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BRIEF REPORT

Dendritic cell PIK3C3/VPS34 controls the pathogenicity of CNS autoimmunity independently of LC3-associated phagocytosis

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ABSTRACT

PIK3C3/VPS34 is a key player in macroautophagy/autophagy and MAP1LC3/LC3-associated phagocytosis (LAP), which play critical roles in dendritic cell (DC) function. In this study, we assessed the contribution of PIK3C3 to DC function during experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS). We found that *Pik3c3*-deficient DCs exhibit attenuated capacity to reactivate encephalitogenic T cells in the central nervous system, leading to reduced incidence and severity of EAE in DC-specific *Pik3c3*-deficient mice. Additionally, animals with a DC-specific deficiency in *Rb1cc1/Fip200* but not *Rubcn* were protected against EAE, suggesting that the EAE phenotype of DC-specific *Pik3c3*-deficient mice is due to defective canonical autophagy rather than LAP. Collectively, our studies have revealed a critical role of PIK3C3 in DC function and the pathogenicity of these cells during EAE, with important implications for the development of immunotherapies for autoimmune diseases such as MS.

Abbreviations: ATG: autophagy-related; CNS: central nervous system; DC: dendritic cell; DEG: differentially expressed gene; EAE: experimental autoimmune encephalomyelitis; LAP: LC3-associated phagocytosis; MAP1LC3/LC3: microtubule-associated protein 1 light chain 3; MHC: major histocompatibility complex; MOG: myelin oligodendrocyte glycoprotein; MS: multiple sclerosis; PIK3C3/VPS34: phosphatidylinositol 3-kinase catalytic subunit type 3; ROS: reactive oxygen species

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Introduction

Macroautophagy (called autophagy hereafter) is an evolutionarily highly conserved nutrient-sensing system that promotes the degradation of cytoplasmic proteins and damaged organelles by lysosomes [1]. The resulting degradation products are then used in cellular remodeling and in regenerating molecular building blocks in normal physiology and during conditions of stress. This process is orchestrated by over 30 autophagy-related (ATG) gene products that were initially identified in yeast but are largely conserved in higher eukaryotes.

Dendritic cells (DCs) are professional antigen-presenting cells that are critical for capturing, processing, and presenting antigens to major histocompatibility complex (MHC)-restricted T cells, which requires high levels of endocytic and lysosomal activity that is tightly linked to autophagy. DCs play a critical role in the induction and progression of a variety of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE), an animal model of human multiple sclerosis (MS). The role of DC-specific autophagy in the pathogenicity of EAE has been explored previously. Bhattacharya *et al.* reported that *Atg7*-deficiency in DCs does not result in gross alterations in global DC function but leads to reduced onset and severity of MOG (myelin oligodendrocyte glycoprotein)-induced EAE by reducing *in vivo* priming of T cells [2]. Keller *et al.* reported that ablation of *Atg5* in DCs results in complete protection from EAE development upon adoptive transfer of primed, encephalitogenic $CD4^+$ T cells [3]. This protection appeared to be due to impaired ATG-regulated phagocytosis of injured oligodendrocytes for MHC class II antigen presentation, leading to reduced accumulation of myelin-specific T cells within the central nervous system (CNS) [3]. Yet, only minor or statistically non-significant differences in disease incidence rates and clinical severity scores were observed in active EAE [3]. In another report, Alissafi *et al.* demonstrated that DCs with a selective deletion of *Atg16l1* are less efficient in priming autoreactive T cells and, as a result, co-transfer of *Atg16l1*-deficient DCs and 2D2 MOG_{35-55} antigen-specific T cells into $rag^{-/-}$ mice leads to less severe EAE disease [4].

The PIK3C3 subunit of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex is critical for the initiation of autophagy [5] and has also been implicated in other cellular processes, including endocytosis, intracellular vesicular trafficking [6], and MAP1LC3/LC3 (microtubule-associated protein 1 light chain 3)-associated phagocytosis (LAP) [7]. Inhibiting PtdIns3K activity has shown promising results in both preclinical and clinical trials for the treatment of immune-related disorders including MS (reviewed in [8,9]). We and others have previously demonstrated that *Pik3c3*-

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deficient T cells, DCs, and myeloid cells exhibit defective autophagy and have remarkably altered cellular functions [10–15]. However, the role of PIK3C3 in DCs in the context of autoimmunity is unknown. In the current study, we show that mice with a DC-specific *Pik3c3*-deficiency exhibit significant resistance to EAE, which was associated with attenuated reactivation of primed T cells in the CNS. These effects of DC-specific PIK3C3 on EAE were independent of LAP, pointing to the autophagy defect of these cells as the cause of EAE resistance. Thus, our findings have revealed a critical role for DC PIK3C3 in $CD4^+$ T cell-mediated autoimmune diseases.

Results

Deletion of Pik3c3 *in DCs reprograms gene expression*

It has been widely acknowledged that autophagy is critical for both MHC class I- and MHC class II-restricted antigen presentation [16,17]. To investigate the role of PIK3C3 in antigen presentation, we previously generated DC-specific *Pik3c3*-deficient mice by breeding mice in which exon 4 of the *Pik3c3* gene was flanked by loxP sites (*Pik3c3*$^{f/f}$) to mice expressing Cre recombinase driven by the *Itgax*/*Cd11c* promoter [11]. *pik3c3*$^{f/f}$*;Itgax-Cre* mice showed a DC-specific *Pik3c3* ablation and DCs in these animals exhibited a partially activated phenotype, with increased surface expression of MHC class I, MHC class II, and co-stimulatory molecules, and spontaneous production of both pro- and anti-inflammatory cytokines [11]. This study also revealed that mice with a DC-specific deficiency in *Pik3c3* exhibit a partial impairment in the homeostatic maintenance of $CD8A^+$ conventional DCs [11], which are specialized in cross-presenting antigens on MHC class I molecules [18]. Additionally, we found a modest increase in the activity of the classical MHC class I and class II antigen presentation pathways, as well as a profound defect in the cross-presentation of dead cell-associated antigens to MHC class I-restricted $CD8A^+$ T cells owing to defective uptake of apoptotic cells [11].

We previously showed that the *pik3c3*$^{f/f}$*;Itgax-Cre* mice exhibit a selective *Pik3c3* deletion in their DCs [11]. DCs in these animals showed defective autophagy compared with WT DCs, as indicated by autophagosomal double-membrane structures revealed by electron microscopy [11] and increased levels of LAMP1, SQSTM1/p62, and total LC3 levels revealed by western blotting (Figure S1). To obtain a better understanding of potential effects of *Pik3c3* deletion on DC function, we investigated the global transcriptomic profiles of FACS-enriched DCs from both *Pik3c3*$^{f/f}$ and *pik3c3*$^{f/f}$*;Itgax-Cre* mice. RNA sequencing analysis revealed significant genome-wide changes in transcript levels. *Pik3c3*-deficiency in DCs significantly induced (n = 367) or reduced (n = 254) gene expression, including reduced *Pik3c3* mRNA expression (3.27-fold reduction, p = 5.73E-14) (Figure 1A). Subsequent pathway enrichment analysis identified the impacted antigen-presenting function in *Pik3c3*-deficient DCs (Figure 1B), as expected. Interestingly, impacted genes in antigen presentation were all associated with the MHC class I pathway (Figure 1C). Genes in the MHC class II antigen presentation pathway were unchanged or slightly downregulated. Furthermore, our RNA sequencing data confirmed the partially activated phenotype in *Pik3c3*-deficient DCs as indicated by enhanced expression of cytokine- and cytokine receptor-associated genes (Figure 1D).

Pik3c3*-deficiency in DCs reduces the incidence and severity of EAE*

DC-specific *Pik3c3*-deficient mice contain a defect in the capacity of $CD8A^+$ DCs to present dead cell-associated antigens to MHC class I-restricted T cells and, as a result, these animals show increased metastases in response to challenge with B16 melanoma cells [11]. However, the role of PIK3C3 in DCs in the context of autoimmunity remains unclear. To test the role of DC-specific PIK3C3 in autoimmunity, we employed the EAE model of MS, by immunizing mice with MOG_{35-55} peptide to induce MOG-specific, MHC class II-restricted $CD4^+$ T cells. We first induced active EAE in *pik3c3*$^{f/f}$*;Itgax-Cre* mice with *Pik3c3*$^{f/f}$ mice as controls. We found reduced severity and delayed onset of EAE in *pik3c3*$^{f/f}$*;Itgax-Cre* mice (Figure 2A). The incidence of EAE was also decreased in *pik3c3*$^{f/f}$*;Itgax-Cre* mice (11 out of 11 in control mice *versus* 5 out of 12 in *pik3c3*$^{f/f}$*;Itgax-Cre* mice from three independent experiments, p = 0.0024, chi-square test) (Figure 2A). Additionally, in *pik3c3*$^{f/f}$*;Itgax-Cre* mice, the cumulative disease score and peak disease score were significantly reduced (Figure 2A).

Previous studies have provided evidence that CNS-resident classical DCs can stimulate effector myelin-specific T cells and that selective depletion of these cells results in reduced numbers of myelin-specific T cells and reduced EAE incidence [19]. Therefore, we immunized wild-type (WT) mice with MOG peptide and transferred primed myelin-specific $CD4^+$ T cells to induce EAE in recipient *Pik3c3*$^{f/f}$ or *pik3c3*$^{f/f}$*;Itgax-Cre* mice to model the effector phase of EAE, which is highly dependent on the presence of antigen-presenting cells within the CNS. Similar to our results for active EAE, *pik3c3*$^{f/f}$*;Itgax-Cre* mice exhibited attenuated disease severity compared with their littermate controls upon adoptive transfer of encephalitogenic T cells (Figure 2B), suggesting that the reduced EAE severity in *pik3c3*$^{f/f}$*;Itgax-Cre* mice is due to the lack of PIK3C3 in DCs from recipient animals.

We have previously reported that DCs from *pik3c3*$^{f/f}$*;Itgax-Cre* mice exhibit an activated phenotype under steady-state conditions [11], which is also reflected in the transcriptomic studies shown in Figure 1. We therefore analyzed the prevalence and activation state of DCs in the CNS of *pik3c3*$^{f/f}$*;Itgax-Cre* mice during EAE. We found that fewer $ITGAX^+$ cells accumulate in the CNS of *pik3c3*$^{f/f}$*;Itgax-Cre* than *Pik3c3*$^{f/f}$ mice during EAE (Figure 2C). Further, these cells from EAE-induced mutant animals were in a less activated state, as revealed by lower surface expression of CD80, CD86, and MHC class I and class II molecules (Figure 2D).

Because the cytokine IL1B plays an important role in EAE, we next analyzed IL1B production by total $PTPRC^+$ leukocytes and $ITGAX^+$ myeloid cells in the CNS of *pik3c3*$^{f/f}$*;Itgax-Cre* mice during EAE. We found fewer $IL1B^+$ cells among total $PTPRC^+$ cells (Figure 2E) but similar frequencies of

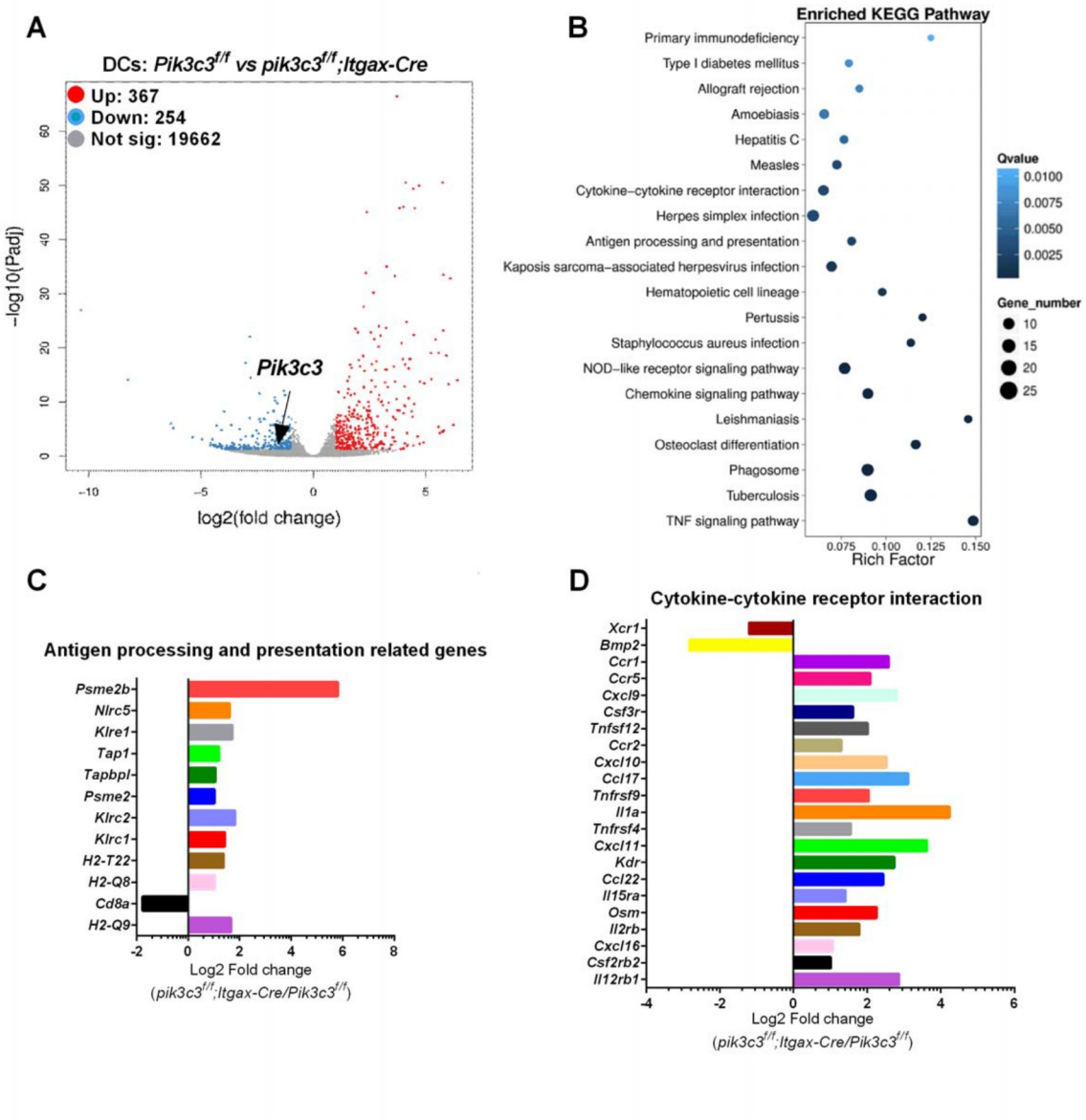

Figure 1. Deletion of *Pik3c3* in DCs reprograms DC gene expression. (A) Clustered heat map of RNA-seq gene counts in *Pik3c3*-deficient DCs and *Pik3c3*-sufficient DCs (n = 2 mice per group). (B) Gene set enrichment analysis showing enriched pathways from differentially expressed genes between *Pik3c3*-deficient DCs and *Pik3c3*-sufficient DCs. (C) Altered gene expression associated with antigen processing and presentation. (D) Altered gene expression associated with cytokine-cytokine receptor interactions.

IL1B$^+$ cells among ITGAX$^+$ cells (Figure 2F) from *pik3c3*$^{f/f}$;*Itgax-Cre* than *Pik3c3*$^{f/f}$ mice. Considering our previous finding that *Pik3c3*-deficient DCs produce higher levels of IL1B upon activation with heat-killed *L. monocytogenes* compared with *Pik3c3*-sufficient DCs [11], we propose that reduced IL1B levels in *pik3c3*$^{f/f}$;*Itgax-Cre* mice during EAE are likely a consequence rather than a cause of ameliorated EAE development in these animals.

Peripheral antigen-specific T cell recall response is unaltered in pik3c3$^{f/f}$;Itgax-Cre *mice*

The finding that *pik3c3*$^{f/f}$;*Itgax-Cre* mice showed specific defects in CD4$^+$ T cell-mediated EAE suggested the possibility that *Pik3c3*-deficiency in DCs results in reduced CD4$^+$ T cell priming. To test this possibility, we isolated splenocytes from *Pik3c3*$^{f/f}$ and *pik3c3*$^{f/f}$;*Itgax-Cre* mice following EAE induction (day 10) and incubated them with MOG peptide (20 µg/ml) to measure antigen-specific cytokine production. Non-significant differences were observed for the frequency of IL17A$^+$ CD4$^+$ and IFNG$^+$CD4$^+$ T cells, although high inter-group variation was seen (Figure 3A,B). Since CD8A$^+$ T cells can also contribute either positively or negatively to the pathogenesis of EAE in mice [20–22], we further analyzed the antigen-specific cytokine production of CD8A$^+$ T cells. However, no difference in cytokine production by CD8A$^+$ T cells derived from MOG-immunized *Pik3c3*$^{f/f}$ and *pik3c3*$^{f/f}$;*Itgax-Cre* mice was observed after *in vitro* MOG restimulation in our system (Figure 3B). To determine the extent of CNS inflammation, we examined frequencies of CNS-

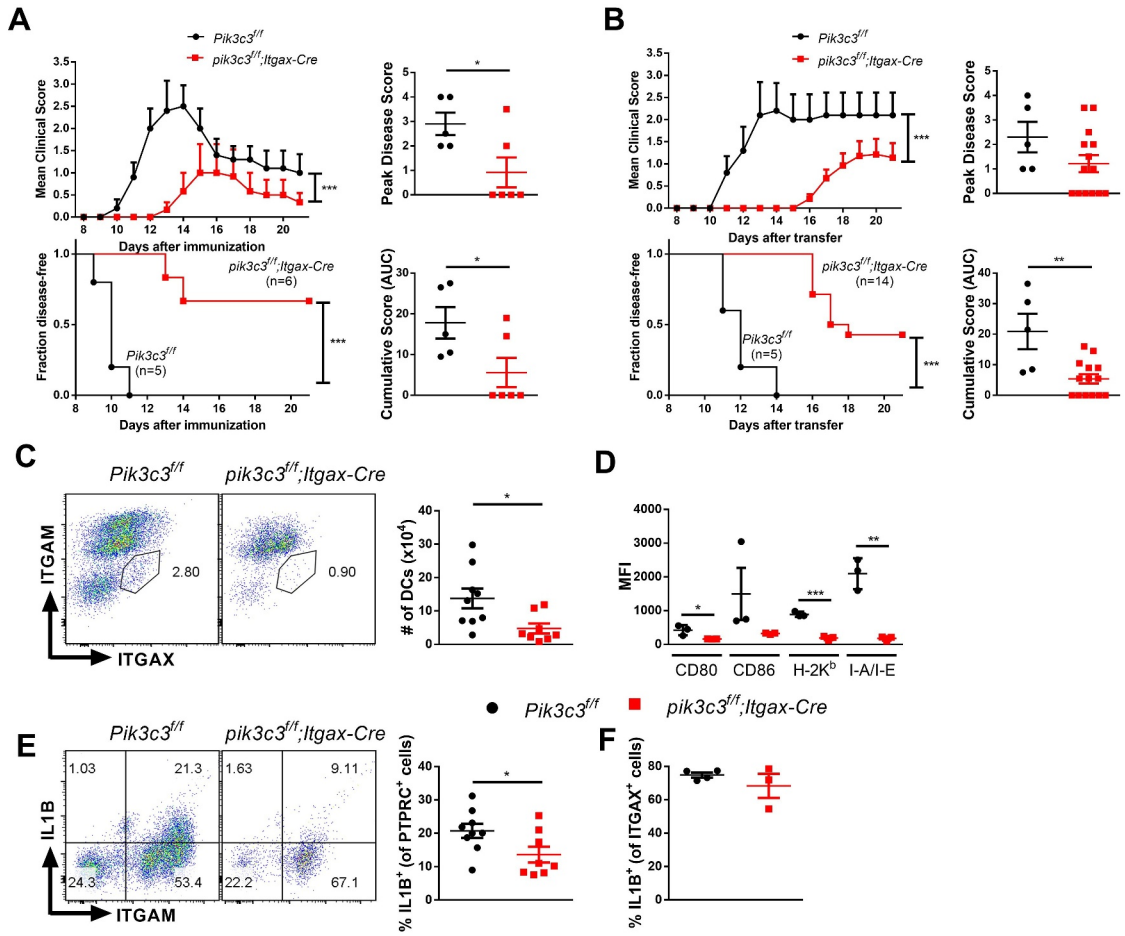

Figure 2. *Pik3c3*-deficiency attenuates incidence and severity of EAE. (A) EAE induced by active immunization with MOG_{35-55} peptide. (B) EAE induced by adoptive transfer of myelin-specific T cells. Results show one experiment that is representative of three independent experiments. (C) Flow cytometric analysis of DCs in the brain of *pik3c3*$^{f/f}$*;Itgax-Cre* mice at 14 days after EAE induction. Results from three independent experiments were pooled. (D) CNS leukocytes were prepared from mice, stained with anti-ITGAX, -ITGAM, -CD80, -CD86, -H-2Kb, and I-A/-E antibodies, and analyzed by flow cytometry. Results show one experiment that is representative of three independent experiments. (E) IL1B production by CNS leukocytes. Cells were isolated from the CNS and IL1B-producing ITGAM$^+$ cells were assessed by flow cytometry. Results from two independent experiments were pooled. (F) IL1B production by ITGAX$^+$ cells in the CNS. Results show one experiment that is representative of two independent experiments. The data shown are the average ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

infiltrating T cells in *pik3c3*$^{f/f}$*;Itgax-Cre* mice compared with *Pik3c3*$^{f/f}$ littermates. To this end, mice were sacrificed at day 14 following active EAE induction and T cells were analyzed by flow cytometry. *pik3c3*$^{f/f}$*;Itgax-Cre* mice showed substantially lower total and CD44$^+$ CNS-infiltrating CD4$^+$ T cells (Figure 3C) as well as IFNG$^+$ CD4$^+$ T cells, but no significant difference in the prevalence of IL17A$^+$ CD4$^+$ T cells (Figure 3D).

To determine whether *Pik3c3*-deficiency in DCs impairs CD4$^+$ T cell pathogenicity, we immunized *pik3c3*$^{f/f}$*;Itgax-Cre* mice with MOG peptide and transferred primed myelin-specific CD4$^+$ T cells to induce EAE in WT recipient mice. We found that all mice receiving T cells from *pik3c3*$^{f/f}$*;Itgax-Cre* or *Pik3c3*$^{f/f}$ donor animals develop similar clinical manifestations of EAE (Figure 3E). Further, antigen-specific cytokine production by T cells analyzed at day 10 after EAE induction did not correlate with peak disease score (Figure 3F) but correlated with the kinetics of disease onset (Figure 3G).

Taken together, these data suggest that peripheral CD4$^+$ T cell priming is largely unaltered but that accumulation of CD4$^+$ T cells in the CNS is reduced in the absence of PIK3C3 in DCs, suggesting that effects of *Pik3c3*-deficiency on EAE might be due to DCs in the CNS rather than the periphery.

The EAE phenotype of DC-specific Pik3c3*-deficient mice is associated with the lack of canonical autophagy rather than LAP*

Although canonical autophagy and LAP share many molecular components including PIK3C3, LAP proceeds independently of the autophagy preinitiation complex, which is composed of ATG13, RB1CC1/FIP200, ATG101 and ULK1 [23]. In addition, RUBCN, in association with the BECN1/Beclin1-PIK3C3-UVRAG-containing PtdIns3K complex mediates LAP but not autophagy [7]. While basal autophagy activation was observed in *rubcn*$^{-/-}$ mice [24], our previous study showed that *rubcn*$^{-/-}$ mice develop signs of EAE similar to WT control mice [15], suggesting that the slight elevation in autophagy of these mice does not have a major impact on EAE disease progression. In the present study, we further transferred primed MOG-specific CD4$^+$ T cells to induce EAE in *rubcn*$^{-/-}$ recipient mice. Again, all of the examined *rubcn*$^{-/-}$ mice developed clinical disease manifestations

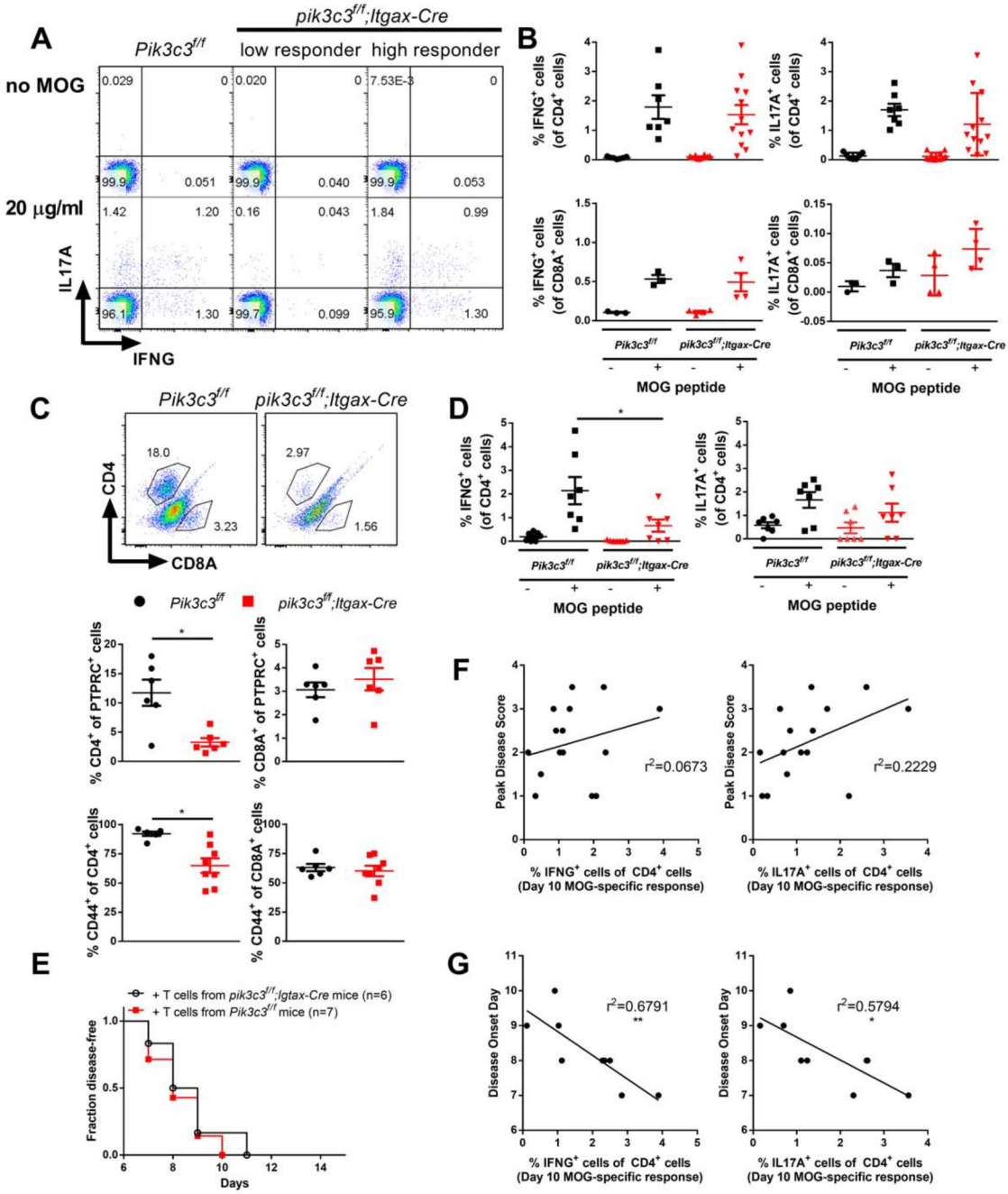

Figure 3. Peripheral antigen-specific T cell recall response in EAE is unaltered in *pik3c3*$^{f/f}$*;Itgax-Cre* mice. (A) Splenocytes from *Pik3c3*$^{f/f}$ and *pik3c3*$^{f/f}$*;Itgax-Cre* mice induced for EAE (day 10) were cultured with MOG_{35-55} peptide (20 μg/ml). After 18 h of culture, cells were harvested and intracellular staining was performed (IL17A and IFNG), and cytokine-producing $CD4^+$ T and $CD8^+$ T cells were analyzed by flow cytometry. (B) Quantification of antigen-specific cytokine production by T cells from spleen. (C) Flow cytometric analysis of $CD4^+$ and $CD4^+CD44^+$ T cells in the brain of *pik3c3*$^{f/f}$*;Itgax-Cre* mice at 14 days after EAE induction. (D) Quantification of antigen-specific cytokine production by CNS-invading $CD4^+$ T cells. (E) Disease onset of WT mice after receiving MOG_{35-55}-specific T cells from *pik3c3*$^{f/f}$*;Itgax-Cre* or *Pik3c3*$^{f/f}$ mice. (F,G) Correlation analyses of the percentage of $IFNG^+$ cells or $IL17A^+$ cells within $CD4^+$ cells with EAE peak disease score (F) and disease onset (G) after adoptive transfer EAE induction. Results from two-four independent experiments were pooled and plotted. The data shown are the average ± SEM. *p < 0.05, **p < 0.01.

similar to WT control mice (Figure 4A). To test the role of RUBCN in DCs more directly, we induced EAE in *rubcn*$^{f/f}$*;Itgax-Cre* mice. We found that *rubcn*$^{f/f}$*;Itgax-Cre* mice exhibit disease symptoms similar to WT mice in both active and passive EAE models (Figure 4B,C). To confirm that the resistance of DC-specific *Pik3c3*-deficient mice to EAE is indeed due to defective autophagy, we induced EAE in *rb1cc1*$^{f/f}$*;Itgax-Cre* mice. Similar to *pik3c3*$^{f/f}$*;Itgax-Cre* mice, *rb1cc1*$^{f/f}$*;Itgax-Cre* mice were resistant to EAE induction (Figure 4D,E). Collectively, these results suggest that the EAE phenotype of DC-specific *Pik3c3*-deficient mice is associated with the lack of canonical autophagy rather than LAP.

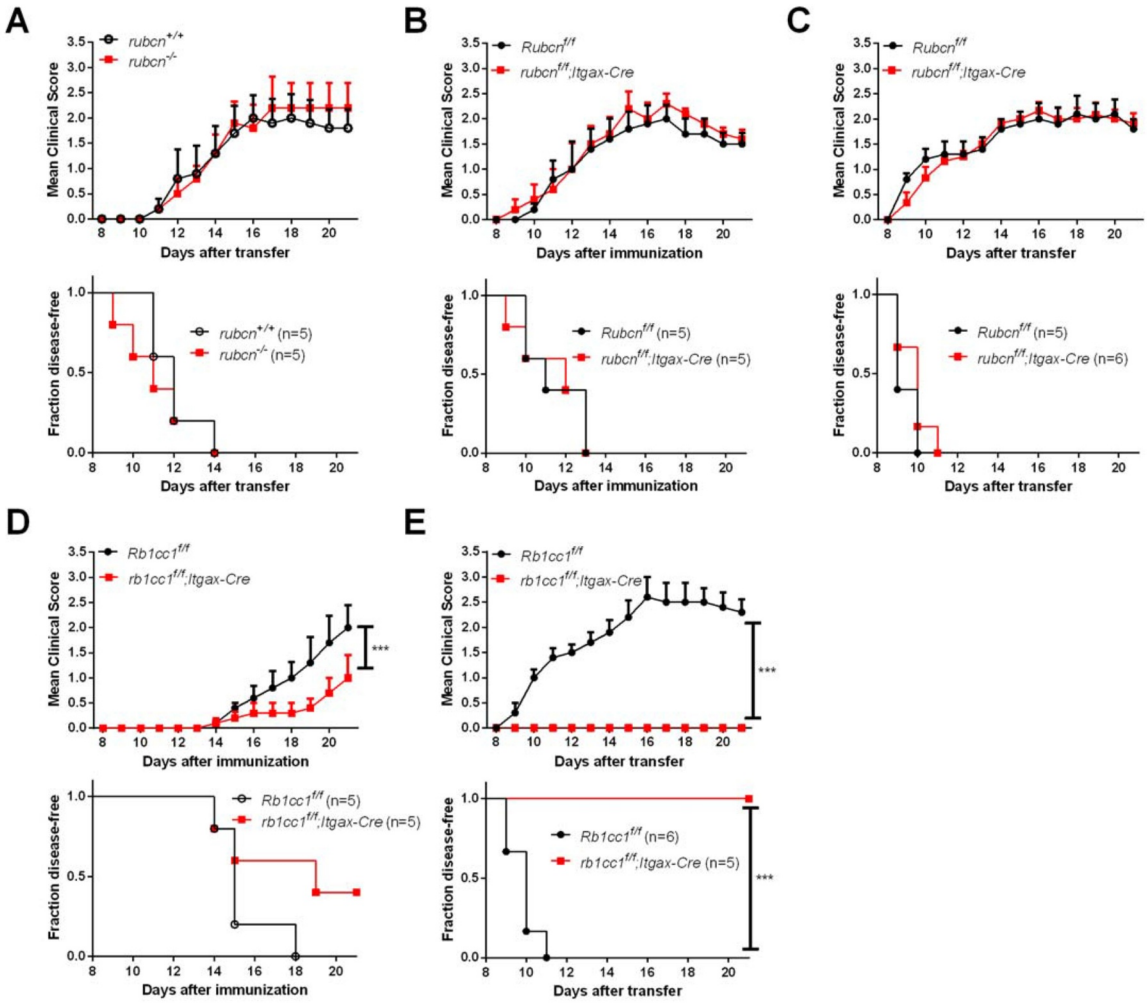

Figure 4. DC autophagy but not LAP is required for EAE. (A–C) EAE was induced in *rubcn*$^{-/-}$, *rubcn*$^{f/f}$*;Itgax-Cre*, and respective littermate control mice. (D,E) EAE was induced in *rb1cc1*$^{f/f}$*;Itgax-Cre* and littermate control mice. Results show one experiment that is representative of two independent experiments. The data shown are the average ± SEM. ***p < 0.001.

Pik3c3-*deficiency in DCs impairs their T cell stimulatory capacity*

To investigate whether the absence of *Pik3c3* in DCs impairs their T cell stimulatory capacity, DCs were sorted from *pik3c3*$^{f/f}$*;Itgax-Cre, rb1cc1*$^{f/f}$*;Itgax-Cre, rubcn*$^{f/f}$*;Itgax-Cre* and their respective littermate control mice. Isolated splenic DCs were pulsed with MOG_{35-55} peptide or a longer fragment of MOG (MOG_{1-125}) that requires antigen processing for its presentation on MHC class II, and then cocultured with naïve $CD4^+$ T cells expressing a transgenic T cell receptor specific for the MOG_{35-55} peptide (2D2 cells). We observed similar proliferation levels of 2D2 cells cocultured with MOG_{35-55}-primed WT, *Pik3c3*-, *Rb1cc1*-, and *Rubcn*-deficient DCs (Figure 5A,B). However, MOG_{1-125}-primed WT and *Rubcn*-deficient DCs induced a more prominent effector T cell response than *Pik3c3*- and *Rb1cc1*-deficient DCs (Figure 5A,B). Together, these data suggest that autophagy is required for autoantigen processing and that autophagy-deficiency impairs the T cell stimulatory capacity of DCs.

Discussion

The "holy grail" strategy to treat MS and other autoimmune diseases is to induce tolerance against the inciting self-antigens without affecting the capacity to respond and protect against pathogenic organisms. However, effective drugs currently available are broadly disease-modifying treatments rather than disease-specific treatments that selectively dampen autoantigen-specific immune responses, which leaves patients vulnerable to infections and possibly cancer [25]. Thus, there is an urgent need to explore new modalities to treat MS and other autoimmune diseases. The most common mouse model of MS is EAE, which can be actively induced through immunization with myelin-derived antigens suspended in complete Freund's adjuvant, followed by injection of pertussis toxin to break the blood-brain barrier. The pathogenesis of actively-induced EAE consists of an induction and an effector phase. EAE can also be induced by adoptive transfer of activated myelin-specific $CD4^+$ T cells, which models the effector phase of EAE disease.

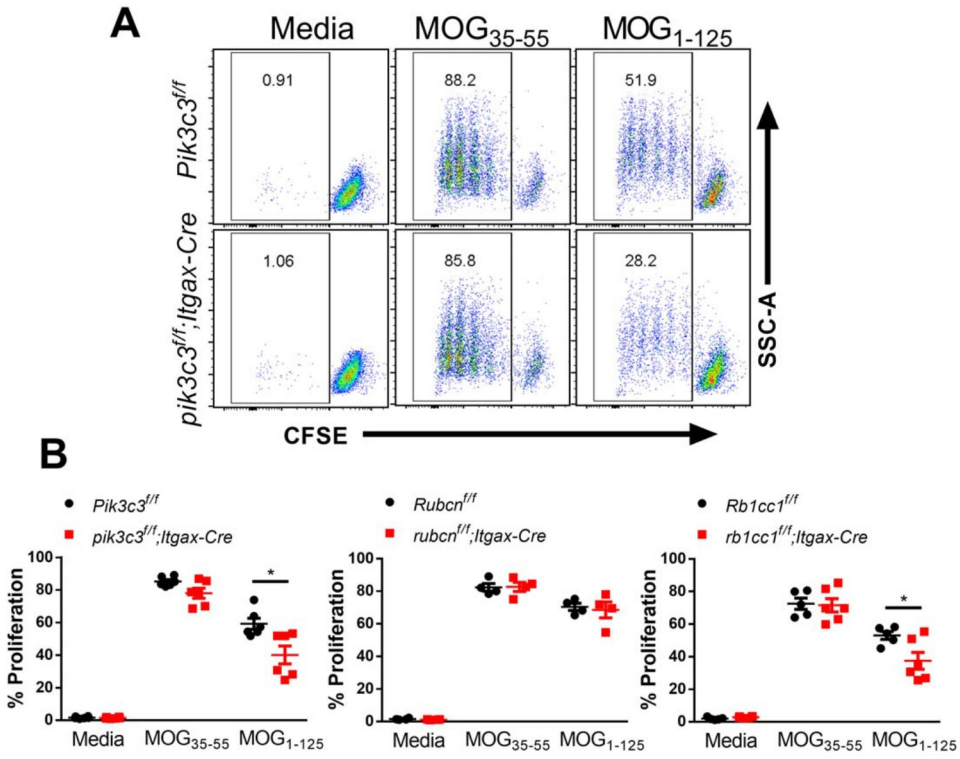

Figure 5. *Autophagy*-deficient DCs display impaired capacity to present myelin antigens to T cells. Splenic DCs were isolated from *pik3c3*$^{f/f}$*;Itgax-Cre, rb1cc1*$^{f/f}$*;Itgax-Cre, rubcn*$^{f/f}$*;Itgax-Cre*, and their respective littermate control mice. Sorted DCs (2×10^4) were pulsed with 20 µg/ml MOG_{35-55} or MOG_{1-125} for 4 h in complete medium and washed. CFSE-labeled 2D2 $CD4^+$ T cells (1×10^5) were added and cocultured for 72 h to measure T cell proliferation by flow cytometry. (A) Representative FACS plots depicting proliferation of 2D2 $CD4^+$ T cells upon coculture with MOG_{35-55}- or MOG_{1-125}-pulsed *pik3c3*-sufficient and -deficient DCs. (B) Quantification of 2D2 $CD4^+$ T cell proliferation under different conditions. Results from two-three independent experiments were pooled and plotted. The data shown are the average ± SEM. *$p < 0.05$.

In this study, we found that *Pik3c3*-deficiency in DCs significantly reduces the onset and severity of actively induced EAE. The underlying mechanism is likely due to a disrupted effector response, as evidenced by attenuated disease severity upon adoptive transfer of encephalitogenic T cells into *pik3c3*$^{f/f}$*;Itgax-Cre* mice. These data are consistent with previous studies suggesting that DCs require ATG5 to reactivate primed myelin-specific $CD4^+$ T cells during the effector phase of EAE [3]. Our results demonstrate that DC autophagy is required for the priming of autoreactive $CD4^+$ T cells *in vivo* and development of EAE. Autophagy is a critical regulator of intracellular antigen loading to MHC class II molecules and antigen presentation by DCs [17]. Interestingly, the autophagy machinery also contributes to MHC class II presentation of endocytosed MOG_{35-55} antigen. In support, a previous study showed suppressed 2D2 T cell responses after treating MOG_{35-55}-pulsed DCs with NH_4Cl (an inhibitor of autophagosome fusion with lysosomes), wortmannin (a PI3K inhibitor), or MRT67307 (a ULK1 inhibitor) [4]. However, our results show similar proliferation levels of 2D2 cells cocultured with MOG_{35-55}-primed WT, *Pik3c3*-, or *Rb1cc1*-deficient DCs. The underlying reasons for these divergent findings are currently unknown and may involve differences in experimental details and/or generic differences when protein function is disrupted by genetic deletion versus chemical inhibition. Instead, we found that autophagy is required for effective exogenous processing of recombinant MOG_{1-125} by DCs for presentation to MOG_{35-55}-specific T cells. This finding is in agreement with a previous study showing that *Atg16l1*-deficient DCs exhibit defective processing of intact ovalbumin proteins for presentation to antigen-specific $CD4^+$ T cells [4]. The mechanism involved in MOG_{1-125} protein processing by DCs is currently unknown and requires further investigation. Overall, we propose that *Pik3c3*-deficient DCs exhibit attenuated capacity to reactivate encephalitogenic T cells *in vivo*, leading to reduced incidence and severity of EAE in DC-specific *Pik3c3*-deficient mice.

The incomplete protection afforded by conditionally knocking out *Pik3c3* in DCs could be due to incomplete deletion of *Pik3c3* by *Itgax-Cre* in plasmacytoid DCs [26], which contribute significantly to priming T cells during EAE [27], or the involvement of other types of antigen-presenting cells that contribute to EAE development [14,28]. Conversely, protection against EAE is unlikely due to defects in the homeostasis of the cDC1 compartment in these mice since *batf3*$^{-/-}$ mice lacking all peripheral ITGAM/CD11b$^{low/-}$ DCs show robust Th cell priming after subcutaneous immunization and are susceptible to EAE [29].

It is now widely acknowledged that most, if not all, components of the autophagy apparatus can also contribute to non-autophagic functions, including LAP, endocytosis, melanogenesis, cytokinesis, and Golgi apparatus to ER transport

[30]. Of note, PIK3C3, BECN1, ATG7, ATG5, and ATG16L1 are shared between the autophagy pathway and LAP, whereas RB1CC1 is involved in autophagy but not LAP [30]. It has recently been proposed that LAP in DCs licenses encephalitogenic $CD4^+$ T cells to initiate and sustain EAE induction [3,31,32]. This conclusion was based on the finding that DC-specific deletion of *Cybb/Nox2* or *Atg5* inhibits LC3 decoration to phagosomes, antigen presentation to encephalitogenic $CD4^+$ T cells, CNS immune cell invasion, and clinical disease development. However, these data do not conclusively address the involvement of LAP in DCs during EAE for several reasons. First, DCs produce low levels of reactive oxygen species (ROS) and express relatively low levels of CYBB [33]. Second, CYBB loss in DCs could be compensated for by other NOX family members, or possibly other forms of ROS as reported in other cell types [34,35]. Third, reduced CYBB-mediated ROS production in *Cybb*-deficient DCs may cause defects in neutrophil degranulation, resulting in reduced EAE severity [36]. Moreover, CYBB may influence DC functions in various LAP-independent pathways [37,38]. Finally, deficiency of *Cybb* causes reduced cellular proliferation and leukocyte accumulation in response to tissue injury [39], which could contribute for the attenuated EAE phenotype in $atg5^{f/f}$;*Itgax-Cre* and $cybb^{f/f}$;*Itgax-Cre* mice but was not assessed in the studies that suggested a role for LAP [3,31].

We demonstrated that $rb1cc1^{f/f}$;*Itgax-Cre* mice exhibit reduced susceptibility to EAE induction. However, mice with systemic deficiency or DC-specific deficiency in RUBCN, which in association with the BECN1-PIK3C3-UVRAG-containing PtdIns3K complex mediates LAP but not autophagy [7], showed WT levels of EAE susceptibility, suggesting that resistance of EAE induction in $pik3c3^{f/f}$;*Itgax-Cre* mice is not caused by defective LAP. This conclusion is further supported by a report showing that mice deficient in the gene encoding IRGM1, an inducer of autophagy, are resistant to myelin basic protein-induced EAE [40]. Nevertheless, because PIK3C3 has pleiotropic effects [6], our current results cannot exclude the possibility that resistance to EAE in $pik3c3^{f/f}$; *Itgax-Cre* mice is mediated by cellular defects besides canonical autophagy that involve PIK3C3 and other components of the autophagy machinery such as ATG5 or CYBB. We also cannot exclude the possibility that $ITGAM^+$ $ITGAX^+$ myeloid cells contribute to this partial protection. Indeed, we recently showed reduced EAE severity in animals with myeloid cell-specific deficiency of *Pik3c3* [14].

In conclusion, our data indicate that PIK3C3 in DCs has a critical role in the effector phase of EAE and potentially other autoimmune diseases. Interestingly, we recently observed a delayed EAE disease progression in mice treated with SAR405 [14], a selective PIK3C3 inhibitor. These findings have important implications for the development of immunotherapies for autoimmune diseases such as MS by targeting PIK3C3 complexes.

Materials and methods

Mice

$Pik3c3^{f/f}$ [10,41], $rubcn^{-/-}$ [7], and $Rubcn^{f/f}$ [42] mice have been described. $Rb1cc1^{f/f}$ mice were obtained from Dr. Jun-Lin Guan (University of Cincinnati, Cincinnati, OH). B6.Cg-Tg (Itgax-cre)1–1Reiz/J (designated *Itgax-Cre*, Stock No: 008068) and C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J (designated 2D2 TCR^{MOG}, Stock No: 006912) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). DC-specific deletion of *Pik3c3, Rubcn*, and *Rb1cc1* was achieved by crossing the $Pik3c3^{f/f}$, $Rubcn^{f/f}$, and $Rb1cc1^{f/f}$ mice with *Itgax-Cre* transgenic mice. Six- to 12-week-old animals of both sexes were used in this study. All breeder and experimental mice were housed under specific pathogen-free conditions in compliance with guidelines from the Institutional Animal Care and Use Committee at Vanderbilt University.

Preparation of spleen cells

Cells from spleens were obtained by mashing the organ through a 70-μm cell strainer (Fisher Scientific International, Inc, 22–363-548) into RPMI 1640 medium (Fisher Scientific International, Inc, MT10040CM) supplemented with 5% fetal bovine serum (Sigma-Aldrich Corporation, F2442) followed by erythrocyte lysis with ACK lysing buffer (Lonza bioscience,10–548E).

Immunoblotting analyses

DCs from spleen were purified by magnetic sorting (Miltenyi Biotec, 130-100-875) and washed three times with PBS (Corning Cellgro, 21-031-CV) before preparing cellular proteins for western blotting. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd, 500-0116). Protein was separated by 10–15% SDS-PAGE and transferred to nitrocellulose membranes. Blot membranes were subsequently blocked with 5% milk, incubated with primary and secondary antibodies, and then visualized using ECLTM western blotting detection reagents (Amersham Bioscience) and exposure to the film. Antibodies against PIK3C3 (D9A5), LC3B (2775), SQSTM1 (5114), RUBCN (D9F7), and ACTB (13E5) were purchased from Cell Signaling Technology. Antibody against LAMP1 (1D4B) was from BioLegend.

Gene expression profiling

Fresh DCs were sorted from spleen samples using a BD FACSAria III cell sorter at Vanderbilt Technologies for Advanced Genomics. Sorted cells were pelleted and lysed with RNA lysis buffer from Quick-RNA^{TM} MiniPrep (ZYMO Research, R1054) to extract RNA. RNA quantity and purity were measured with an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was isolated from biological replicates and provided to the Beijing Genomics Institute for library construction and sequencing following standard protocols. The library products were sequenced using a BGISEQ-500. Briefly, poly-A tailed RNA was purified using paramagnetic oligo-dT beads (Thermo Fisher Scientific, 61002) from 1 μg of total RNA. The purified mRNA was chemically fragmented and reverse transcribed to double-stranded cDNA using random primers. The adapter-ligated DNA library was amplified and sequenced. Standard

bioinformatic analysis was performed by the Beijing Genomics Institute. Briefly, gene expression levels were quantified using RSEM after reads cleaning and genome mapping. Differentially expressed genes (DEGs) were detected with DEseq2, which is based on the negative binomial distribution. DEGs were classified according to official classification with the KEGG annotation result. Pathway enrichment analysis was performed using phyper, a function of R. The raw sequence data have been submitted to the Sequence Read Archive under SRA accession: PRJNA557158.

Induction and evaluation of active EAE

Six- to eight-week-old animals were used for EAE induction. Under isoflurane anesthesia, the animals were subcutaneously injected at two sites into the flank with 200 µl of 1 mg/ml MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) peptide (Biomatik, 51716) emulsified in Freund's complete adjuvant (2 mg/ml *Mycobacterium tuberculosis* extract H37Ra in incomplete Freund's adjuvant [BD bioscience, 263910]). Immediately after immunization and 48 h later, all mice received 400 ng of pertussis toxin (Calbiochem, 516560) by i.p. injection. Mice were scored daily in a blind manner according to the following scale: 0, no clinical signs; 0.5, partially limp tail; 1, paralyzed tail; 1.5, paralyzed tail and hind leg inhibition; 2, loss in coordinated movement, hind limb paresis; 2.5, one hind limb paralyzed; 3; both hind limbs paralyzed; 3.5, hind limbs paralyzed, weakness in forelimbs; 4, forelimbs paralyzed; 5, moribund.

Induction of passive EAE

For induction of adoptively transferred EAE, *pik3c3*$^{f/f}$*;Itgax-Cre, rubcn*$^{-/-}$, *rb1cc1*$^{f/f}$*;Itgax-Cre, rubcn*$^{f/f}$*;Itgax-Cre* mice, and their respective littermate controls were used as either donors or recipients for adoptive transfer of T cells. Active EAE was induced in mice as described above. At day 10, spleens were harvested, and single-cell suspensions were prepared. The cells were stimulated with 50 µg/ml MOG_{35-55} at 1×10^7 cells/ml in 10 ml RPMI 1640 complete medium in a Petri dish. After 72 h, cells were harvested and resuspended in PBS (1×10^8 cells/ml) for adoptive transfer. Recipient mice (8 weeks of age) were irradiated sublethally at 400 rads to generate a lymphopenic environment 6 h prior to injection of MOG_{35-55}-specific T cells. Cells were injected i.p. (300 µl/mouse). The clinical scores were graded similarly to active EAE.

T cell proliferation assay

Splenic DCs from *pik3c3*$^{f/f}$*;Itgax-Cre, rb1cc1*$^{f/f}$*;Itgax-Cre, rubcn*$^{f/f}$*;Itgax-Cre* and their respective littermate control mice were isolated using a DC isolation kit according to the manufacturer's protocols. MOG_{35-55}-specific $CD4^+$ T cells were isolated from 2D2 TCR-transgenic mouse splenocytes using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, 130-104-453) according to the manufacturer's protocols. T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) according to the manufacturer's instructions (Thermo Fisher Scientific, C34554). DCs were pulsed with 20 µg/ml MOG_{35-55} or MOG_{1-125} (AnaSpec Inc, AS-55158-100) for 4 h and cultured with T cells for 96 h at a ratio of 1:5 with media. T cell proliferation was assessed at the end of the culture.

Flow cytometry

In all experiments, dead cells were excluded from the analysis by electronic gating. Fluorescently labeled mAbs against CD3E, CD4, CD8A, CD44, PTPRC, IFNG, IL17A, ITGAM, ITGAX, IL1B, and appropriate isotype controls were obtained from BD Biosciences or eBioscience. For measuring MOG-specific IFNG and IL17A production during EAE, splenocytes or CNS leukocytes from *Pik3c3*$^{f/f}$ and *pik3c3*$^{f/f}$*;Itgax-Cre* mice induced for EAE (day 10) were cultured with MOG peptide (20 µg/ml) for 18 h in the presence of GolgiStop (BD Biosciences, 554724). Flow cytometric analyses were performed using a Canto II flow cytometer (BD Biosciences). The acquired data were analyzed using FlowJo software (Version 10.0.7, Treestar, Palo Alto, CA).

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software). Throughout the manuscript, the distribution of data points is expressed as mean ± SEM. EAE clinical scores were analyzed using ANOVA and Kaplan-Meier curves by log-rank test. EAE cumulative score, peak disease score, and cytokine production were analyzed by Student's t-test, and non-parametric comparisons were performed by the Mann-Whitney U test. Two-tailed Pearson correlation test was applied for the correlation analysis. $p < 0.05$ was considered significant.

Disclosure statement

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

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