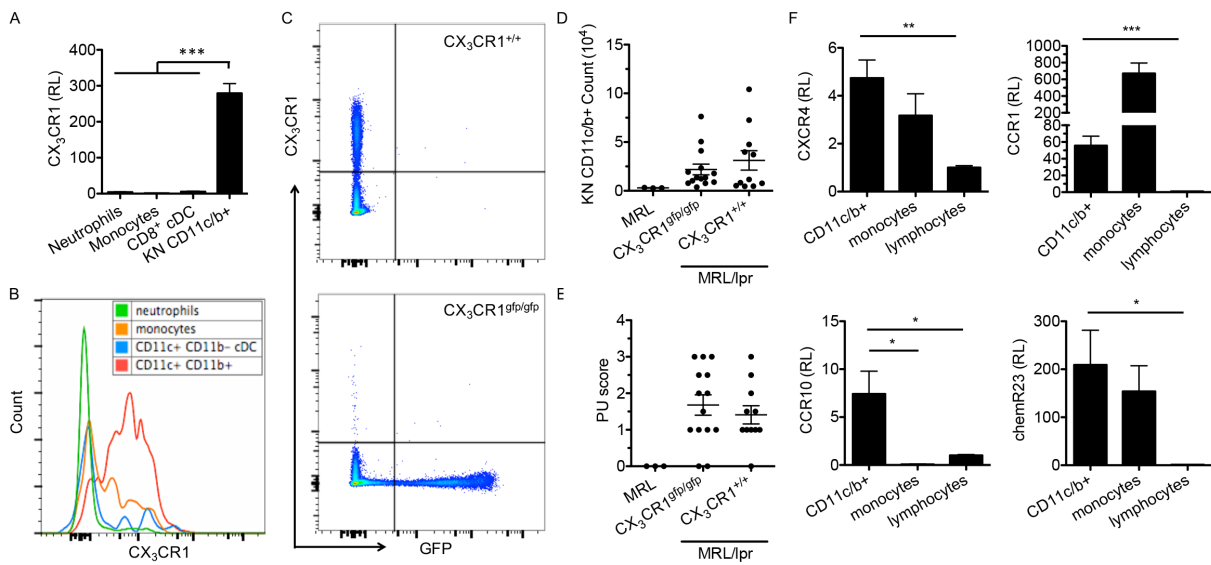




**Figure 2. Phenotype and cytokine/chemokine profile of renal-infiltrating CD11c<sup>+</sup> cells.** (A-F) The surface MFI of (A) MHC-II, (B) F4/80, (C) CD103, (D) CD115, (E) Ly6C and (F) CCR2 on renal-infiltrating CD11c<sup>+</sup> cells (CD11c<sup>+</sup>CD11b<sup>+</sup>, red), CD11b<sup>-</sup> cDC (defined as CD11c<sup>+</sup>CD11b<sup>-</sup>MHC-II<sup>+</sup>, blue), monocytes (defined as CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup>SSC-H<sup>low</sup>, orange) and neutrophils (defined as CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>mid</sup>SSC-H<sup>high</sup>, green) from 4-month-old MRL/lpr mice as determined by flow cytometry. Representative flow cytometry histograms are shown. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA. Data are shown as mean + SEM, n=3 mice in each group. (G) Relative transcript levels of selected cytokines and chemokines as

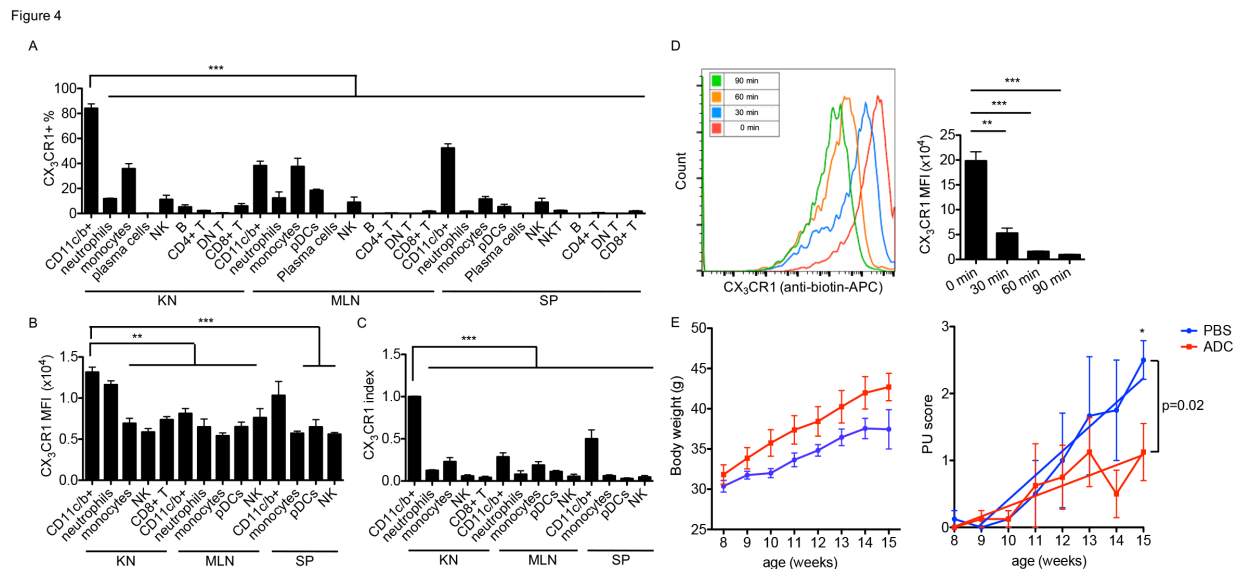
determined by RT-qPCR in bone marrow monocytes (DAPI<sup>-</sup>CD11c<sup>-</sup>CD11b<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>high</sup>), bone marrow neutrophils (DAPI<sup>-</sup>Ly6G<sup>+</sup>CD11b<sup>+</sup>), splenic CD8<sup>+</sup> cDCs (DAPI<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>+</sup>CD8<sup>+</sup>MHC-II<sup>+</sup>) and kidney (KN)-infiltrating CD11c<sup>+</sup> cells (DAPI<sup>-</sup>CD45<sup>+</sup>Lin<sup>-</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>) sorted from 4-month-old MRL/lpr mice. A heat map is shown. Red, higher expression level; green, lower expression level. n=3 mice in each group.

Figure 3



**Figure 3. CX<sub>3</sub>CR1 highly expressed on renal-infiltrating CD11c<sup>+</sup> cells but dispensable for their infiltration into the nephritic kidney.** (A) The transcript level of CX<sub>3</sub>CR1 as determined by RT-qPCR in bone marrow monocytes (DAPI<sup>-</sup>CD11c<sup>-</sup>CD11b<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>high</sup>), bone marrow neutrophils (DAPI<sup>-</sup>Ly6G<sup>+</sup>CD11b<sup>+</sup>), splenic CD8<sup>+</sup> cDC (DAPI<sup>-</sup>CD11b<sup>-</sup>CD11c<sup>+</sup>CD8<sup>+</sup>MHC-II<sup>+</sup>) and kidney (KN)-infiltrating CD11c<sup>+</sup> cells (DAPI<sup>-</sup>CD45<sup>+</sup>Lin<sup>-</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>) sorted from 4-month-old MRL/lpr mice. (B) The surface expression of CX<sub>3</sub>CR1 on renal-infiltrating CD11c<sup>+</sup> cells (CD11c<sup>+</sup>CD11b<sup>+</sup>, red), CD11b<sup>-</sup> cDC (CD11c<sup>+</sup>CD11b<sup>-</sup>MHC-II<sup>+</sup>, blue), monocytes (CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup>SSC-H<sup>low</sup>, orange) and neutrophils (CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>mid</sup>SSC-H<sup>high</sup>, green)

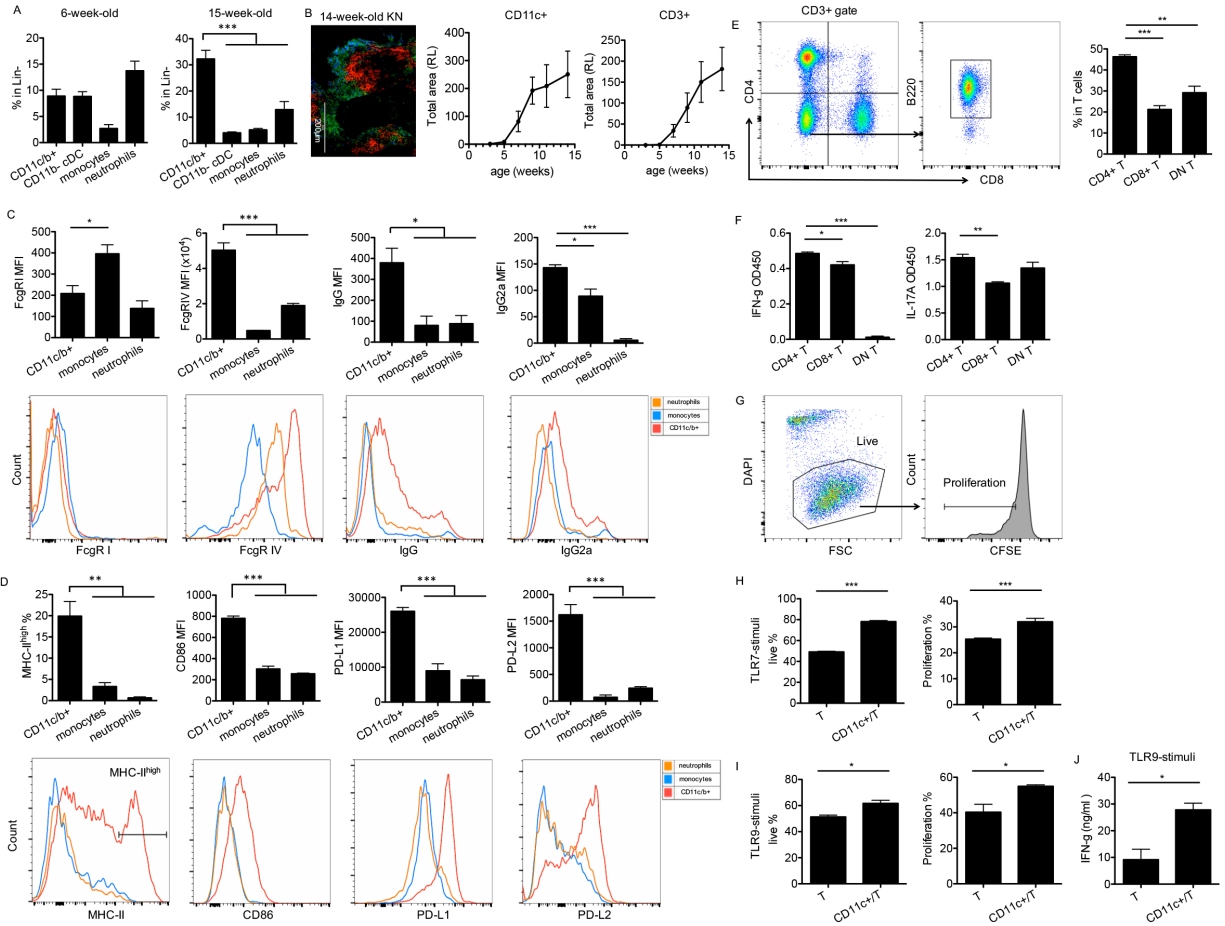
from 4-month-old MRL/lpr mice as determined by flow cytometry. A representative flow cytometry histogram is shown. (C) The expression of CX<sub>3</sub>CR1 and GFP by peripheral blood mononuclear cells from the 5<sup>th</sup> generation of MRL/lpr-CX<sub>3</sub>CR1<sup>+/+</sup> and MRL/lpr-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> littermate mice as determined by flow cytometry. Representative flow cytometry plots are shown. (D) The absolute number of renal-infiltrating CD11c<sup>+</sup> cells in 15-week-old MRL, and the 5<sup>th</sup> generation of MRL/lpr-CX<sub>3</sub>CR1<sup>+/+</sup> and MRL/lpr-CX<sub>3</sub>CR1<sup>gfp/gfp</sup> mice as determined by flow cytometry. (E) The proteinuria (PU) scores of the same 3 groups of mice. (F) The transcript levels of CXCR4, CCR1, CCR10 and chemR23 in renal-infiltrating CD11c<sup>+</sup> cells (DAPI<sup>-</sup>CD45<sup>+</sup>Lin<sup>-</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>), monocytes (DAPI<sup>-</sup>CD45<sup>+</sup>Lin<sup>-</sup>CD11c<sup>-</sup>CD11b<sup>+</sup>Ly6C<sup>high</sup>SSC-H<sup>low</sup>) and lymphocytes (DAPI<sup>-</sup>CD45<sup>+</sup>Lin<sup>+</sup>) sorted from the same kidneys of 4-month-old MRL/lpr mice as determined by RT-qPCR. RL, relative level. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA. Data are shown as mean + or ± SEM, n≥3 mice in each group.



**Figure 4. The pathogenic role of renal-infiltrating CD11c<sup>+</sup> cells *in vivo*.** (A and B) The percentage of CX<sub>3</sub>CR1<sup>+</sup> cells (A) and the MFI of CX<sub>3</sub>CR1 in the CX<sub>3</sub>CR1<sup>+</sup> subpopulation (B) in

each type of leukocytes from the kidney (KN), MLN and spleen (SP) of 4-month-old MRL/lpr mice as determined by flow cytometry. (C) CX<sub>3</sub>CR1 expression index calculated as 
$$\frac{[(\%CX_3CR1^+)_{\text{cell type}} \times (CX_3CR1 \text{ MFI})_{CX_3CR1^+ \text{ part of cell type}}]}{[(\%CX_3CR1^+)_{\text{renal CD11c}^+ \text{ cells}} \times (CX_3CR1 \text{ MFI})_{CX_3CR1^+ \text{ part of renal CD11c}^+ \text{ cells}}]}$$
. (D) Internalization of surface CX<sub>3</sub>CR1 by renal-infiltrating CD11c<sup>+</sup> cells from 4-month-old MRL/lpr mice as determined by flow cytometry. Cells were stained with anti-mouse CX<sub>3</sub>CR1-biotin and cultured for different period of time at 37°C, followed by staining with anti-biotin-APC. A representative flow cytometry histogram is shown. (E) Body weight (left) and PU scores (right) of ADC- (red) or PBS- (blue) treated MRL/lpr mice. Mice were treated from 8-week-old to 15-week-old. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA or linear regression. Data are shown as mean + or ± SEM, n≥3 mice in each group.

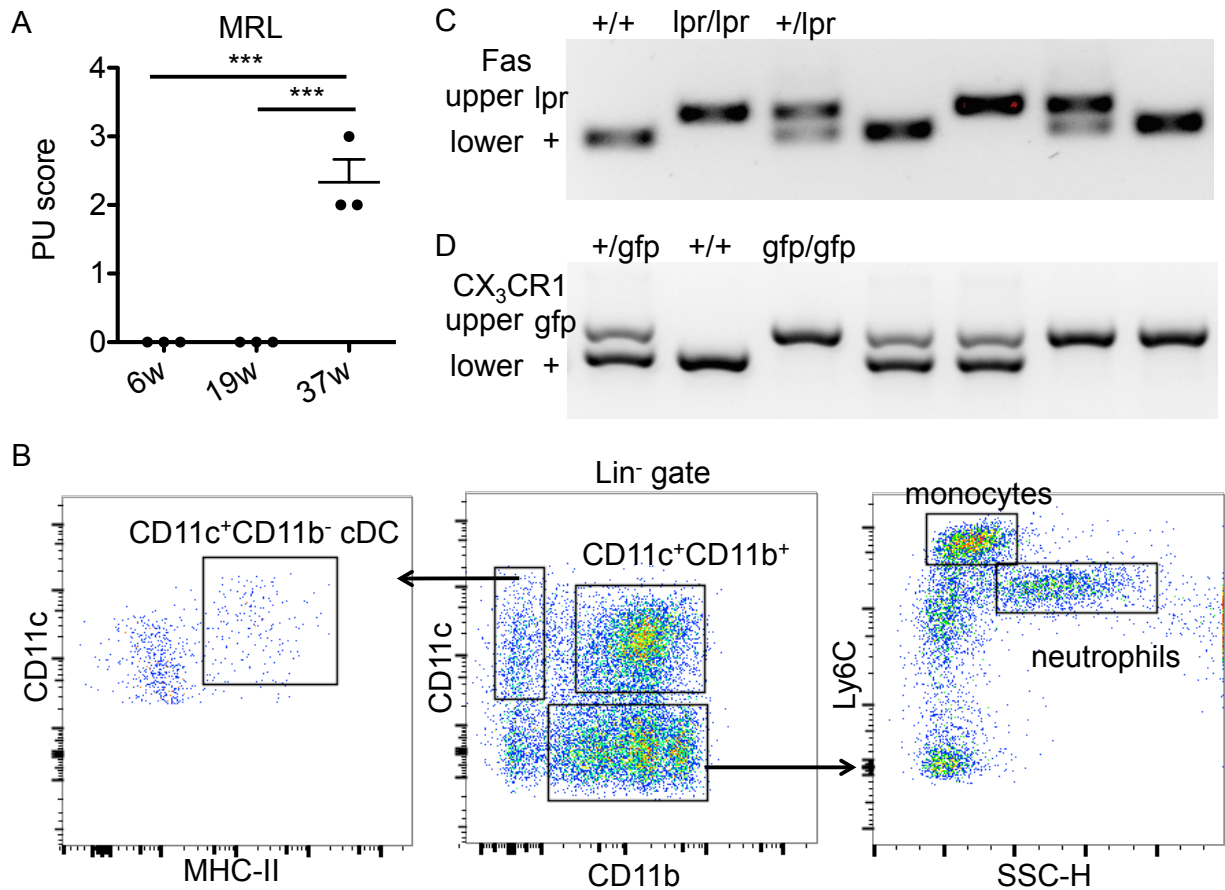
Figure 5



**Figure 5. The interaction between renal-infiltrating CD11c<sup>+</sup> cells and renal-infiltrating CD4<sup>+</sup> T cells.** (A) The percentages of renal-infiltrating CD11c<sup>+</sup>CD11b<sup>+</sup> cells, CD11b<sup>-</sup> cDCs, monocytes and neutrophils in Lin<sup>-</sup> population of 6-week- and 15-week-old MRL/lpr mice. CD11c<sup>+</sup>CD11b<sup>+</sup> cells were the predominant population in the kidney of 15-week-old mice with active LN. (B) Total renal infiltration areas of CD11c<sup>+</sup> cells (red) and CD3<sup>+</sup> T cells (green) from 3-, 5-, 7-, 9-, 11-, 14-week-old MRL/lpr mice as determined by IHC and ImageJ quantification. RL, relative level. A representative image of the kidney (KN) of a 14-week-old MRL/lpr mouse is shown. Bar equals 200 μm. Blue, DAPI. (C and D) The surface level of FcγR I, FcγR IV, IgG, IgG2a, MHC-II, CD86, PD-L1 and PD-L2 on renal-infiltrating CD11c<sup>+</sup> cells (red), monocytes (orange) and neutrophils (blue) in the kidney of 4-month-old MRL/lpr mice as determined by

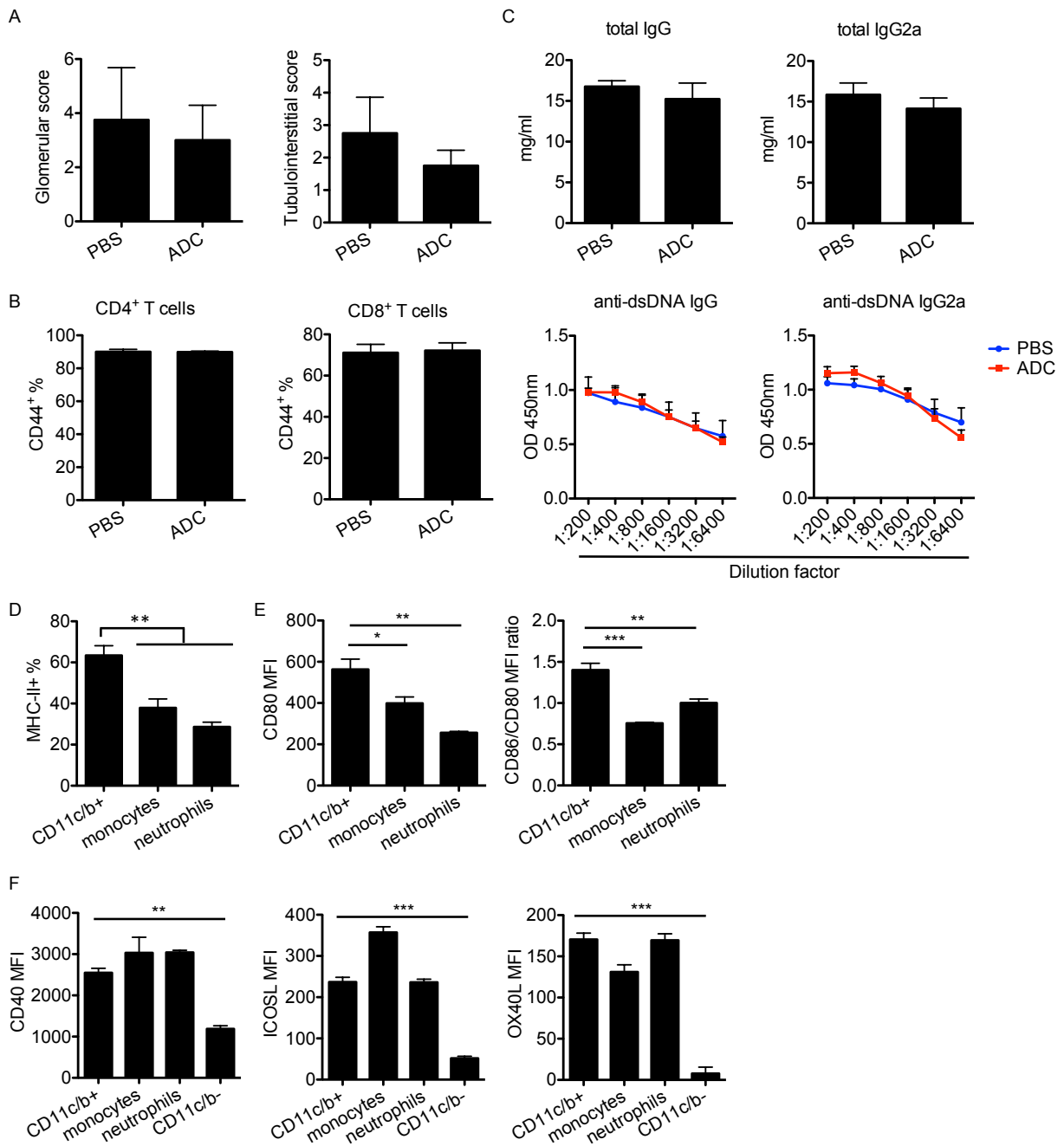
flow cytometry. Representative flow cytometry histograms are shown. (E) The percentages of renal-infiltrating CD4<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup>B220<sup>+</sup> DN T cells in total renal-infiltrating CD3<sup>+</sup> T cells of 4-month-old MRL/lpr mice as determined by flow cytometry. The gating strategy is shown. (F) IFN $\gamma$  and IL-17a levels in the culture supernatant of CD4<sup>+</sup>, CD8<sup>+</sup> and DN T cells stimulated with anti-CD3/CD28 as determined by ELISA. (G-I) The percentages of live cells (DAPI<sup>-</sup>) and proliferating cells (CFSE<sup>low</sup>) in renal-infiltrating CD4<sup>+</sup> T cells cultured alone or co-cultured with renal-infiltrating CD11c<sup>+</sup> cells that were stimulated with anti-CD3/CD28 and M-CSF in the presence of (H) TLR7 agonist, Imiquimod or (I) TLR9 agonist, ODN 1585 CpG. Representative flow cytometry plots and the gating strategy are shown in (G). (J) IFN $\gamma$  levels in the culture supernatant of renal-infiltrating CD4<sup>+</sup> T cells cultured alone or co-cultured with renal-infiltrating CD11c<sup>+</sup> cells that are stimulated with anti-CD3/CD28, M-CSF and ODN 1585 CpG as determined by ELISA. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA for (A-F) and student's *t*-test for (H-J). Data are shown as mean + or  $\pm$  SEM, n=3 mice in each group.

Figure S1



**Figure S1.** (A) Proteinuria (PU) scores of 6-, 19- and 37-week-old MRL mice. \*\*\*  $P < 0.001$ , one-way ANOVA. Data are shown as mean + SEM,  $n = 3$  mice in each group. (B) Gating strategy for renal-infiltrating  $CD11c^+$  cells ( $Lin^-CD11c^+CD11b^+$ ),  $CD11b^-$  cDC ( $Lin^-CD11c^+CD11b^-MHC-II^+$ ), monocytes ( $Lin^-CD11c^-CD11b^+Ly6C^{high}SSC-H^{low}$ ) and neutrophils ( $Lin^-CD11c^-CD11b^+Ly6C^{mid}SSC-H^{high}$ ) as determined by flow cytometry. Representative flow cytometry plots are shown. (C) Genotyping of wild type (+) and mutated (*lpr*) *Fas* gene. (D) Genotyping of wild type (+) and mutated (*gfp*) *Cx3cr1* gene.

Figure S2



**Figure S2.** (A) Glomerular (left) and tubulointerstitial (right) scores of ADC- or PBS-treated MRL/lpr mice at 15-week-old. (B) The percentages of CD44<sup>+</sup> activated cells in splenic CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells of ADC- or PBS-treated MRL/lpr mice at 15-week-old as determined by flow cytometry. (C) Total IgG and IgG2a levels (top row) and anti-dsDNA IgG and IgG2a levels



(bottom row) in the plasma of ADC- or PBS-treated MRL/lpr mice at 15-week-old as determined by ELISA. (D-E) The percentages of the MHC-II<sup>+</sup> population (D) and the expression of CD80 and the ratio of CD86 and CD80 MFI (E) in renal-infiltrating CD11c<sup>+</sup> cells, monocytes and neutrophils of 4-month-old MRL/lpr mice as determined by flow cytometry. (F) The expression of CD40, ICOSL and OX40L on renal-infiltrating CD11c<sup>+</sup> cells, monocytes, neutrophils and CD11c<sup>-</sup>CD11b<sup>-</sup> cells (mainly lymphocytes) of 4-month-old MRL/lpr mice as determined by flow cytometry. \* P<0.05, \*\* P<0.01, \*\*\* P<0.001, one-way ANOVA. Data are shown as mean + SEM, n<sub>≥</sub>3 mice in each group.

## TABLES

**Table S1.** List of primers for RT-qPCR.

Name	Primer sequence	Name	Primer sequence
IL-1 $\beta$ -fw	GAAATGCCACCTTTTGACAGTG	IL-1 $\beta$ -bw	TGGATGCTCTCATCAGGACAG
TNF- $\alpha$ -fw	CCCTCACACTCAGATCATCTTCT	TNF- $\alpha$ -bw	GCTACGACGTGGGCTACAG
IL-21-fw	GGACCCTTGTCTGTCTGGTAG	IL-21-bw	TGTGGAGCTGATAGAAGTTCAGG
IL-10-fw	GCTCTTACTGACTGGCATGAG	IL-10-bw	CGCAGCTCTAGGAGCATGTG
BAFF-fw	ACACTGCCCAACAATTCCTG	BAFF-bw	TCGTCTCCGTTGCGTGAAATC
IFN $\gamma$ -fw	ATGAACGCTACACACTGCATC	IFN $\gamma$ -bw	CCATCCTTTTGCCAGTTCCTC
IL-12a-fw	AGACATCACACGGGACCAAAC	IL-12a-bw	CCAGGCAACTCTCGTTCCTGT
IL-17a-fw	TTTAACTCCCTTGCGCAAAA	IL-17a-bw	CTTCCCTCCGCATTGACAC
TGF $\beta$ 1-fw	CTCCCGTGGCTTCTAGTGC	TGF $\beta$ 1-bw	GCCTTAGTTTGGACAGGATCTG
IL-6-fw	TAGTCCTTCTACCCCAATTTCC	IL-6-bw	TTGGTCCTTAGCCACTCCTTC-
CCL2-fw	TTAAAAACCTGGATCGGAACCAA	CCL2-bw	GCATTAGCTTCAGATTTACGGGT
CCL3-fw	TTCTCTGTACCATGACACTCTGC	CCL3-bw	CGTGGAATCTTCCGGCTGTAG
CCL9-fw	CCCTCTCCTTCCTCATTCTTACA	CCL9-bw	AGTCTTGAAAGCCCATGTGAAA
CXCL13-fw	GGCCACGGTATTCTGGAAGC	CXCL13-bw	GGGCGTAACTTGAATCCGATCTA
CCL5-fw	GCTGCTTTGCCTACCTCTCC	CCL5-bw	TCGAGTGACAAACACGACTGC
CCL20-fw	GCCTCTCGTACATACAGACGC	CCL20-bw	CCAGTTCTGCTTTGGATCAGC
IL-18-fw	GACTCTTGCGTCAACTTCAAGG	IL-18-bw	CAGGCTGTCTTTTGTCAACGA
CX3CR1-fw	GAGTATGACGATTCTGCTGAGG	CX3CR1-bw	CAGACCGAACGTGAAGACGAG
CXCR4-fw	GACTGGCATAGTCGGCAATG	CXCR4-bw	AGAAGGGGAGTGTGATGACAAA
CCR1-fw	CTCATGCAGCATAGGAGGCTT	CCR1-bw	ACATGGCATCACCAAAAATCCA
CCR10-fw	GGACTTTACTCCGGGTACGAT	CCR10-bw	CAGGGAGACTGGGTTGGA
chemR23-fw	ATGGAGTACGACGCTTACAACG	chemR23-bw	GGTGGCGATGACAATCACCA
L32-fw	AAGCGAAACTGGCGGAAAC	L32-bw	TAACCGATGTTGGGCATCAG

**Table S2.** List of primers for speed congenic PCR.

<b>Name</b>	<b>Primer sequence</b>	<b>Name</b>	<b>Primer sequence</b>
D4Mit17-fw	TGGCCAACCTCTGTGCTTCC	D4Mit17-bw	ACAGTTGTCCTCTGACATCC
D4Mit9-fw	GGCTTTGGAATGCTATGCAT	D4Mit9-bw	TGGCAGGAGGTATGACAGAA
D4Mit146-fw	AAAAATGACAGCATTATGTTGGG	D4Mit146-bw	CTCCCTCAGTCTTGCTTTGG
D5Mit145-fw	TATCAGCAATACAGACTCAGTAGGC	D5Mit145-bw	TGCCCTTAAATTCATGGTC
D5Mit13-fw	CATCGTTGCTCTTGACAGGA	D5Mit13-bw	CCGGGAGAACCCAAATAAGT
D5Mit356-fw	CCATGCCCAGTCTGGTATCT	D5Mit356-bw	TTTTACTTTCCACTAAGCATTCCC
D7Mit82-fw	GGACACGGTGTCCATCAAG	D7Mit82-bw	CTGAGTAGAAAGCATGTGGGG
D7Mit211-fw	CAATGAGAACTATAGAATGCTCAGC	D7Mit211-bw	TCATCTACTCGCTTTGGCCT
D7Nds1-fw	GAGATCTTCATACTCATATT	D7Nds1-bw	TAGATAGTGTTAACAGTGACC
D7Mit39-fw	ACATGCAGAGGCACACAAAC	D7Mit39-bw	AAGTATGAGCTATGGCACCTAGC
D10Mit51-fw	CTTGAGCCTACGTGACCACA	D10Mit51-bw	TGCAGTCCCTCACACATATAACA
D10Mit20-fw	CACCCTCACACAGATATGCG	D10Mit20-bw	GCATTGGGAAGTCCATGAGT
D10Mit11-fw	GAGAAGTCACTGGGAGCTGG	D10Mit11-bw	TTGCCAGGTTGCTCTTCTTT
D17Mit16-fw	ACCCAGAAGACAGCATTCCA	D17Mit16-bw	TGTATGTCAGGGCTAGTTGACAGG
TNF-fw	GTTTCAGTTCTCAGGGTCCTA	TNF-bw	CAGGATTCTGTGGCAATCTGG
CX3CR1 wild type-fw	GTCTTACGTTCCGGTCTGGT	CX3CR1 common-bw	CCCAGACACTCGTTGTCCTT
CX3CR1 mutant-fw	CTCCCCCTGAACCTGAAAC		
Fas common-fw	GTAAATAATTGTGCTTCGTCA G	Fas wild type-bw	CAAATCTAGGCATTAACAGTG
		Fas mutant-bw	AGAAAGGTGCACGGGTGTG

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## Chapter 7. Discussion

Overall, in these studies, we have demonstrated that vitamin A, particularly its functional metabolite, tRA, has both anti-inflammatory effects to ameliorate glomerulonephritis and pro-inflammatory effects to deteriorate lupus disease in the tubulointerstitial region of kidney and other organs. The pro-inflammatory effects of tRA is dependent on the pre-existing inflammatory environment established in the body, suggesting that tRA treatment alone can only ameliorate part of the inflammation in lupus disease. Other targeting treatment strategies to eliminate additional inflammation contributors are required.

During the investigation of inflammation contributors in lupus, we have found that although pDCs are capable of producing large amounts of IFN $\alpha$  at the early stage of lupus development, they gradually lose this ability at the later stage, suggesting that pDC-targeted treatment of lupus should be performed at the initiation stage of the autoimmune responses in lupus rather than after the symptoms have been established. However, this pDC targeting strategy will need the support of improved early disease diagnosis. Therefore, directly targeting IFN $\alpha$  rather than pDCs, or identifying late-stage IFN $\alpha$  producers for specific targeting, appear to be more practical and more translatable into future clinical treatments.

Different from the contribution of pDCs at the early stage of lupus development, we have identified a subpopulation of renal-infiltrating cDCs that significantly accumulate at the late stage and possess a phenotype that suggests its mature monocyte origin. Although CX3CR1, a chemokine receptor highly expressed by these cDCs, is dispensable for their migration into the kidney, the use of anti-CX3CR1-saporin conjugates to directly targeting these cells has shown



beneficial effects against LN in MRL/lpr mice, suggesting that these cDCs play critical pathogenic roles in promoting LN at the late stage. It is worthwhile to investigate whether similar renal-infiltrating cDCs, which could be a new therapeutic target, exist in human patients with LN.

There are several pitfalls in these studies that can be improved in the future. In general, all these studies were performed in mouse models instead of SLE patients. The similarity of the immune system between mice and humans makes them a great model to study the detailed immune mechanisms that may be involved in human diseases. However, there are still many differences between these two species, which may lead to failures of clinical trials based on discoveries in mice. To overcome this hurdle, it is worthwhile to establish humanized lupus mouse models aiming to transfer the immune system of SLE patients into mice. Besides this common pitfall, in the first project, although we used MRL mice as a control to suggest that the pro-inflammatory effects of tRA in MRL/lpr mice were dependent on the pre-existing inflammatory environment, we still cannot exclude the possibility that it was due to the unique characteristics of MRL/lpr mouse strain. In the second project, although we demonstrated the deficiency of IFN $\alpha$  production by pDCs from late stage lupus mice *ex vivo*, the similar *in vivo* situation was not confirmed. In addition, pDCs can produce IFN $\alpha$  by both TLR7 and TLR9 stimulations. However, in our studies, the deficiency of IFN $\alpha$  production in pDCs was only evaluated in response to TLR9 stimulation. In the third project, the surface phenotype of renal-infiltrating CD11c<sup>+</sup> cells suggested their mature monocyte origin, but we didn't demonstrate it *in vivo*. In antibody-drug conjugate (ADC) treatment study, although ADC treated group had higher body weight and lower proteinuria scores, the pathological scores of both glomerular and tubulointerstitial regions

were not significantly lower, suggesting that a higher dose of ADC may be required to show more beneficial effects. In addition, we only used PBS administration as the control group, which cannot exclude the off-target effect of ADC through FcγRs on other cells. However, in lupus mice, this off-target effect should be minimal because a large amount of auto-IgG already exists in the body to block FcγRs.

These studies have enhanced our understanding of the pathogenesis of SLE. The paradoxical effects of tRA in lupus mice not only suggests that nutritional supplementation could be a novel therapy but also reflects that no one single treatment can completely reduce the complicated pathogenesis of SLE, as it is due to many aspects of the autoimmune responses with different mechanisms. The functional changes of pDCs with lupus disease progression further suggest the dynamic changes of immune responses which require different targeting strategies at different stages of lupus development. Moreover, the identification of a pathogenic cDC subset in the nephritic kidney of lupus mice suggests that effector immune responses in non-immune peripheral tissues are critical in the pathogenesis of SLE and can be targeted without affecting the initiation of autoimmune responses in immune tissues.

In summary, SLE is a complicated autoimmune disease that requires a combination of natural and specific interventions to effectively suppress autoimmune responses while simultaneously preserving the normal immune responses. Based on these studies, I propose the use of combination therapies against SLE as shown in Figure 1. tRA administration as a natural way shows beneficial effects on glomerulonephritis but detrimental effects on tubulointerstitial nephritis and other organs. Therefore, kidney-specific delivery of tRA should be performed by

using chemically modified nanoparticles containing tRA with kidney-enrichment property. To further minimize the detrimental effects of tRA on tubulointerstitial nephritis, a method targeting critical inflammatory contributors should be performed as a compensation. We demonstrated that pDCs were able to produce a large amount of IFN $\alpha$  at the early stage of lupus but lost this ability at the late stage of lupus. Therefore, pDC-targeted therapy could be applied at the early stage of lupus progression, together with tRA administration as an early combination therapy. For the late stage of lupus progression, IFN $\alpha$  or other inflammatory cells could be targeted. We identified a renal-infiltrating cDC population that was pathogenic in the late stage of LN in lupus mice that can be targeted based on the high expression of CX3CR1. If a similar population exists in the nephritic kidney of SLE patients, these renal-infiltrating cDCs could be a therapeutic target at the late stage of lupus progression, together with tRA administration as a late combination therapy. Compared to current treatments, the advantage of these combination therapies is that they are much less cytotoxic and less immunosuppressive, but are able to control lupus progression efficiently. However, there are still some hurdles to overcome. One is that nanotechnology needs to be improved to produce the nanoparticles more physiologically degradable with a consistent size. Another is that the early combination therapy depends on the improvement of early diagnosis with the detection of reliable biomarkers far before the onset of symptoms. In addition, pDCs although critical for lupus development are also involved in the immune responses against pathogens. How to balance the reduced lupus risk and the increased infection risk by targeting pDCs requires further investigations. Finally, as it is difficult to find a surface marker uniquely expressed by our target cells, the potential off-target effects need to be evaluated carefully.

As a future direction, the safety and efficacy of combination therapies can be evaluated in lupus-prone mice that involve nanoparticle-based tRA delivery into the kidney and specific DC-targeting at different stages of lupus progression. In addition, we will investigate whether there is a similar pathogenic cDC population in the nephritic kidney of SLE patients as what we have found in lupus mice. Based on the safety and efficacy studies in mouse models and clinical relevant studies with human samples, the combination therapies will then be evaluated in clinical trials on SLE patients to finally exam their safety and efficacies compared to those of current treatments.

