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NLRC3 expression in dendritic cells attenuates CD4$^+$ T cell response and autoimmunity

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Abstract

NOD-like receptor (NLR) family CARD domain containing 3 (NLRC3), an intracellular member of NLR family, is a negative regulator of inflammatory signaling pathways in innate and adaptive immune cells. Previous reports have shown that NLRC3 is expressed in dendritic cells (DCs). However, the role of NLRC3 in DC activation and immunogenicity is unclear. In the present study, we find that NLRC3 attenuates the antigen-presenting function of DCs and their ability to activate and polarize CD4$^+$ T cells into Th1 and Th17 subsets. Loss of NLRC3 promotes pathogenic Th1 and Th17 responses and enhanced experimental autoimmune encephalomyelitis (EAE) development. NLRC3 negatively regulates the antigen-presenting function of DCs via p38 signaling pathway. Vaccination with NLRC3-overexpressed DCs reduces EAE progression. Our findings support that NLRC3 serves as a potential target for treating adaptive immune responses driving multiple sclerosis and other autoimmune disorders.

Keywords autoimmunity; dendritic cells; NLRC3; p38; vaccination
Subject Categories Immunology
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Introduction

CD4$^+$ T cells play a key role in regulating adaptive response during infection and tumor conditions (Godfrey *et al*, 2018). However, the dysregulated activity of the Th1 and Th17 subsets of CD4$^+$ T cells triggers tissue inflammation and autoimmunity, including multiple sclerosis, and its animal model, experimental autoimmune encephalomyelitis (EAE) (Simmons *et al*, 2013). Dendritic cells (DCs) are potent and versatile antigen-presenting cells that play a crucial role at the interface of innate and adaptive immunity (Murphy *et al*, 2016). On stimulation by antigens, DCs increase the expression of surface co-stimulatory molecules and proinflammatory or anti-inflammatory cytokines to direct differentiation of naive CD4$^+$ T cells (Lombardi *et al*, 2009; Shan *et al*, 2009; Mascanfroni *et al*, 2013; Segura *et al*, 2013). Alterations in the functioning of DC play a major or even key role in allergy and autoimmune diseases like lupus erythematosus, inflammatory bowel diseases, and EAE (Worbs *et al*, 2017). Thus, it is important to study the detailed molecular mechanisms that mediate DC activation and immunogenicity for identifying mechanisms of autoimmune disease pathogenesis and developing new approaches of therapeutic intervention.

Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) are cytoplasmic receptors that mediate immune regulation during infection or cellular stress (Elinav *et al*, 2011; Motta *et al*, 2015). However, dysregulation in the functioning of multiple NLR family members, such as NLR family pyrin domain containing 3 (NLPP3), NLR family CARD domain containing 4 (NLRC4), and NLR family pyrin domain containing 12 (NLRP12), has been linked to autoinflammatory disease (Romberg *et al*, 2014; Coll *et al*, 2015; Lukens *et al*, 2015). NLRs contribute to diverse biological functions by regulating multiple downstream signals, such as promoting inflammatory responses (Kanneganti *et al*, 2007; Rathinam *et al*, 2012; Van Gorp *et al*, 2014), activating nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs) and PI3K-AKT pathways (Geddes *et al*, 2009; Griebel *et al*, 2014; Karki *et al*, 2016), and altering transcriptional activity (Motta *et al*, 2015) in different cell types. In recent years, studies to understand the roles of NLRs in regulating DC responses have increased. Previous study has shown that NOD2 induces autophagy to regulate generation of major histocompatibility complex (MHC) class II antigen-specific CD4$^+$ T-cell responses in DCs (Cooney *et al*, 2010). Previous report has shown that NLRP10 governs DC migration and is essential for the initiation of adaptive immunity through DCs (Eisenbarth *et al*, 2012), but subsequent research finds that the role of NLRP10 is due to an unexpected mutation in Dock8 harbored by the original *Nlrp10* knockout mice (Krishnaswamy *et al*, 2015). NLRP10 deficiency in DCs leads to lower level of IL-12 due to a substantial decrease in NF-κB activation, which further decreases IFN-γ production by CD4$^+$ T cells

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(Vacca *et al*, 2017). However, the precise role of most of the NLR family members in DCs has not been characterized.

NLRC3, an intracellular member of NLR family, was first detected in T cells and is reported to be expressed in various immune tissues and immune cell populations (Conti *et al*, 2005). As a negative regulator of inflammatory signaling pathways, NLRC3 inhibits NF-κB and stimulator of interferon genes (STING) pathways on lipopolysaccharide (LPS) stimulation or pathogen infection in macrophages (Schneider *et al*, 2012; Zhang *et al*, 2014; Guo *et al*, 2017; Tocker *et al*, 2017; Li *et al*, 2019). In T cells, NLRC3 attenuates the activation, proliferation, and cytokine production of $CD4^+$ T cells (Hu *et al*, 2018; Uchimura *et al*, 2018). In colorectal cancer, NLRC3 is considered as an inhibitory sensor of the PI3K/AKT/mTOR signaling pathway that protects against tumorigenesis (Karki *et al*, 2016, 2017). Previous reports have shown that NLRC3 is expressed in DCs (Conti *et al*, 2005; Hu *et al*, 2018). However, the role of NLRC3 and the mechanisms underlying its effect on DC activation and immunogenicity remain unclear. The present study focused on investigating the functions and molecular mechanisms underlying NLRC3-mediated regulation of DC activation and immunogenicity in autoimmune diseases. Our results revealed that NLRC3 attenuates the antigen-presenting function of DCs and their ability to activate and polarize naïve $CD4^+$ T cells into Th1 and Th17 subsets and thereby restricts EAE progression. NLRC3 has a negative effect via regulating p38 signal pathway in DCs *in vitro* and *in vivo*. Significantly, vaccination with DCs overexpressing NLRC3 regulated the development of EAE. Our findings suggest that NLRC3 serves as a potential target for treating misguided adaptive immune responses that induce allergy and autoimmunity.

Results

NLRC3 deficiency promotes antigen-presenting function of DCs *in vitro*

Although NLRC3 has been demonstrated as a negative regulator in macrophages and T cells (Schneider *et al*, 2012; Zhang *et al*, 2014; Hu *et al*, 2018), the specific role of NRLC3 in DCs still remains poorly understood. In the presence of DC-maturing stimuli, DCs uptake antigens and mature to become the most potent antigen-presenting cells accompanied by upregulation of MHC II and co-stimulatory molecules (Guermonprez *et al*, 2002). To investigate the role of NLRC3 in activating DCs, we first sorted the DCs from the spleens of wild-type (WT) and *Nlrc3*$^{-/-}$ mice and then investigated the expression of NLRC3. As expected, NLRC3 was expressed in DCs from WT naïve mice, but not in DCs from *Nlrc3*$^{-/-}$ naïve mice (Appendix Fig S1). Further, we stimulated DCs with LPS and found that NLRC3 deficiency in DCs led to significantly enhanced expression of MHC II and co-stimulatory molecules, such as CD40, CD86, and CD80 (Fig 1A and Appendix Fig S2A). It has been shown that DCs play a crucial role in $CD4^+$ T-cell differentiation via by regulating the secretion of polarizing cytokines (van Panhuys *et al*, 2014). We found that on LPS stimulation, DCs from *Nlrc3*$^{-/-}$ mice showed higher expression of IL-12, IL-6, and IL-23 while lower expression of IL-27 compared to the IL-12, IL-6, IL-23, and IL-27 expression found in DCs from WT mice (Fig 1B). We did not observe any effect of NLRC3 deficiency on expression of IL-4, TGF-β, IL-10, or IL-1β (Appendix Fig S2B) in LPS-stimulated DCs. Similarly, DCs from the spleen of *Nlrc3*$^{-/-}$ mice showed enhanced expression of MHC II and co-stimulatory molecules CD40, CD86, and CD80 on stimulation with CD40 ligand, soluble form (CD40L) compared to the MHC II and co-stimulatory molecule expression in DCs from the spleen of WT mice (Appendix Fig S3A and B). Further, we also found consistency that on stimulation with CD40L, NLRC3 deficiency in DCs led to enhanced expression of IL-12, IL-6, and IL-23, and reduced expression of IL-27 while IL-4, TGF-β, IL-10, or IL-1β expression was unaltered (Appendix Fig S3C). Overall, the data suggested that NLRC3 negatively regulates the production of cytokines that promote the differentiation of Th1 and Th17 cells and has no effect on the production of anti-inflammatory cytokines in DCs.

Further, we investigated the ability of NLRC3 to regulate DCs in activating and polarizing $CD4^+$ T cells into specific subsets. DCs from WT and *Nlrc3*$^{-/-}$ mice were activated with LPS and co-cultured with naïve TCR transgenic 2D2 $CD4^+$ T cells in the presence of myelin oligodendrocyte glycoprotein (MOG)(35–55), a type of cognate target antigen. Analysis of intracellular IFN-γ and IL-17A expression revealed that NLRC3 deficiency promoted the ability of DCs to polarize $CD4^+$ T cells into Th1 and Th17 subsets (Fig 1C and Appendix Fig S4A). Similarly, naïve 2D2 $CD4^+$ T cells co-cultured with DCs from *Nlrc3*$^{-/-}$ mice were found to secrete enhanced expressions of IFN-γ and IL-17A (Fig 1D). Moreover, DCs with NLRC3 deficiency were found to enhance proliferation of naive 2D2 $CD4^+$ T cells based on thymidine incorporation (Fig 1E) or carboxyfluorescein succinimidyl ester (CFSE) dye dilution (Fig 1F and Appendix Fig S4B). Overall, the data indicated that NLRC3 deficiency promotes antigen-presenting function of DCs and their ability to activate and polarize $CD4^+$ T cells into Th1 and Th17 subsets.

NLRC3-deficient aggravates EAE development

Production of the DC-mediated pathogenic Th1 and Th17 cell response is associated with the progression of central nervous system (CNS) autoimmunity (Greter *et al*, 2005). Given that DCs show enhanced ability to activate and polarize $CD4^+$ T cells into Th1 and Th17 subsets under NLRC3-deficient conditions, we investigated the role of NLRC3 in EAE development. We found a significant aggravation of EAE in *Nlrc3*$^{-/-}$ mice (Fig 2A). NLRC3 deficiency did not affect the number of $CD4^+$ T cells and $CD8^+$ T cells in CNS (Appendix Fig S5A). However, $CD4^+$ T cells isolated from CNS of *Nlrc3*$^{-/-}$ mice showed enhanced expression of intracellular IFN-γ and IL-17A on re-stimulating with MOG(35–55) compared to IFN-γ and IL-17A expression by $CD4^+$ T cells isolated from WT mice (Fig 2B). Further, we found that NLRC3 deficiency showed no effect on intracellular expression of IFN-γ in $CD8^+$ T cells (Appendix Fig S5B). To investigate the role of DCs in NLRC3-deficient EAE progression, we isolated DCs from WT and *Nlrc3*$^{-/-}$ mice 26 days after EAE induction. Analysis of expression of surface molecules revealed that *Nlrc3*$^{-/-}$ DCs showed upregulation of MHC II and co-stimulatory molecules CD40, CD86 and CD80 compared to MHC II and co-stimulatory molecule expression in WT DCs (Fig 2C and Appendix Fig S5C). To detect intracellular genetic expression in DCs, we did not actually detect NLRC3 expression in isolated DCs from *Nlrc3*$^{-/-}$ mice (Fig 2D). NLRC3 deficiency in DCs induced upregulation of IL-12, IL-6, and IL-23, while downregulation of

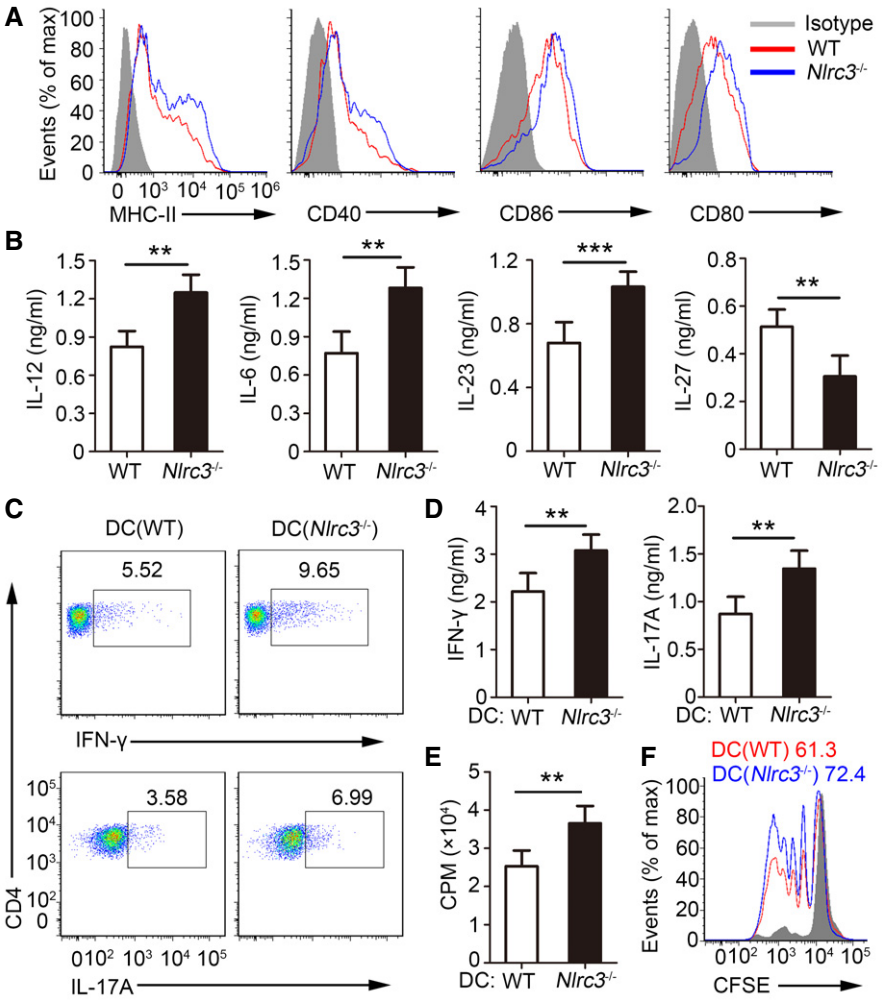

Figure 1. NLRC3 deficiency promoted antigen-presenting function of DCs.

A Representative flow cytometry data showing surface phenotypes of DCs sorted from spleens of wild-type (WT) or *Nlrc3*$^{-/-}$ mice and treated with LPS (100 ng/ml) for 48 h.

B ELISA of cytokines in culture supernatants of DCs treated as in (A).

C–F The intracellular production of IFN-γ and IL-17 by CD4^{+} T cells (C), cytokines in culture supernatants (D), thymidine incorporation proliferation assay (E), and CFSE proliferation assay (F) among naive 2D2 CD4^{+} T cells stimulated with MOG(35–55) plus DCs treated as in (A).

Data information: Data (n = 5 in B, D, and E) shown are the mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ by an unpaired t-test. Data are representative of three independent experiments with similar results.

IL-27 (Fig 2D). However, the expression of IL-4, TGF-β, IL-10, and IL-1β did not show any significant difference in the DCs isolated from WT and *Nlrc3*$^{-/-}$ mice (Appendix Fig S5D). The results suggested that NLRC3 deficiency in DCs contributes to the worsening of EAE in *Nlrc3*$^{-/-}$ mice.

NLRC3 expression in DC restricts EAE progression

It has been reported that NLRC3 is expressed by many types of cells, including T cells (Hu *et al*, 2018; Uchimura *et al*, 2018) that influence activity of DCs via their interactions. Thus, it still remains unclear about the direct contribution of NLRC3 regulates DCs during EAE progression. To further study the role of NLRC3 in DCs during EAE, we used a chimera-based approach to generate mice lacking NLRC3 expression in DCs (Appendix Fig S6A) as described (D'Amico & Wu, 2003; Mascanfroni *et al*, 2013). The mice that reconstituted their DC compartment with DC precursors from WT mice were defined as DC(WT) mice while those mice which reconstituted their DC compartment from *Nlrc3*$^{-/-}$ mice were defined as DC(NLRC3-KO) mice. The NLRC3 expression was significantly lower in DCs from DC(NLRC3-KO) mice compared to NLRC3 expression in DCs from DC(WT) mice. However, there was no difference in the monocytes between DC (NLRC3-KO) mice and DC(WT) mice (Appendix Fig S6B). The frequency or absolute number of DCs was not altered by NLRC3 deficiency (Appendix Fig S6C). Consistent with *Nlrc3*$^{-/-}$ mice, DC (NLRC3-KO) mice reached significantly higher disease scores than did DC(WT) groups after immunization with MOG(35–55)

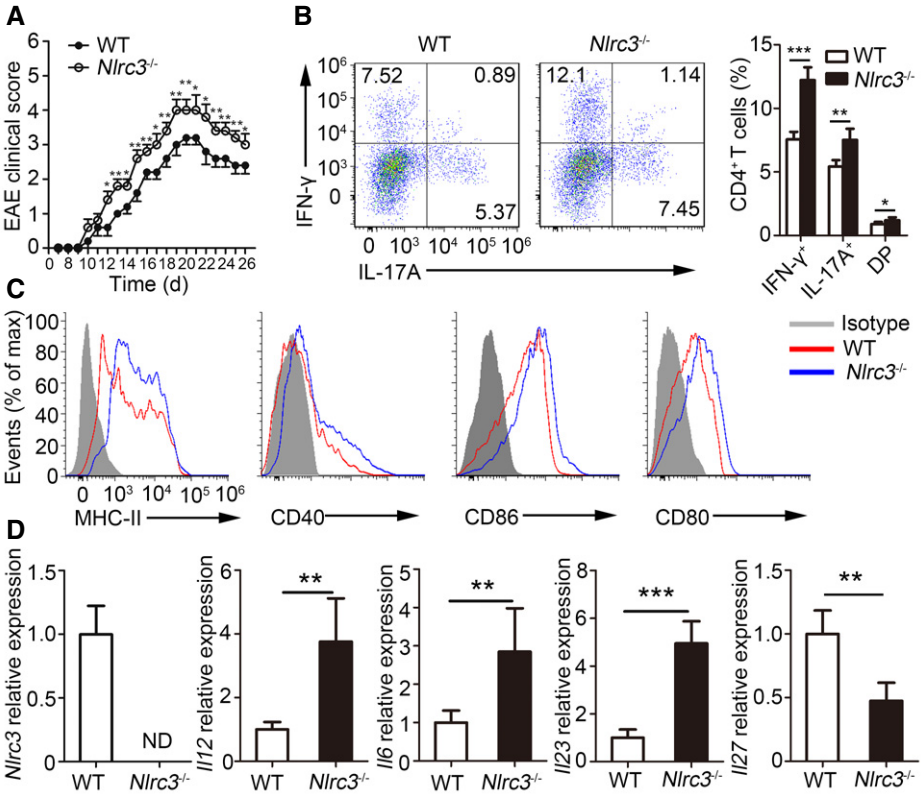

Figure 2. NLRC3 deficiency promoted EAE development.

WT and *Nlrc3*$^{-/-}$ mice were immunized with MOG(35–55) peptide in CFA adjuvant and pertussis toxin to induce EAE.

A Mean clinical scores of EAE in immunized WT (n = 10) and *Nlrc3*$^{-/-}$ mice (n = 10).

B Representative flow cytometry data showing intracellular production of IFN-γ and IL-17A by CD4$^+$ T cells from the spinal cord and brain of WT or *Nlrc3*$^{-/-}$ mice 26 days after EAE induction after restimulation with MOG(35–55) peptide. Pooled data are presented in the right panel.

C Representative flow cytometry data showing surface phenotypes of DCs from spleens of WT or *Nlrc3*$^{-/-}$ mice 26 days after EAE induction.

D Expression of *Nlrc3*, *Il12*, *Il6*, *Il23*, and *Il27* mRNA in DCs sorted from WT and *Nlrc3*$^{-/-}$ mice 26 days after EAE induction, presented relative to that of *Gapdh*.

Data information: Data (n = 5 in B right and D) shown are the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 by an unpaired t-test. Data are representative of three independent experiments with similar results.

(Fig 3A). Intracellular staining showed a higher expression of IFN-γ$^+$ and IL-17A$^+$ CD4$^+$ T cells in CNS of DC(NLRC3-KO) mice compared to IFN-γ$^+$ and IL-17A$^+$ CD4$^+$ T cells in CNS of DC (WT) mice (Fig 3B and Appendix Fig S6D). Moreover, we performed the splenic CD4$^+$ T-cell thymidine incorporation assays and found that splenic CD4$^+$ T cells isolated from DC(NLRC3-KO) mice had a higher recall proliferative response to MOG(35–55) compared to recall proliferative response of splenic T cells from DC(WT) mice (Fig 3C).

To study the effects of NLRC3 in DCs during EAE progression, we isolated DCs from DC(WT) and DC(NLRC3-KO) mice 26 days after EAE induction. DCs with NLRC3-deficiency upregulated expression of IL-12, IL-6, and IL-23, concomitant with down-regulated expression of IL-27 (Fig 3D). Moreover, we found that DCs from DC(NLRC3-KO) mice promoted proliferation of 2D2 CD4$^+$ T cells based on CFSE dye dilution (Fig 3E and Appendix Fig S6E) along with increased production of IFN-γ and IL-17 (Fig 3F). Together, these results demonstrated that NLRC3 negatively regulated the antigen-presenting function of DCs *in vivo* to regulate the progression of pathogenic CD4$^+$ T cells and EAE.

NLRC3 negatively regulates p38 signaling pathway in DCs *in vitro*

The specific molecular pathways that mediate the role of NLRC3 in DCs are still unknown. To reveal these pathways, DCs were isolated from WT and *Nlrc3*$^{-/-}$ mice and stimulated with LPS. We found that NLRC3 deficiency in DCs caused enhanced phosphorylation of p38 (Fig 4A and Appendix Fig S7A). However, the phosphorylation of p65, protein kinase B (AKT), extracellular signal-regulated kinases (ERK), and c-Jun N-terminal kinase (JNK) had no difference between the two groups (Fig 4A and Appendix Fig S7A). Similarly, we observed enhanced phosphorylation of p38, however, no change in the phosphorylation of p65, AKT, ERK, and JNK in DCs from *Nlrc3*$^{-/-}$ mice compared to the phosphorylation patterns observed in DCs from WT mice on stimulation with CD40L (Appendix Fig S7B). Further, we assessed the activation of the two crucial p38 targets, mitogen- and stress-activated protein kinase 1 (MSK1) and MAPK-activated protein kinase 2 (MK2), and found that they were enhanced in DCs from *Nlrc3*$^{-/-}$ mice compared to MSK1 and MK2 expression in DCs from WT mice (Appendix Fig S8A). Additionally, phosphorylation of cAMP-response element binding protein (CREB),

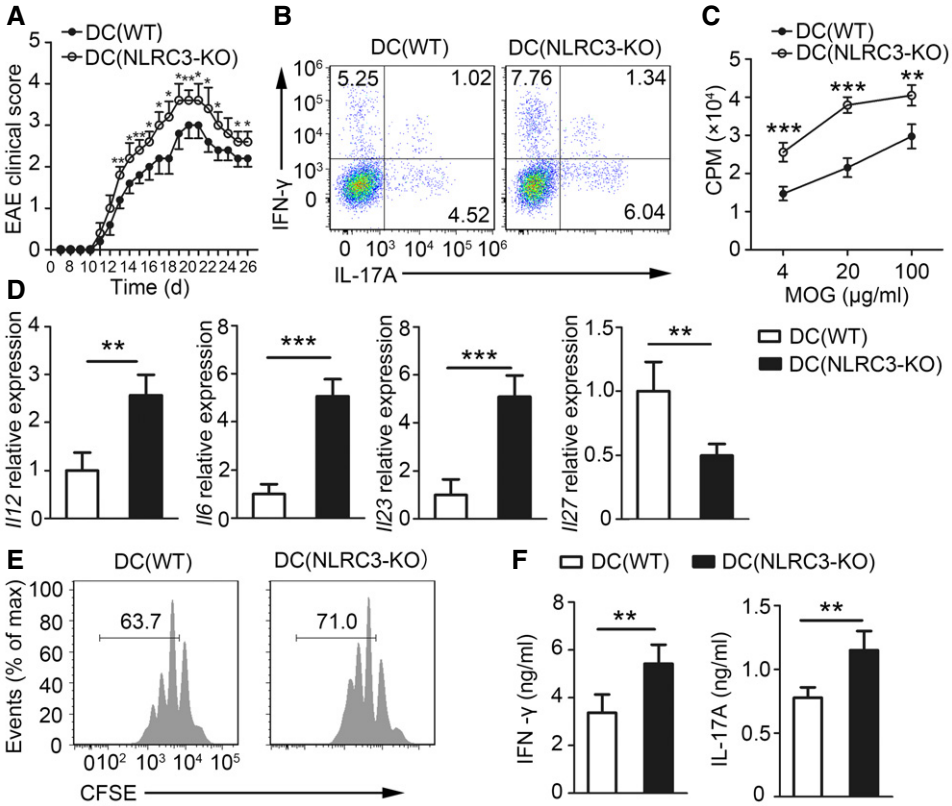

Figure 3. NLRC3 deficiency in DC promoted EAE development.

DC(WT) and DC(NLRC3-KO) mice were immunized with MOG(35–55) peptide in CFA adjuvant and pertussis toxin to induce EAE.

A Mean clinical scores of EAE in immunized DC(WT) (n = 5) and DC(NLRC3-KO) mice (n = 5).

B Representative flow cytometry data showing intracellular production of IFN-γ and IL-17A by $CD4^+$ T cells in the spinal cord and brain from DC(WT) and DC(NLRC3-KO) mice 26 days after EAE induction after restimulation with MOG(35–55) peptide.

C Recall response to MOG(35–55) by splenocytes isolated from DC(WT) and DC(NLRC3-KO) mice 26 days after EAE induction.

D Expression of *Il12*, *Il6*, *Il23*, and *Il27* mRNA in DCs sorted from DC(WT) and DC(NLRC3-KO) mice 26 days after EAE induction, presented relative to that of *Gapdh*.

E, F CFSE proliferation assay (E) and cytokine secretion (F) of naive 2D2 $CD4^+$ T cells stimulated with MOG(35–55) plus DCs sorted from DC(WT) and DC(NLRC3-KO) mice 26 days after EAE induction.

Data information: Data (n = 5 in C, D, and F) shown are the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 by an unpaired t-test. Data are representative of three independent experiments with similar results.

a transcription factor important for IL-6 production, was found to be significantly enhanced in DCs from *Nlrc3*$^{-/-}$ mice compared to phosphorylation of CREB in DCs from WT mice (Appendix Fig S8A).

Next, we investigated the effect of inhibiting p38 phosphorylation on ability of DCs. Cytokine detection revealed that the p38 MAP kinase inhibitor (SB203580) significantly retuned increased expressions of IL-12, IL-6, and IL-23 caused by NLRC3 deficiency in DCs (Fig 4B). While the expressions of IL-27 were increased in DCs treated with SB203580 (Fig 4B). Importantly, these cytokine expressions showed no difference in the DCs isolated from WT mice and *Nlrc3*$^{-/-}$ mice (Fig 4B). However, the NF-κB inhibitor (JSH-23) did not retune significantly enhanced expression of IL-12, IL-6, and IL-23 caused by NLRC3 deficiency in DCs (Appendix Fig S8B). Furthermore, treatments with pharmacological inhibition of p38 signaling pathway treatments lowered the ability of *Nlrc3*$^{-/-}$ mice-isolated DCs to induce production of IFN-γ and IL-17A by naïve 2D2 $CD4^+$ T cells (Fig 4C) and also to activate proliferation of naïve 2D2 $CD4^+$ T cells (Fig 4D and Appendix Fig S8C). These results demonstrated that NLRC3 negatively regulates antigen-presenting function of DCs via p38 signaling pathway *in vitro*.

NLRC3 expression in DCs restricts EAE progression via negatively regulating p38 signaling pathway *in vivo*

To analyze whether NLRC3 regulated p38 signal pathway in DCs during EAE progression, we isolated DCs from spleens of DC(WT) and DC(NLRC3-KO) mice with EAE and detected the activation of p38 signaling pathway via flow cytometry. The results showed that DCs from DC(NLRC3-KO) mice had an enhanced phosphorylation of p38 compared to p38 phosphorylation in DCs from DC(WT) mice (Fig 5A). *In vitro* assays showed that NLRC3 negatively regulated the antigen-presenting function of DCs via p38 signaling pathway. We validated whether the same mechanism exists *in vivo*. Thus, we generated mice lacking NLRC3 and p38 expression in DCs. Concretely, we first crossed *Mapk14*$^{fl/fl}$ mice with mice expressing Cre

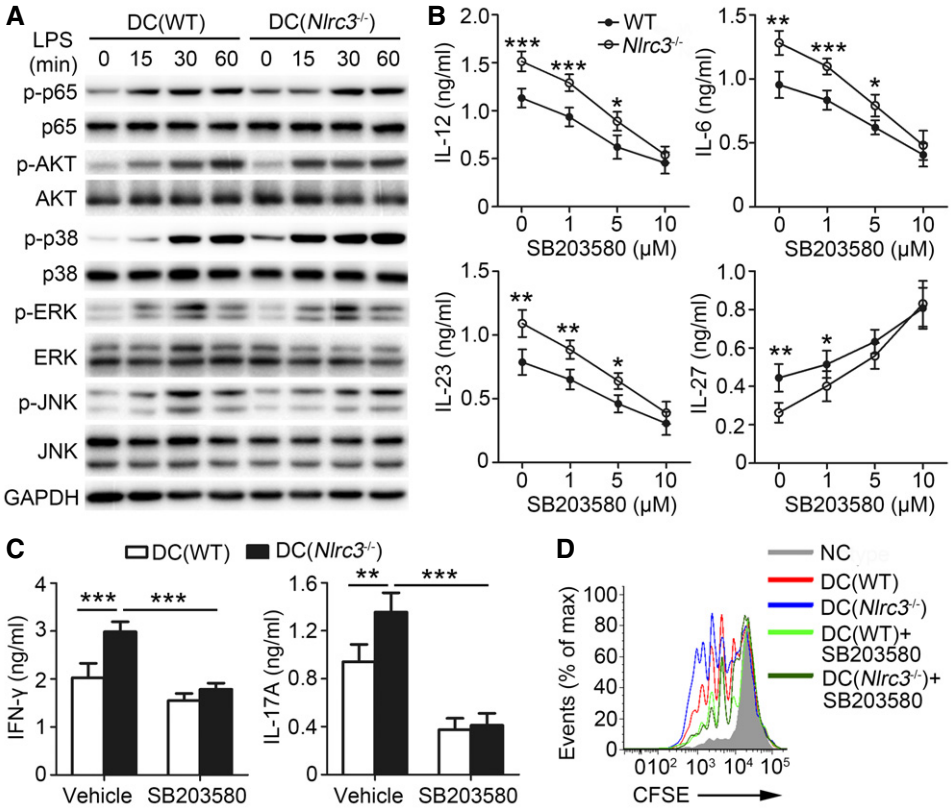

Figure 4. NLRC3 negatively regulated p38 signaling pathway in DC.

DCs were sorted from spleens of WT or *Nlrc3*$^{-/-}$ mice.

A Purified DCs were treated with LPS (100 ng/ml) for specified time. DC lysates were probed for phosphorylated p65 (p-p65), total p65, p-AKT, AKT, p-p38, p38, p-ERK, ERK, p-JNK, JNK, and GAPDH.

B–D Purified DCs were treated for 48 h with LPS (100 ng/ml) in the presence or absence of the p38 inhibitor SB203580 (10 μM or indicated concentrations). (B) Concentrations of IL-12, IL-6, IL-23, and IL-27 in supernatants were detected by ELISA. Cytokines in culture supernatants (C) and CFSE proliferation assay (D) among naive 2D2 CD4$^+$ T cells stimulated with MOG(35–55) plus DCs. NC: negative control.

Data information: Data (n = 5 in B and C) shown are the mean ± SD. *P < 0.05, **P < 0.01, and ***P < 0.001 by an unpaired t-test. Data are representative of three independent experiments with similar results.
Source data are available online for this figure.

recombinase driven by the Cd11c promoter (CD11c-Cre mice) to generate mice lacking p38 expression in DCs (defined as p38$^{\Delta DC}$ mice). Further, these p38$^{\Delta DC}$ mice were crossed with *Nlrc3*$^{-/-}$ mice to generate mice lacking p38 expression in DCs and completely lacking NLRC3 expression (defined as *Nlrc3*$^{-/-}$;p38$^{\Delta DC}$ mice here). Finally, we used the chimera-based approach to generate mice lacking p38 or both p38 and NLRC3 expression in DCs (defined as DC(p38-KO) or DC(p38 + NLRC3-KO) mice, respectively) (Appendix Fig S9A). The Western blot analysis of isolated DCs revealed that the expression of p38 was deficient in DCs of DC(p38-KO) mice while p38 and NLRC3 were deficient in DCs of DC(p38 + NLRC3-KO) mice (Appendix Fig S9B). No observable defects were found in the development or homeostasis of the immune system when p38 was deleted in DCs (Appendix Fig S9C).

To immunize these mice with MOG(35–55), we found that the DC(p38 + NLRC3-KO) mice developed a disease similar to that of DC(p38-KO) mice (Fig 5B). The expression level of Th1 and Th17 cells in the CNS showed no significant difference between DC(p38 + NLRC3-KO) and DC(p38-KO) mice (Fig 5C). Further, we isolated the DCs from DC(p38 + NLRC3-KO) and DC(p38-KO) mice with EAE. Quantitative PCRs were performed and showed that there was no differential expression in IL-12, IL-6, IL-23, and IL-27 on NLRC3 deficiency when p38 expression was knocked out in DCs (Fig 5D and Appendix Fig S9D). These results demonstrated that NLRC3 negatively regulated the antigen-presenting function of DCs *in vivo* to restrict the progression of pathogenic T cells and EAE in a dependent on p38 signaling pathway manner.

DC immunogenicity was significantly impaired on overexpression of NLRC3

Earlier in this study, we showed that NLRC3 is a key negative modulator in DCs using NLRC3-deficient models. Further, we examined the effect of NLRC3 overexpression on the DC immunogenicity *in vitro* and *in vivo*. DCs were transduced with either lentiviral vector encoding GFP and NLRC3 (LV-NLRC3) (defined as DC(NLRC-OE)) or only GFP (LV-Ctrl) (defined as DC(Ctrl)), and NLRC3 overexpression in DCs was confirmed by Western blotting (Fig 6A). We

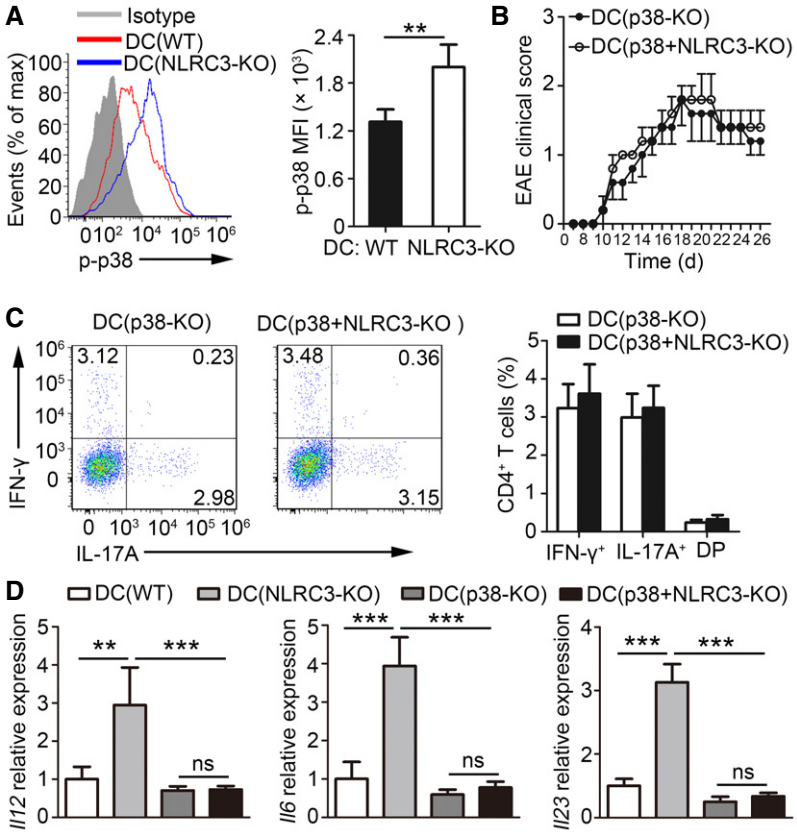

Figure 5. NLRC3 deficiency in DC promoted EAE development via p38 signaling pathway.

A Activity phosphorylation of p38 was detected in DCs in the spleens from DC(WT) and DC(NLRC3-KO) mice 26 days after EAE induction. Pooled data are presented in the right panel.

B–D DC(p38-KO) and DC(p38 + NLRC3-KO) mice were immunized with MOG(35–55) peptide in CFA adjuvant and pertussis toxin to induce EAE. (B) Mean clinical scores of EAE in immunized DC(WT) (n = 5) and DC(NLRC3-KO) mice (n = 5). (C) Frequencies of $CD4^+$ T cells that express IFN-γ and IL-17A in the spinal cord and brain from DC(WT) and DC(NLRC3-KO) mice 26 days after EAE induction after restimulation with MOG(35–55) peptide. Pooled data are presented in the right panel. (D) Expression of *Il12*, *Il6*, and *Il23* mRNA in DCs sorted from spleens of DC(WT), DC(NLRC3-KO), DC(p38-KO), and DC(p38 + NLRC3-KO) mice 26 days after EAE induction, presented relative to that of *Gapdh*.

Data information: Data (n = 5 in A, B, C right, and D) shown are the mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ by one-way ANOVA test. Data are representative of three independent experiments with similar results.

found that NLRC3 overexpression reduced the activity of p38 (Fig 6A). Secretion of IL-12, IL-6, and IL-23 by DCs was also significantly decreased on NLRC3 overexpression (Fig 6B), concomitant with an increased IL-27 expression (Fig 6B). Consistent with the altered expression of cytokines, NLRC3 overexpression significantly reduced the ability of DCs to induce production of IFN-γ and IL-17 by 2D2 $CD4^+$ T cells (Fig 6C). Flow cytometry showed that 2D2 $CD4^+$ T-cell proliferation was also reduced when they were co-cultured with DCs overexpressing NLRC3 (Fig 6D). These results further confirmed that NLRC3 might be an intrinsic negative regulator of DC immunogenicity to maintain immune homeostasis *in vivo*.

Vaccines with DCs overexpressing NLRC3 attenuate EAE progression

As an intrinsic negative regulator of DC immunogenicity, NLRC3 can be considered to have the therapeutic potential to treat disorders mediated by the immune system. Previous reports have shown that DC vaccine promotes the immune response in anti-tumor and pathogenic conditions (Tacken *et al*, 2007); however, tolerogenic DC vaccine reduces the immune response during an autoimmune disease (Mascanfroni *et al*, 2013). Thus, we investigated the therapeutic effects of vaccines with DCs overexpressing NLRC3 during EAE. Bone marrow-derived DCs (BMDCs) were overexpressed with NLRC3 using lentiviral vector, cultured for 4 h with 20 μg MOG(35–55), washed extensively, and administered intravenously (2×10^6 cells per mouse) into WT and $Nlrc3^{-/-}$ mice induced EAE on day 10 for four times, once every 4 days (Fig 7A and Appendix Fig S10A). Our results showed that vaccinating with DCs that did not overexpress NLRC3 or were not loaded with peptide had no significant effect on the disease development in WT or $Nlrc3^{-/-}$ mice induced EAE (Fig 7A and B, and Appendix Fig S10A and B). However, DC vaccine with NLRC3 overexpression loaded with the peptide significantly reduced EAE progression (Fig 7A and B, and Appendix Fig S10A). Healthy WT and $Nlrc3^{-/-}$ mice when administered with DC vaccine had a similar trend in weight in comparison with mice that

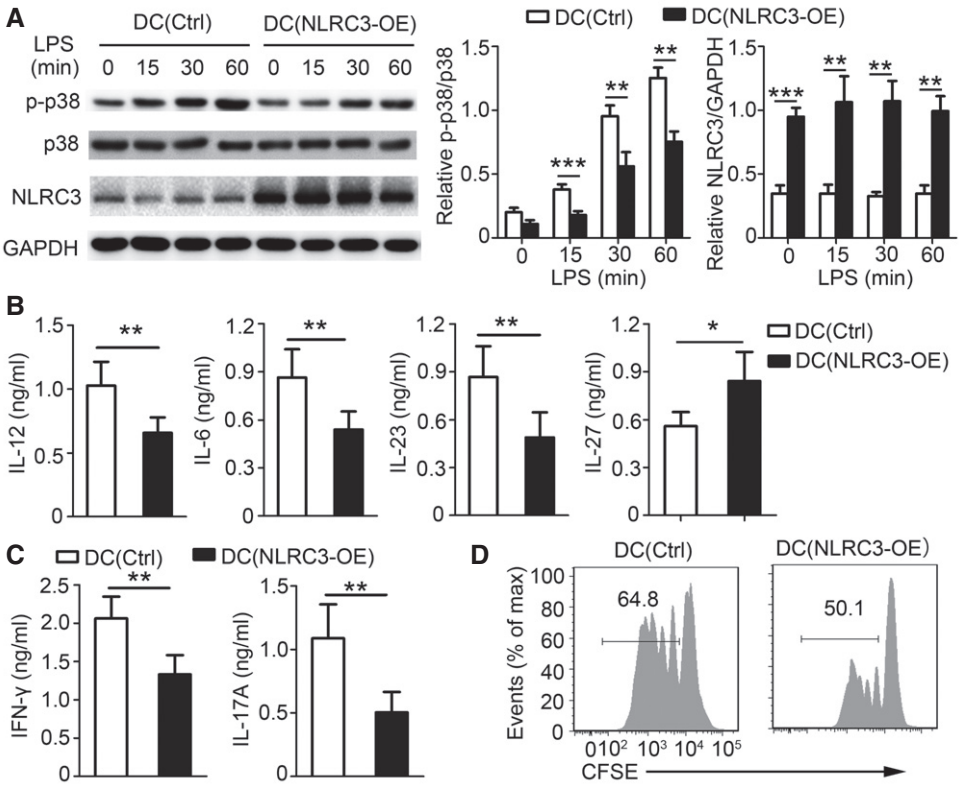

Figure 6. NLRC3-overexpression attenuated antigen-presenting function of DCs.

DC(Ctrl) and DC(NLRC3-OE) were stimulated with LPS (100 ng/ml) for specified time.

A DC lysates were probed for p-p38, p38, NLRC3, and GAPDH. Densitometry quantification of band intensity was presented in the right panel.

B Enzyme-linked immunosorbent assay of cytokines in culture supernatants of DCs treated with LPS for 48 h.

C, D Cytokines in culture supernatants (C) and CFSE proliferation assay (D) among naive 2D2 $CD4^+$ T cells stimulated with MOG(35–55) plus DCs treated with LPS for 48 h.

Data information: Data ($n = 3$ in A right; $n = 5$ in B and C) shown are the mean ± SD. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ by an unpaired t-test. Data are representative of three independent experiments with similar results.
Source data are available online for this figure.

were not administered DC vaccine. This showed that these DC vaccines did not bring a negative effect on the health of mice (Appendix Fig S10C and D). Furthermore, splenic T cells from mice treated by NLRC3 overexpressed DC vaccine loaded with peptide showed a reduced recall proliferative response to MOG(35–55) (Fig 7C) and decreased secretion of IFN-γ and IL-17A (Fig 7D) compared to the recall proliferative response, and IFN-γ and IL-17A expression in spleen T cells of other mice. Together, these data demonstrated that vaccinating with overexpressing NLRC3 can regulate the progression of EAE.

Discussion

Increasing evidence suggests that NLRs play a vital role in regulating DC activation and immunogenicity and thereby mediate $CD4^+$ T-cell activation and differentiation (Lombardi *et al*, 2009; Cooney *et al*, 2010; Eisenbarth *et al*, 2012; Murphy *et al*, 2016; Corridoni & Simmons, 2017; Fekete *et al*, 2018). However, the role of NLRC3 and the mechanisms underlying its effects on DC activation and immunogenicity still remain unclear. In the present study, we focused on elucidating the roles of NLRC3 and the mechanisms in regulating DCs. We demonstrated that NLRC3 attenuated the antigen-presenting function of DCs and their ability to activate and polarize naïve $CD4^+$ T cells into Th1 and Th17 subsets and further protect against EAE progression via negatively regulating p38 signaling pathway.

IL-12 secreted by DC induces polarization of naïve $CD4^+$ T cells to Th1 subset that predominantly expresses the cytokine IFN-γ, while IL-4 is a key cytokine involved in Th2 differentiation (Langenkamp *et al*, 2000; Walsh & Mills, 2013). Previous studies have shown that IL-6, IL-23, and TGF-β mediate IL-17 expression in lymphoid cells (Hu *et al*, 2017). In this study, we found that NLRC3 deficiency in DCs promotes expression of Th1-differentiation-related cytokine, IL-12; and Th17-differentiation-related cytokine, IL-6 and IL-23; however, there is no change in the expression of IL-4 or TGF-β. Thus, this suggested that NLRC3 in DCs might be a negative regulator of Th1 and Th17 differentiation, which was consistent with the *in vivo* results where enhanced Th1 and Th17 differentiation proportions were observed in *Nlrc3*$^{-/-}$ mice and mice with NLRC3-deficient DCs during EAE progression. The dysregulated activity of the Th1 and Th17

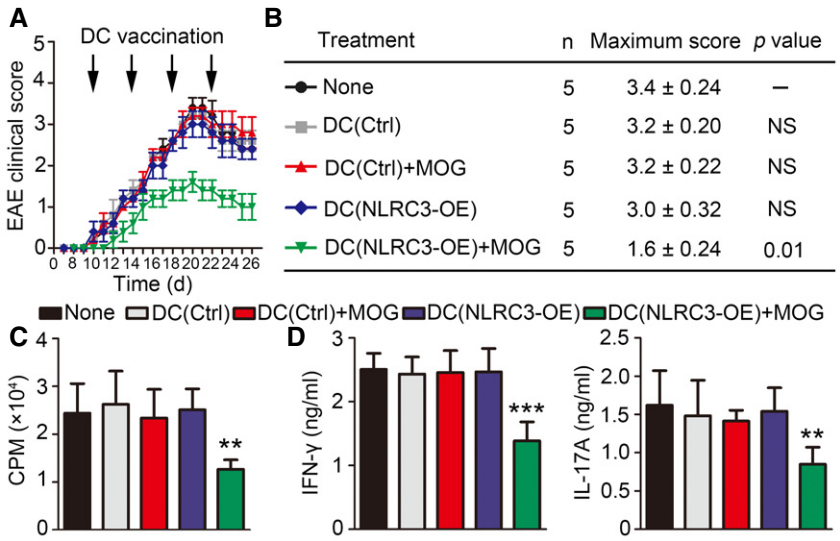

Figure 7. Vaccination with NLRC3-overexpression DCs suppressed EAE.

EAE was induced by immunization of naive B6 mice with MOG(35–55), and the mice were randomly divided into five groups. BMDCs transduced with either lentiviral vector encoding GFP and NLRC3 (LV-NLRC3) or only GFP (LV-Ctrl) were administered i.v. 4 times, once every 4 days, starting at day 10 after EAE induction.

A Mean clinical scores of EAE (n = 5 mice per group). Arrows indicate DC vaccine administration.

B Effects of therapeutic DC vaccination on B6 EAE. The maximum score: Mean of the maximum scores per mouse in each group. Data shown are the mean ± SD. Determination of statistical differences was performed using by a one-way ANOVA test.

C, D Recall proliferative (C) and cytokine response (D) to MOG(35–55) in splenocytes taken from DCs-treated mice 26 days after EAE induction.

Data information: Data shown (n = 5 in A, C, and D) are the mean ± SD. $^{**}P < 0.01$ and $^{***}P < 0.001$ by an one-way ANOVA test. Data are representative of three independent experiments with similar results.

subsets of $CD4^+$ T cells trigger the tissue inflammation and autoimmunity (Simmons *et al*, 2013) while enhanced IL-4 produced by $CD4^+$ T cells induces atypical neuroinflammatory disease symptoms (ataxia and issues with balance) during EAE (Lukens *et al*, 2015). Consistent with these finding, we observed conventional ascending paralysis in *Nlrc3*$^{-/-}$ mice and mice with NLRC3-deficient DCs during EAE progression. In addition, previous study has shown that IL-27 signaling in DC plays a vital role in the control of the T-cell response and CNS autoimmunity (Huang *et al*, 2012; Mascanfroni *et al*, 2013). We found that NLRC3 regulated the expression of IL-27 in DCs. Thus, IL-27 might contribute to the suppressive effects of NLRC3 in DCs.

Previous studies have revealed that NLRC3 plays a significant role in the negative modulation of many signaling pathways. NLRC3 deficiency in T cells or macrophages results in increased NF-κB p65 phosphorylation (Schneider *et al*, 2012; Hu *et al*, 2018). In cancer, NLRC3 has been found to be an inhibitory sensor of PI3K-mTOR pathways, which mediates protection against colorectal cancer (Karki *et al*, 2016, 2017). It has also been shown that NLRC3 suppresses the activation of $CD4^+$ T via negatively modulating the ERK signaling pathway in $CD4^+$ T cells (Hu *et al*, 2018). However, we did not observe that NLRC3 altered NF-κB, PI3K-AKT, or ERK signaling pathways in DCs. MAPK p38 pathway is a vital component of MAPK signaling pathway, which mediates an evolutionarily conserved mechanism for cellular responses to extracellular signals (Huang *et al*, 2009). Excessive activation of p38 pathway in DCs leads to enhanced development of autoimmune neuroinflammation (Huang *et al*, 2012). In this study, we observed enhanced p38 phosphorylation in NLRC3-deficient DCs, which has not been reported in previous studies. We also showed that pharmacological inhibition or gene deficiency of p38 retuned enhanced ability of DCs which were induced by NLRC3 deficiency in promoting development pathogenic $CD4^+$ T cells and EAE. However, the underlying mechanism of NLRC3 in regulating p38 signaling pathway in DCs remains unclear. Further studies are required to clarify these regulatory mechanisms.

Immunostimulatory properties of DCs are associated with their maturation state (Dhodapkar *et al*, 2001). Previous reports have shown that mature DC vaccine promotes the immune response in anti-tumor and pathogenic conditions (Tacken *et al*, 2007); however, tolerogenic immature DC inhibits effector T-cell functioning and reduces the immune response during an autoimmune disease (Dhodapkar *et al*, 2001; Mascanfroni *et al*, 2013). Thus, as an intrinsic negative regulator of DC immunogenicity, NLRC3 might have a potential therapeutic role in disorders mediated by the immune system. Our results revealed that vaccination with NLRC3-overexpressed DCs regulated EAE progression. In addition, activation of NLRC3 with its ligand has in theory the ability to limit the development of autoimmune disease. However, the typical ligands of NLRC3 are still unclear. Thus, to study whether NLRC3 regulates p38 signaling pathways via recognizing certain ligands will be very meaningful to enhance our understanding and application for its function in various disorders.

In summary, we identified that NLRC3 negatively regulates DC activation and immunogenicity, thereby limiting the programming Th1 and Th17 cell differentiation and further restricts the

development of autoimmune disorders through a mechanism that is dependent on p38 signaling pathway. Our study could have important implications in the development of therapies for multiple sclerosis and other autoimmune disorders.

Materials and Methods

Mice

C57BL/6 mice were from the Lab Animal Center of Southern Medicine University (Guangzhou, China). NLRC3-deficient (*Nlrc3*$^{-/-}$) mice were built by the Shanghai Research Center for Model Organisms (Shanghai, China), and regular cross on WT mice. 2D2, CD11c-Cre, CD11c-DTR, and *Mapk14*$^{fl/fl}$ mice were from the Jackson Laboratories. All mice are all the C57BL/6 background. All mice were used at an age of 6–12 weeks and were randomly divided into different groups. To generate mice, lacking NLRC3 expression in DCs was as described (D'Amico & Wu, 2003; Mascanfroni *et al*, 2013). In detail, lethally irradiated WT mice first were reconstituted with 5×10^6 bone marrow cells isolated from donor femora and tibiae of CD11c-DTR mice. After 2 months, the chimeric mice were inoculated intraperitoneally with 16 ng DTx per gram body weight for 2 weeks, once every 2 days. At last, these chimeric mice were reconstituted their DC compartment (CX3CR1$^+$) isolated from donor femora and tibiae of WT mice (to generate "DC(WT)" mice) or from *Nlrc3*$^{-/-}$ mice (to generate "DC(NLRC3-KO)" mice). These reconstituted mice were used after 2 months. All mice were maintained in the Lab Animal Center of Southern Medicine University under specific pathogen-free conditions. All animal experiments in this study were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All experimental protocols were reviewed and approved by the Medical Ethics Board and the Biosafety Management Committee of Southern Medical University (approval number SMU-L2015123).

Isolation of DCs

Spleens were isolated under sterile conditions. A single-cell suspension was obtained crushing each spleen individually through a 70-μm cell strainer with the back of a sterile 5-ml syringe, followed by erythrocytes lysing. DCs (F4/80$^-$ CD11c$^+$ MHC-II^{hi}) (Thermo Fisher Scientific, USA) were purified by FACs sorting. DCs were cultured for 48 h with LPS (100 ng/ml; *E. coli* strain 0111:B4; InvivoGen, USA). Parallel cultures maintained without stimuli were used as controls.

Generation of bone marrow-derived DCs (BMDCs)

Bone marrow cells isolated from the tibias and femurs of naive mice were cultured for 7 days in the presence of the cytokine GM-CSF (20 ng/ml; Peprotech, USA) and IL-4 (10 ng/ml; Peprotech). On day 7, cells were purified with CD11c$^+$ magnetic beads (Miltenyi, Germany) and then were cultured with LPS (100 ng/ml; *E. coli* strain 0111:B4; InvivoGen, USA) for the specified time. Parallel cultures maintained without stimuli were used as controls.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected. Cytokine production was measured by enzyme-linked immunosorbent assay of mouse IL-12, IL-4, TGF-β, IL-6, IL-23, IL-27, IL-10, IFN-γ, or IL-17A (ExCell Bio, China) according to the manufacturer's protocol.

Flow cytometry staining and analysis

For surface staining, cells were harvested, washed, and stained for 30 min on ice with mixtures of fluorescently conjugated mAbs or isotype-matched controls. mAbs of mouse were as follows: PE-Cy7-anti-CX3CR1 (Clone: 2A9-1; eBioscience), PE-anti-MHC-II (Clone: HIS19; eBioscience), APC-anti-CD40 (Clone: 1C10; eBioscience), PE-Cy7-anti-CD86 (Clone: GL1; eBioscience), FITC-anti-CD80 (Clone: 16-10A1; eBioscience), APC-Cy7-anti-CD3 (Clone: 145-2C11; eBioscience), PE-Cy7-anti-CD4 (Clone: GK1.5; eBioscience), APC-anti-CD8 (Clone: 53-6.7; eBioscience). For intracellular cytokine detection, isolated cells were cultured for 6 h with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml) in the presence of GolgiPlug (BD Pharmingen). And then, cells were stained for surface molecules, fixed 20 min in IC Fixation buffer (Thermo Fisher Scientific), and incubated for 1 h in permeabilization buffer (Thermo Fisher Scientific) with appropriate mAbs of mice: Pacific Blue-IFN-γ (Clone: XMG1.2; eBioscience), PE-IL-17A (Clone: eBio17B7; eBioscience), and PerCP-eFluor 710-anti-p-p38 (Clone: 4NIT4KK; eBioscience). Cell phenotype was analyzed by flow cytometry on a flow cytometer (BD LSR II) (BD Biosciences, USA) or Attune NxT (Thermo Fisher Scientific). Data were acquired as the fraction of labeled cells within a live-cell gate and analyzed using FlowJo software (Tree Star). All gates were set on the basis of isotype-matched control antibodies.

Proliferation assays

For *in vitro* experiments, DC activated for 48 h with LPS was used (at a ratio of 1:10) to stimulate naïve T cells from wild-type or 2D2 mice. For *in vivo* experiments, splenic cells were obtained from DC(WT) or DC(NLRC3 KO) mice at day 26 after immunization with MOG(35–55) and were restimulated *in vitro* for 3 days in the presence of MOG(35–55). For thymidine incorporation assay, during the last 8 h of stimulation, T cells were pulsed with [^{3}H]thymidine and the amount of incorporated [^{3}H]thymidine was measured as counts per minute (CPM). For (5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester) CFSE CD4$^+$ T-cell proliferation assay, purified naïve T cells were labeled with 2.5 μM CFSE and then 5×10^4 T cells/well were cultured for 72 h and proliferation was determined by flow cytometry analysis of CFSE dilution.

Quantitative PCR analysis

Total RNA was isolated with Trizol (Thermo Fisher Scientific) according to the manufacturer's instructions. 1 mg of RNA was reverse-transcribed to cDNA with random RNA-specific primers using the high-capacity cDNA reverse transcription kit (Applied Biosystems). An Eppendorf Master Cycle Realplex2 and a SYBR Green PCR Master Mix (Applied Biosystems, USA) were used for

real-time PCR (40 cycles). The primer sequences used for PCR are in Appendix Table S1.

Western blotting

Cells were washed three times with ice-cold PBS and then lysed in a lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) protease inhibitor cocktail (Sigma Aldrich, USA), and 1 mM DTT. Equal amounts (20 mg) of cell lysates were resolved using 8–15% polyacrylamide gels transferred to PVDF membrane. Membranes were blocked in 5% non-fat dry milk in PBS-T and incubated overnight with their respective primary antibodies at 4°C. These respective primary antibodies list are as follows: phospho-NF-κB p65 (Ser536) (Clone: 93H1; CST, USA), NF-κB p65 (Clone: D14E12; CST), phospho-Akt (Ser473) (Clone:D9E; CST), Akt (Clone: C67E7; CST), phospho-p38 MAPK (Thr180/Tyr182) (Clone: D3F9; CST), p38 MAPK (Clone:D13E1; CST), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Clone: D13.14.4E; CST), p44/42 MAPK (Erk1/2) (Clone: 137F5; CST), phospho-JNK (Thr183/Tyr185) (Clone: G9; CST), SAPK/JNK (CST), GAPDH (Clone: D16H11; CST), phospho-MSK1 (Ser376) (CST), phospho-MK2 (Thr222) (Clone:9A7; CST), and phospho-CREB (Ser133) (Clone: 87G3; CST). The membranes were incubated at room temperature for 1 h with appropriate HRP-conjugated secondary antibodies and were visualized with Plus-ECL (PerkinElmer, CA) according to the manufacturer's protocol.

NLRC3 overexpression in DC

DCs from spleens of mice or BMDCs were transduced with either lentiviral vector encoding GFP and NLRC3 (LV-NLRC3) or only GFP (LV-Ctrl) at multiplicities of infection of 5 in the presence of Polybrene (Santa Cruz Biotechnology, USA) (5 μg/ml) for 48 h. Transduction efficiency was assessed by flow cytometry through determination of the frequency of cells positive for GFP, and then, the cells positive for GFP were purified by FACs sorting. The overexpression of NLRC3 was assessed via Western blot. The DCs transduced with LV-NLRC3 or LV-Ctrl were defined as DC(NLRC3-OE) or DC(Ctrl), respectively.

EAE model

WT, *Nlrc3*$^{-/-}$, DC(WT), or DC(NLRC3-KO) mice were immunized subcutaneously with 100 mg MOG(35–55) peptide (MEVGWYRSPFSRVVHLYRNGK) emulsified in CFA (Difco Laboratories, USA) with 500 mg *Mycobacterium tuberculosis* H37Ra on day 0. Mice also received 200 ng of pertussis toxin (Sigma, USA) by intraperitoneal injection on days 0 and 2. Classical disease score was assessed daily by assigning clinical scores according to the following ascending paralysis scale: 0, no disease; 1, tail paralysis; 2, weakness of hind limbs; 3, paralysis of hind limbs; 4, paralysis of hind limbs and severe hunched posture; 5, moribund or death. Classical clinical scores were assigned based on ascending paralysis development. For vaccination with DCs, DCs (Ctrl) or DCs(NLRC3-OV) were cultured for 4 h with 20 μg MOG (35–55) and then (2×10^6 cells per mouse) were extensively washed and administered intravenously four times, once every 4 days.

Statistics

All experiments were performed at least twice. When shown, multiple samples represent biological (not technical) replicates of mice randomly sorted into each experimental group. No blinding was performed during animal experiments. Determination of statistical differences was performed with Prism 5 (GraphPad Software, Inc.) using unpaired two-tailed *t*-tests (to compare two groups with similar variances), or one-way ANOVA with Bonferroni's multiple comparison test (to compare more than two groups).

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Author contributions
YLF, XXZ, LSL, LM, and SFH designed research; YLF, XXZ, YCW, XBJ, ML, YLY, YLH, XLD, and SFH conducted research; YLF, XXZ, XPZ, LSL, LM, and SFH analyzed data; YLF, XXZ, XPZ, and SFH wrote the paper. LSL and LM provided essential reagents or provided essential materials; SFH, LM, and LSL as the corresponding author conducted the experiment. All authors read and approved the final manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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