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SHORT COMMUNICATION

Lung inflammation stalls T_h17-cell migration *en route* to the central nervous system during the development of experimental autoimmune encephalomyelitis

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Abstract

Recruiting pathogenic T cells to the central nervous system (CNS) is a critical step during the development of experimental autoimmune encephalomyelitis (EAE). Here, we report that the absence of autophagy and microtubule-associated protein 1A/1B-light chain 3-associated phagocytosis significantly delayed the onset of EAE in *Atg7* conditional knockout (*Atg7* CKO) mice in myeloid cells. T-helper cell-cell priming appeared to be normal in the *Atg7* CKO mice, but the mice showed significant accumulation of T_h17 cells in the lung. The data suggested that the stalling of T_h17 cells in the lung *en route* to the CNS caused the delay. The lung of *Atg7* CKO mice, in which we previously demonstrated spontaneous mild inflammation, showed high expression of CCL20, a chemokine that attracts T_h17 cells. We have also shown that LPS intranasal instillation delayed EAE onset, suggesting that pulmonary inflammation has an impact on EAE development. Based on our data, therapeutic immunomodulation targeted to the lung, rather than systemically, might be a possible future option to treat multiple sclerosis.

Keywords: autoimmunity, autophagy, CCL20, T-cell recruitment

Introduction

The lung possesses a unique immune environment that maintains homeostasis by inhibiting excessive immune responses against harmless environmental stimuli taken up by inhalation (1). However, it is still largely unknown how pulmonary immune activation impacts the development of autoimmune inflammation in the central nervous system (CNS). A previous report demonstrated that the lung is a 'hub' organ that licenses T cells migrating into the CNS during the development of experimental autoimmune encephalomyelitis (EAE) (2), an animal model of multiple sclerosis (MS). Indeed, epidemiological studies have implied a relationship between the lung and the pathology of MS, such as smoking increasing the risk of MS (3, 4), although the molecular mechanism is not fully elucidated. Therefore, it is highly possible that modulating the lung microenvironment alters the pathology of neuroinflammatory diseases.

ATG7 is required for autophagy and microtubule-associated protein 1A/1B-light chain 3 (LC3)-asscociated phagocytosis (LAP). The authors along with another group have recently found that the lack of ATG7 in myeloid cells can disturb the delicate balance of immune homeostasis and induce spontaneous and subclinical inflammation in the lung (1, 5). Our previous study demonstrated that pulmonary inflammation in *Atg7* myeloid cell conditional knockout (*Atg7* CKO; $Atg7^{fl/fl}$*LysM*-Cre) mice is caused by increased bacterial burdens in the lung during the suckling period (1). (Adult mice do not show detectable levels of bacterial burdens both in wild type (WT) and in *Atg7* CKO mice (1).) In addition, *Atg7* CKO mice showed increased sensitivity of alveolar macrophages (AMs) to TLR4 ligands, suggesting AM hypersensitivity as another cause of the lung inflammation (1). Since intranasally instilled antibiotics ameliorated the spontaneous lung inflammation, bacteria in the lung are a major trigger of inflammation (1). Interestingly, the spontaneous inflammation in *Atg7* CKO mice was limited to the lung and was not observed in other organs including the mesenteric lymph node, colon, small intestine, liver, kidney, skin, spleen, brain and spinal cord (1). The lung-specific inflammatory phenotype of *Atg7* CKO mice is considered to be a reflection of the lung's constant exposure to the outer environment and the subsequent hyperresponsiveness to harmless stimuli.

In this study, we sought to elucidate the impact of lung inflammation on EAE development. CD4+ T-cell priming and T-helper cell polarization were normal in the draining lymph nodes DLNs of *Atg7* CKO mice. However, T-helper cells, and T_h17 cells in particular, were entrapped in the lungs of *Atg7* CKO mice, resulting in delayed EAE onset. Intranasal instillation of LPS in WT mice also resulted in delayed EAE onset with T_h17 accumulation in lungs. Therefore, lung inflammation, rather than autophagy and/or LAP, was sufficient to modulate the disease course of EAE. These results strongly suggest the involvement of the lung in the EAE pathophysiology, particularly in T_h17-cell migration into the CNS. Thus, modulating local immune responses in the lung may be a novel therapeutic approach to treat MS.

Methods

Animals and reagents

All mice used in this study were on the C57BL/6 background. *Atg7*$^{fl/fl}$ mice were described previously (1, 6, 7). Lysozyme M (*LysM*)$^{cre/cre}$ and 2D2 TCR transgenic mice specific to myelin oligodendrocyte glycoprotein (MOG) were purchased from Jackson Laboratories. All the experiments were performed as approved by the Institutional Animal Care and Use Committee. Antibodies against CD45, CD4, CD3, CCR6, IL-17, Foxp3 and IFN-γ were purchased from BioLegend. $MOG_{35–55}$ peptide was synthesized by New England Peptides. Recombinant IL-6 (rIL-6), rIL-23 and rTGF-β were purchased from BD Biosciences. Neutralizing antibody for IFN-γ was purchased from BD Biosciences.

EAE induction and LPS instillation

Active EAE was induced by immunizing the mice with $MOG_{35–55}$ peptide (100 μg) emulsified in complete Freund's adjuvant (Sigma) containing heat-killed *Mycobacterium tuberculosis* H37Ra (200 μg per mouse, DIFCO Laboratories) on day 0, and intraperitoneal injection of 200 ng pertussis toxin (PTx; List Biological Laboratories) on days 0 and 2. To induce T_h17-mediated passive EAE, CD4+ T cells, obtained from 2D2 mice, were polarized with rIL-6 (20 ng ml^{-1}), rIL-23 (10 ng ml^{-1}), rTGF-β (3 ng ml^{-1}) and anti-IFN-γ antibody (4 μg ml^{-1}) on a plate coated with anti-CD3 and anti-CD28 antibodies for 5 days and adoptively transferred to sublethally irradiated (450 rads) WT and *Atg7* CKO mice. PTx (200 ng) was intraperitoneally administrated on days 0 and 2. Intranasal instillation of LPS (2.5 μg per instillation) was performed on days 6 and 9 after EAE induction. EAE severity was scored as previously described (8, 9).

Flow cytometry analyses

Lung tissues were cut into small pieces and incubated in a 1-mg ml^{-1} collagenase D solution at 37°C for 30 min. Cells were then enriched by Percoll gradient (GE Healthcare). After staining with specific antibodies, cells were analyzed with FACS Canto™ II (BD Biosciences) and the FlowJo software (Treestar Inc.). For intracellular cytokine staining, cells were stimulated with PMA (50 ng ml^{-1}) and ionomycin (500 ng ml^{-1}) for 5 h and treated with GolgiPlug (BD Biosciences) for the last 3 h. Cell surface markers were stained first, then intracellular cytokines were stained as previously described (8, 9).

Real-time PCR analysis

Total mRNA were reverse transcribed to cDNA, and gene expression levels were determined by using the $-\Delta\Delta C_t$ method of real-time PCR as previously described (1, 7–9) using primers for *Ccl20* (forward: 5′-AAGACAGATGGCCGATGAAG-3′, reverse: 5′-TCTTGACTCTTAGGCTGAGGA-3′), *Tnfa* (forward: 5′-CCCTCACACTCAGATCATCTTCT-3′, reverse: 5′-GCTACG ACGTGGGCTACAG-3′), *Il6* (forward: 5′-GAGGATACCACT CCCAACAGACC-3′, reverse: 5′-AAGTGCATCATCGTTGTTC ATACA-3′), *Il10* (forward: 5′-GGTTGCCAAGCCTTATCGGA-3′, reverse: 5′-ACCTGCTCCACTGCCTTGCT-3′), *Cxcl1* (forward: 5′-TGGGATTCACCTCAAGAACA-3′, reverse: 5′-TTTCTGAAC CAAGGGAGCTT-3′), *Ccl3* (forward: 5′-TGCTTCTCCTACAGC CGGAAGATT-3′, reverse: 5′-TCAGGCATTCAGTTCCAGGT CAGT-3′) and *Actb* (forward: 5′-GTTACCAACTGGGAC GACA-3′, reverse: 5′-CTGGGTCATCTTTTCACGGT-3′). *Actb* expression was used as the internal control. Results shown are representatives from multiple independent experiments with similar results.

Statistical analysis

The two-tailed Student's *t*-test was used for statistical analyses.

Results and discussion

Atg7 CKO mice delayed EAE onset by accumulating T_h17 cells in the lung

ATG7 is essential for autophagy and LAP (10, 11). To evaluate the impact of ATG7 in myeloid cells during EAE, we induced EAE in *Atg7*$^{fl/fl}$*LysM*$^{cre/+}$ mice (hereafter denoted as '*Atg7* CKO mice') with *LysM*$^{cre/+}$ mice as control (hereafter denoted as 'WT mice'). *Atg7 CKO* mice develop spontaneous, but subclinical, pulmonary inflammation (1). We found that *Atg7* CKO mice showed significantly delayed onset of EAE (Fig. 1A and B). However, on day 9 when T cells have been primed but not infiltrated in CNS, the mice did not show altered numbers of total CD4+ T cells, T_h1, T_h17 (Fig. 1C, D and E) and T_{reg} cells (Fig. 1F and G) in DLNs (8). Even at a later stage (day 21), the number of Tregs in DLNs in *Atg7* CKO mice was comparable with that in WT mice (Fig. 1H). The result suggests that CD4+ T-cell priming and T-helper cell polarization were normal in the DLNs of *Atg7* CKO mice. We had initially expected reduced T_h17 cell development from the lack of autophagy, as autophagy inhibits NLRP3 inflammasome activity and reduces the expression of IL-1β, which induces T_h17 responses (12). However, T_h17 cell development in DLNs appeared to be normal even without ATG7 in myeloid cells. To evaluate the possibility that *Atg7* deficiency in myeloid cells changes T-cell pathogenicity and whether it causes the delayed EAE onset, we induced EAE in WT and *Atg7* CKO mice by adoptively transferring T_h17-polarized MOG-specific 2D2 CD4+ T cells (Fig. 1I and J). *Atg7* CKO recipients exhibited significantly delayed onset, suggesting that the delay of EAE onset in *Atg7* CKO mice is rather due to the lack of ATG7 in recipients.

Notably, a previous report demonstrated that *Atg7* deficiency in DCs (*Atg7*$^{fl/fl}$CD11c-Cre) did not alter the time of EAE onset, but the disease severity was significantly milder than in WT mice (13). The difference between the phenotype of their results and

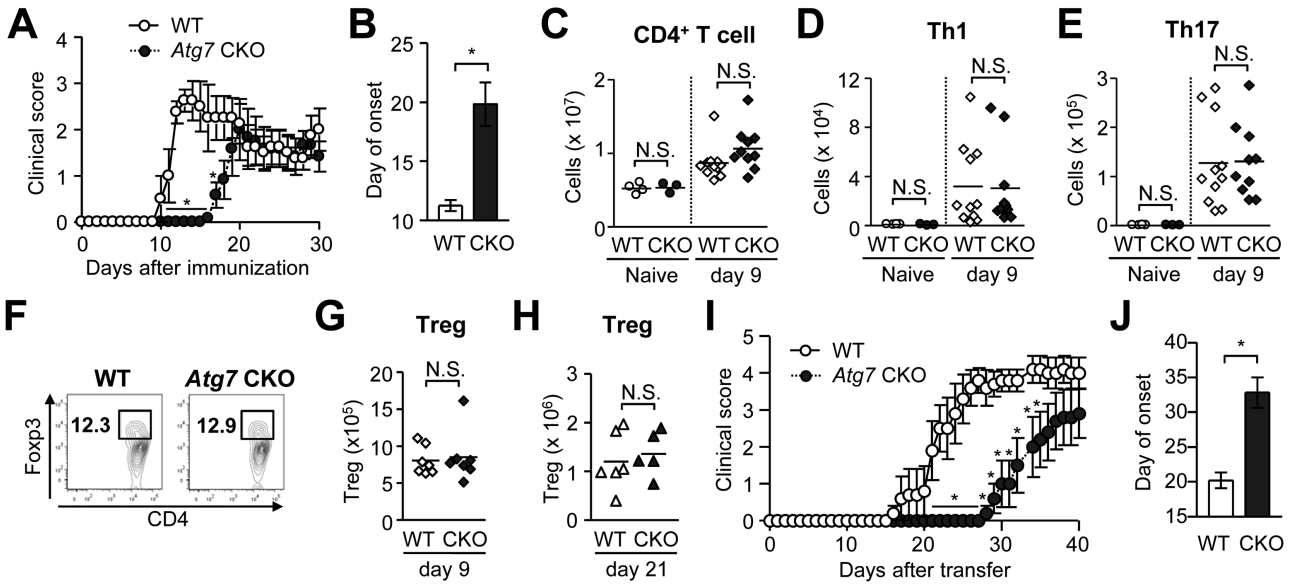

Fig. 1. Delayed onset and mild EAE in *Atg7* CKO mice. (A, B) Time-course of EAE score (A) and average day of EAE onset (B). WT (n = 6). *Atg7* CKO (n = 4). Error bars denote mean ± SD. *$p < 0.05$. (C–E) Numbers of CD4+ T cell (C), T_h1 (D), T_h17 (E) cells in DLNs on days 0 and 9 after EAE induction. (F) Representative flow cytometry charts of CD3-positive pre-gated cells in DLNs on day 9. (G and H) Numbers of Tregs ($Foxp3^+CD4^+$) in DLNs on day 9 (G) or day 21 (H). (I and J) Clinical score (I) and onset (J) of passive EAE. CD4+ T cells obtained from 2D2 mice were cultured with plate-coated anti-CD3 and anti-CD28 antibodies (5 μg ml^{-1} each) in T_h17-polarizing condition for 5 days and adoptively transferred to sub-lethally irradiated WT and *Atg7* CKO recipients. n = 5 per group. One data point reflects a result from one mouse. Horizontal lines denote average values. Data are representative of two independent experiments. N.S.: not significant.

ours is intriguing, but ultimately, they should not be compared due to differences in doses of reagents used in EAE induction. (We have previously demonstrated that the method of EAE induction has significant impact on disease development (9).) Nevertheless, the different outcome in our result from the DC-specific *Atg7*-deficient mice suggests that macrophages, AMs (1, 5) in particular, are involved in delaying EAE onset.

To understand why the onset is delayed in *Atg7* CKO mice, we examined the numbers of total CD4+ T cells, T_h1, and T_h17 cells during EAE development in spinal cords, brains, circulating blood and lungs. We evaluated lungs because a previous publication demonstrated that T cells transiently reside within the lung during migration to the CNS (2). First, we noticed the absence of total CD4+ T cells, T_h1 and T_h17 cells in spinal cords of *Atg7* CKO mice on day 15, although these cells were found on day 21 (Fig. 2A), suggesting a significant delay in T-helper cell migration. *Atg7* CKO brains also showed significant delay in T-cell accumulation, although only with T_h17 cells (Fig. 2B). In contrast, lungs and circulating blood of *Atg7* CKO mice generally showed more CD4+ T cells than WT mice on days 9 and 15 (Fig. 2C and D). A closer look at the time-course data indicated a notable stall of T_h17 cells in lungs of *Atg7* CKO mice (Fig. 2D). Thus, *Atg7* CKO mice accumulated T_h17 cells in the lung, but were yet to fully recruit T_h17 cells in the CNS as WT mice did on day 15 (Fig. 2A, B, D and E). The retention of CD4+ T cells is considered to be mediated mainly by chemokine receptors and adhesion molecules; therefore, T-cell encephalitogenicity itself is most probably not a major factor of their retention in the lung. At a later stage of EAE on day 21, CD4+ T cells, including Tregs, eventually achieved their migration to the CNS (Fig. 2A, B, F). On day 21, neutrophils and monocytes/macrophages also successfully migrated to the CNS in *Atg7* CKO mice to the comparable level to those in WT mice (Fig. 2G–H). In sum, the results strongly suggested that the delay in EAE onset in *Atg7* CKO mice was due to the accumulation of T_h17 cells in the lung.

Ccl20 mRNA is highly expressed in the lung of Atg7 CKO mice

To investigate this 'stalling' of T_h17 cells in the lung of *Atg7* CKO mice, we examined cytokine gene expression in total lung tissues. The genes examined were *Cxcl1*, *Ccl3*, *Il6*, *Tnfa* and *Il10*, and most of them were up-regulated in *Atg7* CKO lungs (Fig. 3A). As we have previously reported, the activation of these genes was due to the spontaneous pulmonary inflammation in *Atg7* CKO mice (1). Because of the accumulation of T_h17 cells in the lung during EAE development (Fig. 2D and E), we then examined the gene expression of CCL20, a T_h17-cell chemoattractant, in *Atg7* CKO lungs. CCL20 is a ligand of CCR6, highly expressed in T_h17 cells, and particularly plays a critical role in EAE development by attracting T_h17 cells to the CNS (13, 14). We found elevated *Ccl20* mRNA expression in lungs of naive (Fig. 3B) and EAE-induced (Fig. 3C) *Atg7* CKO lungs. We also found that T_h17 cells in *Atg7* CKO lungs expressed higher levels of CCR6, compared with T_h17 cells in WT lungs on day 9 (Fig. 3D). CCR6 expression is also critical to progress EAE, as CCR6 deficiency in pathogenic CD4+ T cells abates their recruitment into CNS and EAE development (13, 15). In contrast, circulating T_h17 cells in *Atg7* CKO mice did not show elevated CCR6 expression (Fig. 3E). We also examined other major mucosal tissues that have direct contacts to the outer environment—the small intestine and colon. In contrast to the lung, no difference was found in *Ccl20* mRNA expression in the

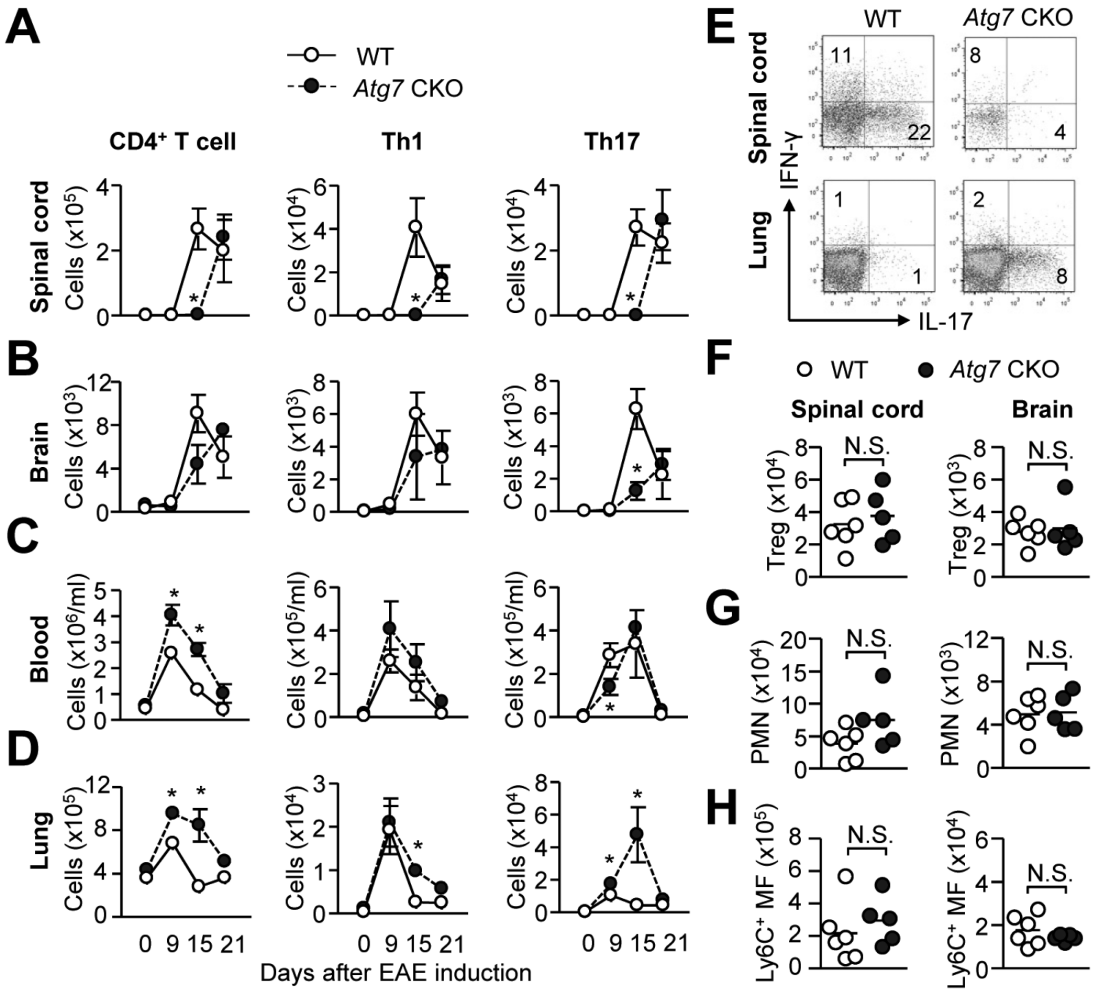

Fig. 2. Time-course of CD4⁺ T-cell numbers during EAE development. Numbers of total CD4⁺ T cells, T_h1 and T_h17 cells were assessed in spinal cords (A), brains (B), circulating blood (C) and lungs (D) on day 0 ($n = 3$), 9 ($n = 8$), 15 ($n = 5$) and day 21 ($n = 3$). (E) Representative flow plots of CD4⁺ T cells (gated on CD45⁺CD3⁺CD4⁺) in the lung and spinal cord of WT and *Atg7* CKO mice on day 15. (F–H) Numbers of Tregs (F), neutrophils (PMN) (G) and Ly6C⁺ monocytes (H) in the spinal cord and brain on day 21. $n = 6$ per group. Data are representative of two independent experiments. Error bars denote mean ± SD. *$p < 0.05$.

gut between WT and *Atg7* CKO mice regardless of EAE induction (Fig. 3F), suggesting that enhanced *Ccl20* mRNA expression in *Atg7* CKO mice is specific to the lung. These results suggest involvement of the CCL20/CCR6 axis in the lung to cause the delay of EAE onset.

Lung inflammation was sufficient to delay EAE onset by stalling T_h17 cells in the lung

We have shown that T_h17-cell stalling in the lung is a mechanism that delays EAE onset in *Atg7* CKO mice. However, it is not clear which is important to stall T_h17 cells in the lung, the absence of autophagy (or LAP) in myeloid cells or just the mild lung inflammation (1). To test whether mild lung inflammation delays the EAE onset, we performed intranasal instillation of a low-dose LPS to WT mice on days 6 and 9 after EAE induction. LPS instillation indeed delayed the onset of EAE in WT mice (Fig. 4A and B), albeit to a lesser extent to that in *Atg7* CKO mice (Fig. 1A). The level of LPS instillation induced transient inflammation and may explain why delayed EAE onset in LPS-treated WT mice was less significant than that seen in *Atg7* CKO mice that have chronic lung inflammation. A previous LPS-induced lung inflammation study showed no change in TNFα levels in plasma after LPS instillation, in which a dose of LPS per mouse weight was similar to our condition (16). The study also showed that TNFα levels in bronchoalveolar lavage fluid (BALF) were transiently up-regulated 4 and 24 h after LPS instillation. This suggests that lung inflammation was ongoing but transient, because BALF TNFα levels came back to normal in 72 h (16). In our study, elevated *Ccl20* mRNA expression and increased T_h17-cell accumulation in lungs were observed in LPS-treated mice on day 9 (Fig. 4C). CCL20 expression in bronchial epithelial cells is known to be induced by a wide variety of stimulations including pathogens, allergens and environmental pollutions (17, 18). Therefore, it is highly possible that bronchial epithelial cells produced CCL20 after LPS instillation. LPS-mediated mild lung inflammation caused lungs to accumulate T_h17 cells, but not total CD4⁺ T and T_h1 cells (Fig. 4D). These results

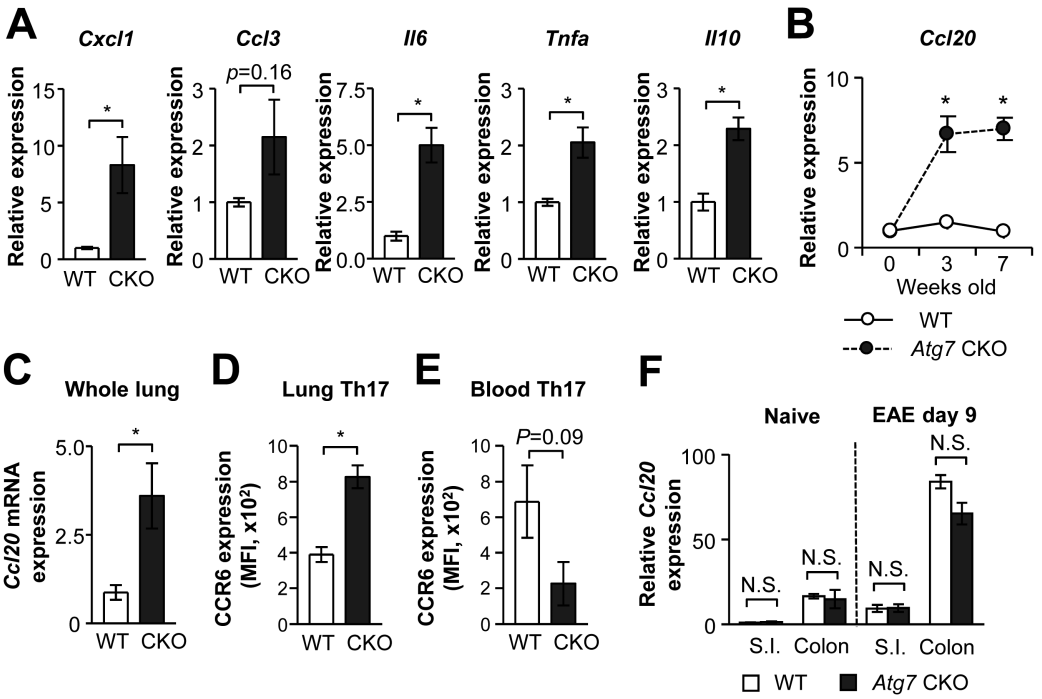

Fig. 3. Expression of *Ccl20* mRNA and CCR6 protein. (A) mRNA expression of *Cxcl1*, *Ccl3*, *Il6*, *Tnfa* and *Il10* in the lungs of WT and *Atg7* CKO mice 9 days after EAE induction. $n = 3$ per group. (B) *Ccl2* mRNA expression in the lungs of 'naive' WT and *Atg7* CKO mice. $n = 3$ per group. (C) *Ccl20* mRNA expression in lungs on day 9 after EAE induction. Three mice per group. (D, E) CCR6 expression on T_h17 cells in lungs (D) and circulating blood (E) on day 9. Three mice per group. (F) *Ccl20* expression in small intestines (S.I.) and colons obtained from days 0 and 9 after EAE induction. $n = 3$ per group. Data are representative of two independent experiments. Error bars denote mean ± SD. $^*p < 0.05$. N.S.: not significant.

suggest that mild lung inflammation is sufficient to stall T_h17 cells in the lung to delay the onset of EAE.

Our previous study showed that *Atg7* deficiency in myeloid cells spontaneously increased bacterial loads in the lung and enhanced TLR4 sensitivity of AMs that results in mild spontaneous pulmonary inflammation (1). In this study, we demonstrated that *Atg7* CKO mice show delayed onset of EAE specifically due to stalling of T_h17 cells in the lung during their migration to the CNS. A very recent article reported that respiratory infection by bacteria reduces CNS inflammation, attenuates clinical symptoms of EAE and delays EAE onset (19). Their study also demonstrated that the pulmonary bacterial infection prevents T-cell infiltration into the CNS (19). Although the study did not particularly show accumulation of T cells in the lung, our results suggest that stalling of T_h17 cells in the lung is possible during the respiratory infection. Indeed, intranasal instillation of a low-dose LPS delayed the EAE onset by the stalling of T_h17 cells in the lung (Fig. 4). During delayed EAE development, *Atg7* CKO mice and LPS-instilled WT mice both increase the expression of *Ccl20* mRNA. Interestingly, cigarette smoking, a risk factor of MS, is known to reduce the expression of *Ccl20* mRNA in human lungs (20), a quality that may allow T_h17 cells to quickly migrate to the CNS. Results from the human study (20) and our data both suggest the involvement of lung CCL20 in controlling autoimmune responses in the CNS. We therefore intended to examine whether local CCL20 expression in the lung is sufficient to delay EAE and intra-tracheally instilled recombinant CCL20 (rCCL20) to WT mice. Unfortunately, the result of the experiment was inclusive, because we were unable to observe a delay in EAE onset after treatment (data not shown). It is still not clear whether the failure was technical (e.g. poor efficiency in rCCL20 delivery, short half-life, or insufficient amount of rCCL20), or whether rCCL20 alone is not sufficient to alter the *in vivo* biology of multiple cell behaviors (e.g. requirement in enhanced expression of adhesion molecules in vascular endothelial cells). Altering multiple factors in the lung may be essential to achieve the T_h17-cell retention.

The majority of treatments for autoimmune diseases involve the administration of immunomodulating drugs that disseminate systemically. To minimize the side-effects during treatment, it is desirable to modulate the immune responses only in a certain organ or a limited area of a body. Our results suggest that the lung might be targeted to modulate T-cell migration to the sites of autoimmunity. In addition, potential therapeutics can be delivered to the lung relatively noninvasively through inhalation, as compared with the current drugs. This study demonstrated that lung inflammation specifically stalls T_h17-cell migration and delays the onset of EAE that may be exploited to create therapeutic opportunities in lung-specific immune modifications to treat CNS inflammatory diseases such as MS.

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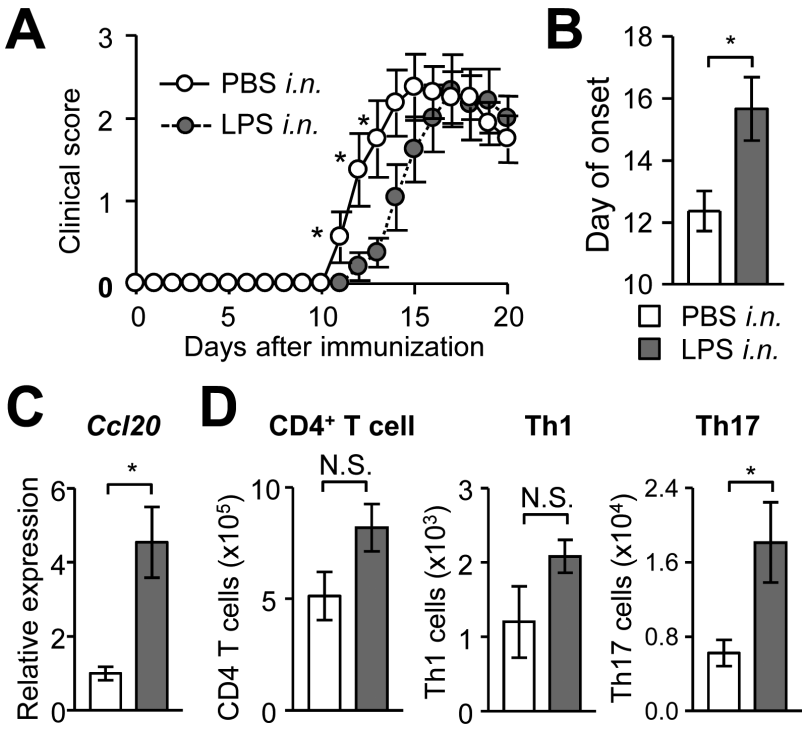

Fig. 4. LPS intranasal instillation delays EAE onset. (A, B) Time-course of EAE score (A) and average day of EAE onset (B). WT mice were intranasally instilled with LPS (2.5 μg per instillation) on days 6 and 9 after EAE induction. LPS-instilled (n = 12) and PBS-instilled control (n = 8) groups are shown. (C) *Ccl20* mRNA expression in lungs on day 9. n = 5 per group. (D) Numbers of CD4+ T cells, T_h1 and T_h17 cells in lungs on day 10. LPS-instilled (n = 6) and PBS-instilled control (n = 7) groups are shown. Data are representative of two independent experiments. White and gray bars denote values obtained from PBS- or LPS-treated mice, respectively (B–D). Error bars denote mean ± SD. *$p < 0.05$. N.S.: not significant.

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Conflict of interest statement: The authors have no financial conflict of interest.

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