Original Article

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IL16 deficiency enhances Th1 and cytotoxic T lymphocyte response against influenza A virus infection

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Summary **Influenza A virus (IAV) is the major cause of seasonal epidemics and flu outbreaks worldwide. Given that interleukin 16 (IL16) can regulate T cell function and is one of the signature markers for virus infection including IAV infection, the impact of IL16 on IAV-induced T cell immune response hasn't been elucidated yet. In this paper, we infected wild type and IL16 knockout (KO) mice with IAV and analyzed the immunity of mice by flow cytometry. We observed an increase in the percentage of T helper (Th) 1 cells in the spleens of IL16 KO mice and elevation of IFN-γ and TNF-α secretion from $CD8^+$ T cells in the lungs and spleens of IL16 KO mice in response to IAV infection. Moreover, the expression of major histocompatibility complex II which represents the maturation of dendritic cells (DCs) was upregulated in the lungs of IL16 KO mice. Taken together, our study suggests that IL16 deficiency enhanced Th1 and cytotoxic T lymphocyte response as well as DC maturation upon IAV infection, which provides new insight into the host regulation of T cell immune responses during IAV infection.**

Keywords: Interleukin 16, influenza A virus, T helper 1, cytotoxic T lymphocyte

1. Introduction

Influenza A virus (IAV), a member of the *Orthomyxoviridae* family, is the major cause of seasonal epidemics and flu outbreaks worldwide. It is estimated that 3 to 5 million severe cases and 250,000 to 500,000 deaths annually are attributed to IAV infection (*1-3*). IAV contains an eight-segmented genome of single-stranded negative sense RNA and can be divided into different subtypes according to the genetic and antigenic properties of hemagglutinin (HA) and neuraminidase (NA) (*4,5*). Two influenza A virus subtypes currently common in general circulation among people are H1N1 and H3N2 (*2,6-8*). The emerging and reemerging threats caused by IAV highlight the necessity to understand more of the host factors limiting or supporting IAV infection.

Upon IAV infection, airway dendritic cells (DCs) recognize viral particles-termed pathogen-associated molecular patterns (PAMPs) through their pattern recognition receptors (PRRs). The recognition subsequently induces the production of interferon (IFN)-α/β and proinflammatory cytokines, which assist DCs to mature into professional antigen presentating cells (APCs) and migrate to peripheral lymphoid organs to initiate a T cell immune response (*9,10*). Noticeably, besides morphological changes, DC maturation triggers the upregulation of major histocompatibility complex (MHC) II on its surface to stimulate a helper T (Th) cell response (*11,12*).

$CD4^+$ T cells and $CD8^+$ T cells have distinct and critical roles in the control of virus infection. Upon activation by APCs, naïve $CD4^+$ T cells, also known as Th0, differentiate into specific subsets including Th1, Th2, Th17 and regulatory T cells (Treg). As was reported, $CD4^+$ T cells routinely exhibit a Th1-

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biased immunity in response to IAV infection (*13,14*). Considered as an assistant of cellular immunity, Th1 not only assists APCs to exert more costimulatory molecules to promote $CD8^+$ T cells to differentiate into cytotoxic T lymphocytes (CTLs), but also restricts viral infection by secreting IFN-γ and tumor necrosis factor (TNF)-α (*15,16*). Therefore, the percentage of Th1 can be regarded as an indicator to evaluate host immune response to IAV and adequate anti-IAV cellular immune response requires timely recognition of APCs, efficient Th1 assistance and robust CTL response.

Interleukin 16 (IL16) is a cytokine with multiple functions including T cell chemotaxis, IL-2Rα upregulation, and T cell transient anergy (*23,24*). An analysis of whole blood samples from patients infected with different viruses including influenza virus revealed that IL16 was a pan-viral biomarker (*25*). The relationship between IL16 and IAV infection hasn't been studied so far. In this paper, we studied the impact of IL16 on T cell response elicited by IAV and found that IL16 deficiency enhanced the Th1 and CTL response as well as DC maturation upon IAV infection.

2. Materials and Methods

2.1. *Viruses, mice and infection*

IAV infections in the study were performed using Influenza virus A/Puerto Rico/8/34 (H1N1) (PR8) strain. The original stock of PR8 virus was offered by Prof. Haikun Wang (Institut Pasteur of Shanghai). The PR8 virus was amplified in 10-day-old embryonated hen's eggs. Briefly, PR8 virus was injected into the allantoic cavity using a 1 ml syringe and incubated in a 37°C incubator with 82% humidity for 48hr. The infected eggs were then cooled overnight at 4°C and the clear allantoic fluid was harvested and stored at -80°C as the final virus stock.

IL16 KO C57BL/6 mice were generated by Shanghai Model Organisms Center. Mice were intranasally infected with PR8 virus at 8,000 PFU/mouse and were humanely euthanized when their weight loss met 25% of the initial weight. All experimental procedures on animals were performed in compliance with the guidelines of the Animal Ethics Committee of Institut Pasteur of Shanghai.

2.2. *Preparation of splenocytes and lung cells*

Lungs and spleens were isolated aseptically on day7 post-infection. Lungs were digested in DMEM supplemented with 20 μg/mL Liberase (Roche, Switzerland) and 25 μg/mL DNase I (Roche, Switzerland) for 30 min at 37°C. Then lungs were ground through a 70 μm nylon mesh to yield single-cell suspensions. Red blood cells were removed using Ammonium-Chloride-Potassium (ACK) lysis buffer (final concentration: 150mM NH_4CL, 10mM $KHCO_3$, 0.1mM Na_2EDTA). Cells were counted and resuspended at 4×10^6cells/mL in DMEM and stimulated with 50 ng/mL phorbol myristate acetate (PMA) (Sigma, Germany) and 1 μg/mL Ionomycin (IM) (Sigma, Germany) for 4 hr. GolgiStop (BD Bioscience, America) was added in the last 2 hr of stimulation at 0.67 μl/mL. Cells were washed twice with sterilized PBS to prepare for further analysis.

2.3. *Flow cytometry analysis*

Splenocytes and lung cells were incubated with anti-CD16/32 (BD Bioscience, USA) for 30min at 4°C in PBS supplemented with 2% FBS (FACS buffer) to block non-specific binding with Fc receptors. Cells were surface-stained with BV650-anti-CD8 (BD-563234, USA), BV711-anti-CD4 (BD-563050, USA), BV510-anti-CD3 (BD-563024, USA), PECY7-anti-CD25 (eBioscience-25-0251-82, USA), PE-anti-F4/80 (BD-565410, USA), BV510-anti-CD11b (BD-562950, USA), APC-anti-CD11c (eBioscience-17-0114-81, USA) for 20 min on ice and washed twice with PBS. For intracellular staining, cells were fixed and permeablized using BD CytoFix/CytoPerm Kit (BD Bioscience, USA) and stained with APC-anti-IL-4 (eBioscience-17-7041-82, USA), PE-anti-IFN-γ (eBioscience-12-7311-82, USA), PE-anti-IL-2 (eBioscience-12-7021-82, USA), BV421-anti-IL-17 (BD-563354, USA), PECY7-anti-TNF-α (BD-557644, USA), Pacific blue-anti-Foxp3 (eBioscience-48-5773-82, USA) for 20min. Cells were washed with 1×BD Perm/Wash buffer and were finally resuspended in 4% paraformaldehyde (PFA) for flow cytometry analysis. Flow cytometry was performed using BD LSR Fortessa (BD Bioscience, USA) and raw data was analyzed using FlowJo software.

2.4. *Statistical analysis*

Statistical analysis was performed using Prism (Graph Pad Software). The data are presented as mean ± SD. Differences between groups of research subjects were analyzed for statistical significance with two-tailed Student's *t* tests. A *p* value of 0.05 was considered significant.

3. Results

3.1. *IL16 deficiency doesn't affect the function of T cells in resting state*

T cell immunity is important in both viral clearance and host recovery (*4,26*). To detect the role of IL16 in T cell response during IAV infection, we first analyzed whether the function of T cells are affected by IL16 deficiency before infection. Flow cytometry analyses revealed that the population of T cells (Figure 1A), the $CD4^+$ T cell subsets (Th0/Th1/Th2/Th17/Treg) (Figure 1B), as well

as the IFN-γ-secreting and TNF-α-secreting $CD8^+$ T cells (Figure 1C) were comparable in the spleens of uninfected wild type (WT) and IL16 KO mice, suggesting that IL16 deficiency doesn't change the populations and cytokine secretion of T cells in the resting state.

3.2. *IL16 deficiency elevates Th1 response after IAV infection*

Given that IL16 doesn't alter T cell functions in the resting state, we next assessed the T cell response in PR8-infected WT and IL16 KO mice. Flow cytometry revealed that the percentage of $CD4^+$ T cells (Figure 2A) and $CD4^+$ T cell subsets (Figure 2B and C) in the lungs of WT mice were comparable to that of IL16 KO mice on day 7 post-infection. Splenocytes didn't show significant differences in the $CD4^+$ T cell population between WT and IL16 KO mice either (Figure 3A). But surprisingly, intracellular staining revealed that the percentage of Th1 in the spleens of IL16 KO mice markedly surpassed that of WT mice, albeit WT mice also displayed a Th1-biased response compared with PBS mock-infected mice (Figure 3B and C). These data suggest that IL16 deficiency enhances Th1 response in

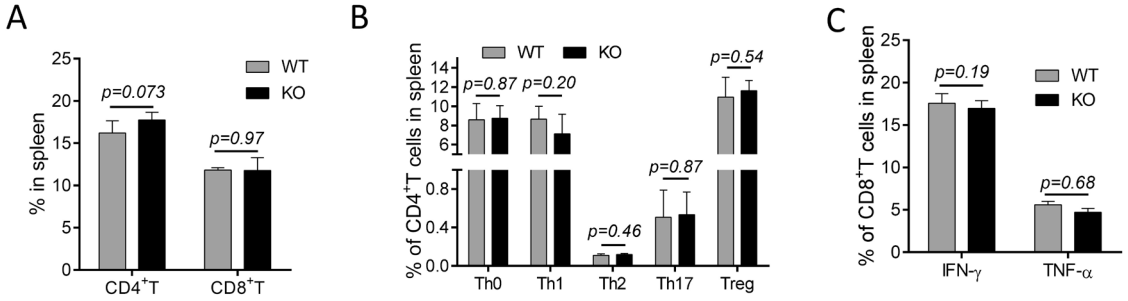

Figure 1. IL16 deficiency doesn't affect T cell populations in spleens of uninfected mice. Splenocytes were isolated from eight-week-old male WT and IL16-KO mice and analyzed by flow cytometry after 4 hr of phorbol myristate acetate (PMA) and Ionomycin (IM) stimulation. The percentage of $CD4^+$T cells and CD8+T cells **(A)**, the percentage of Th0/Th1/Th2/Th17/Treg **(B)**, the secretion of IFN-γ and TNF-α in $CD8^+$T cells **(C)** were analyzed by flow cytometry. A p value of < 0.05 was considered significant. Data shown are representative of 3 independent experiments; $n = 5$ per group per experiment.

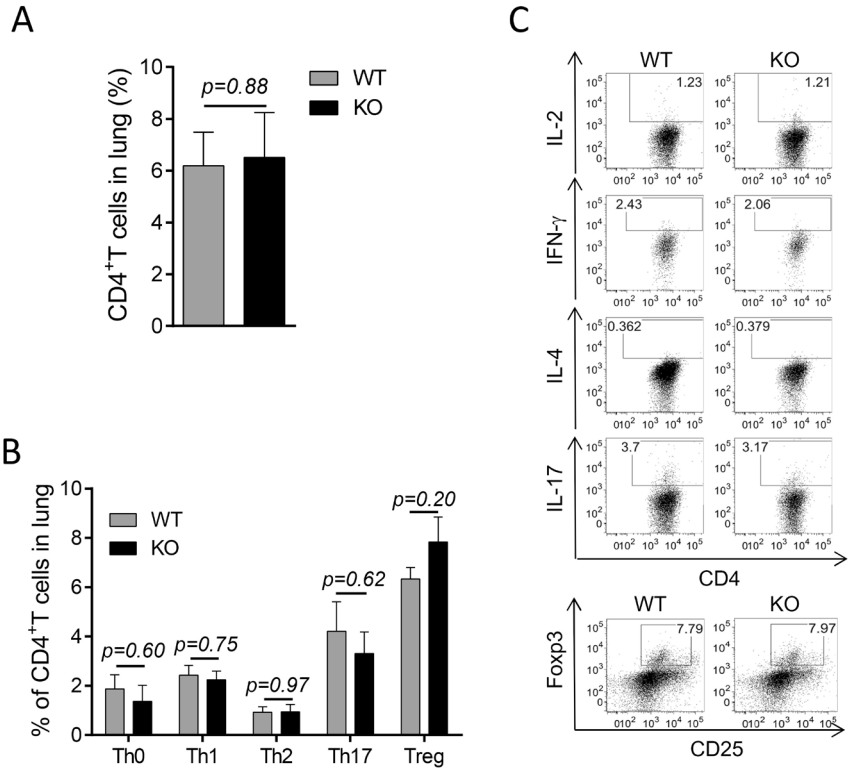

Figure 2. $CD4^+$T cell subsets in lungs of IAV-infected mice aren't significantly changed by absence of IL16. PR8 virus was intranasally inoculated into WT and IL16 KO mice at 8,000 PFU/mouse. The percentage of $CD4^+$T cells **(A)** and the percentage of Th0 ($CD4^+IL\text{-}2^+$), Th1 ($CD4^+IFN\text{-}\gamma^+$), Th2 ($CD4^+IL\text{-}4^+$), Th17 ($CD4^+IL\text{-}17^+$) and Treg ($CD4^+CD25^+Foxp3^+$) **(B** and **C)** in the lungs were detected by flow cytometry on day 7 post infection. A p value of < 0.05 was considered significant. Data shown are representative of 3 independent experiments; $n = 5$ per group per experiment.

the spleen after IAV infection.

3.3. *IL16 deficiency enhances CTL response against IAV*

Since Th1 assists $CD8^+$ T cell differentiation into CTLs, we next examined the CTL-secreted cytokines involved in anti-virus immune response. Flow cytometry revealed that $CD8^+$ T cells recruited in the lungs were reduced (Figure 4A), while IFN-γ-secreting $CD8^+$ T cells were increased in the lungs of IL16 KO mice (Figure 4B and C). Furthermore, $CD8^+$ T cells in the spleen were also decreased because of IL16 deficiency (Figure 5A), but the production of IFN-γ and TNF-α in $CD8^+$ T cells in the spleen of IL16 KO mice were almost twice as many as that of WT mice (Figure 5B and C). Taken together, these data suggest that IL16 deficiency enhanced the

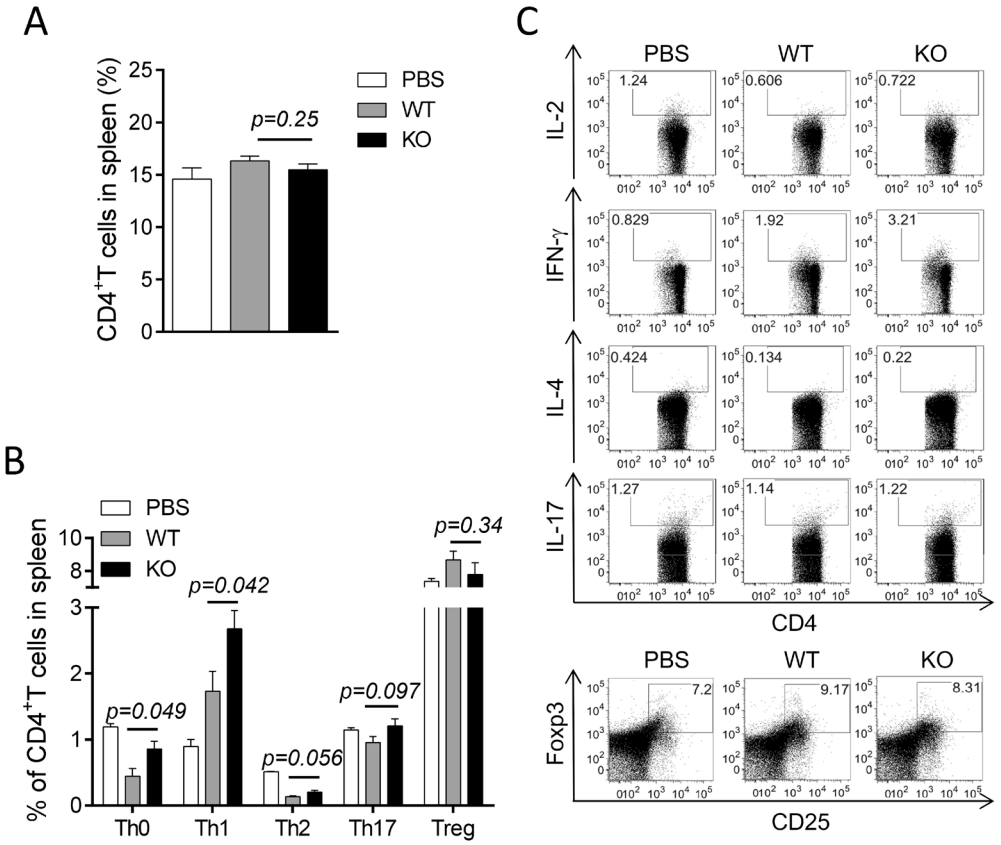

Figure 3. IL16 deficiency promotes Th1 polarization in spleens of IAV-infected mice. Seven-week-old male WT and IL16 KO mice were intranasally infected with PR8 virus, while control mice received mock inoculation with sterilized PBS. The percentage of $CD4^+$T cells **(A)** and the Th0/Th1/Th2/Th17/Treg subsets **(B** and **C)** in the spleens were detected by flow cytometry on day 7 after infection. A p value of < 0.05 was considered significant. Data shown are representative of 3 independent experiments; $n = 5$ per group per experiment.

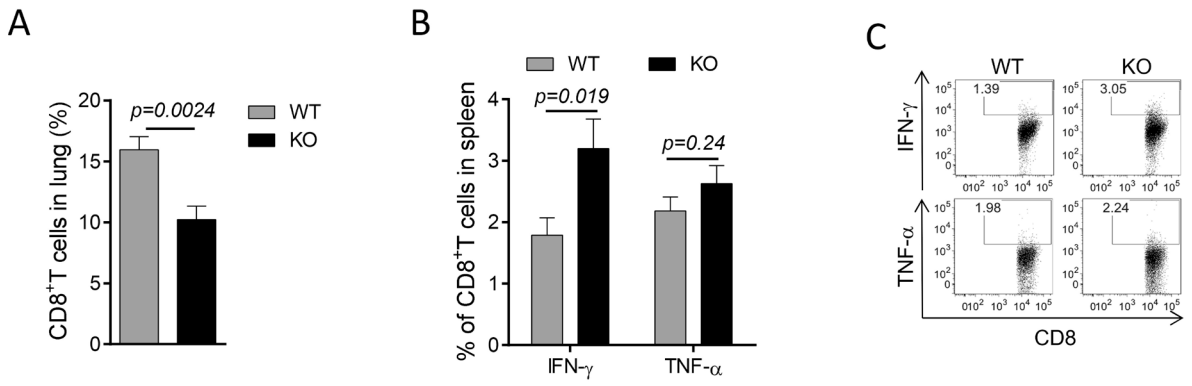

Figure 4. IFN-γ-secreting $CD8^+$T cells are increased in lungs of IL16 KO mice after infection. Mice were intranasally infected with PR8 virus. On day 7 after infection, the percentage of $CD8^+$T cells **(A)**, the IFN-γ-secreting and TNF-α-secreting $CD8^+$T cells **(B** and **C)** in the lungs were detected by flow cytometry respectively. A p value of < 0.05 was considered significant. Data shown are representative of 3 independent experiments; $n = 4$-5 per group per experiment.

CTL response after IAV infection.

3.4. *Absence of IL16 elevates DC maturation in IAV-infected mouse*

MHCII is mainly expressed on the surface of APCs and is upregulated as APCs become mature. With the involvement of the CD4 molecule and T cell receptor (TCR), MHCII on APCs was able to mediate $CD4^+$ T cell activation and differentiation (*27*). Since Th1 response in IL16 KO mice was increased after infection, we next detected the expression of MHCII on the surface of DCs. Flow cytometry analyses revealed that the percentage of lung DCs from IL16 KO mice was decreased (Figure 6A and B), while the MHCII expression on lung DCs was increased in IL16 KO mice (Figure 6C and D). These results indicate that IL16 deficiency promotes DC maturation in response to IAV infection.

4. Discussion

Continuous antigen drift and antigen shift of IAV enable it to evolve fast, which makes it very common for newly-derived strains to escape from host immunity and

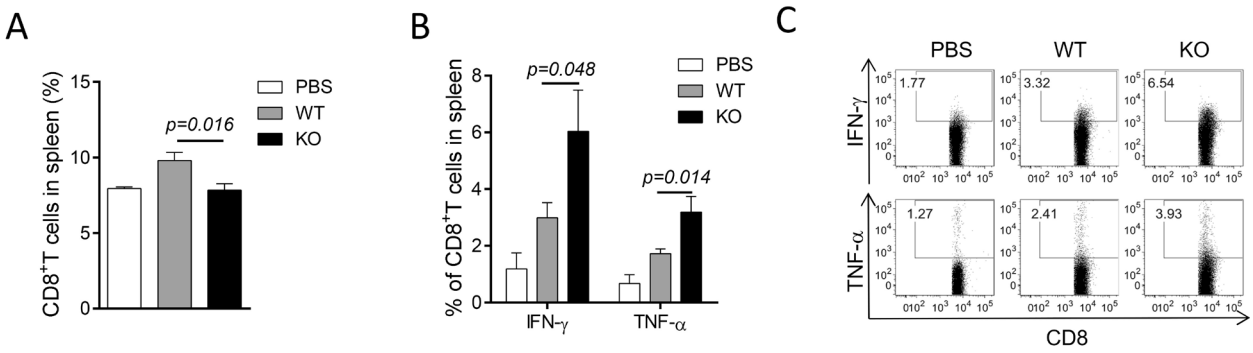

Figure 5. IL16 deficiency enhances both IFN-γ and TNF-α secretion of $CD8^+$T cells in spleens of IAV-infected mice. WT and IL16 KO mice were intranasally infected with PR8 virus and the percentage of $CD8^+$T cells **(A)**, the IFN-γ-secreting and TNF-α-secreting $CD8^+$T cells **(B** and **C)** in the spleens were detected by flow cytometry on day 7 after infection. A p value of < 0.05 was considered significant. Data shown are representative of 3 independent experiments; $n = 4$-5 per group per experiment.

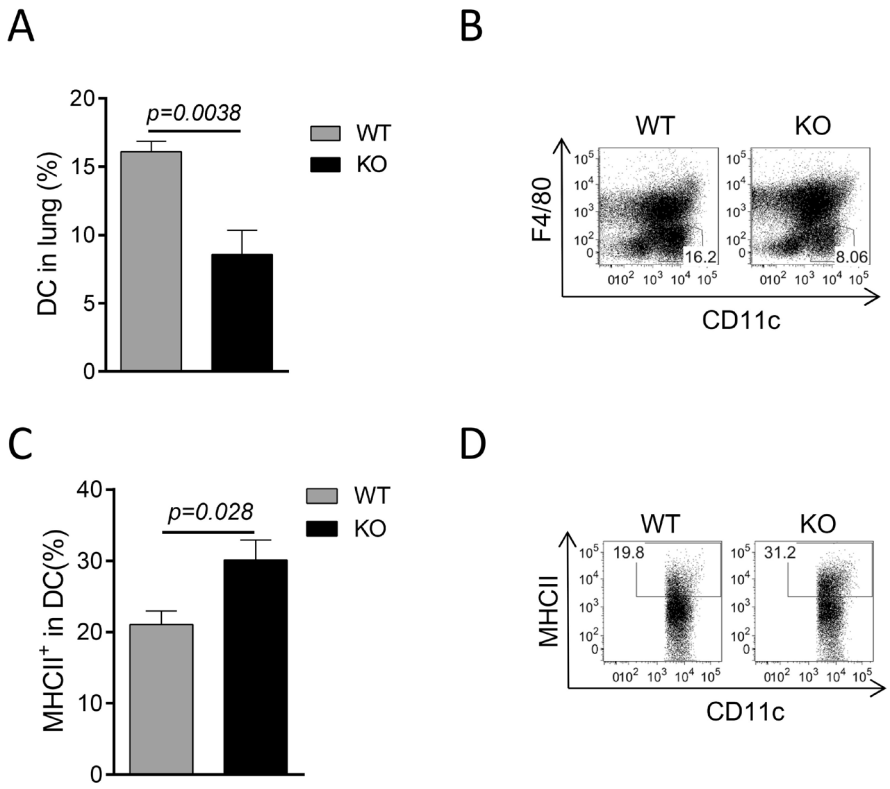

Figure 6. IL16 deficiency promotes DC maturation in lungs after IAV infection. WT and IL16 KO mice were infected with PR8 virus and the lungs were isolated on day 7 post infection. The percentage of DCs ($F4/80^-CD11c^+$) **(A** and **B)** and MHCII expression on the DCs **(C** and **D)** were detected by flow cytometry. A p value of < 0.05 was considered significant. Data shown are representative of 3 independent experiments; $n = 4$-5 per group per experiment.

result in flu pandemics and outbreaks (*28*). A thorough understanding of host factors involved in IAV infection might provide new treatment strategies or indicators for IAV-induced illness. In this study, we identified that IL16 deficiency promotes Th1 polarization and CTL response after IAV infection, in accordance with the relieved lung injury and decreased viral load we observed in IL16 KO mice in our unpublished data. Besides, we found that MHCII was elevated in lung DCs of IL16 KO mice, which might be associated with an elevated T cell response.

Th1 and CTL responses have vital roles in viral clearance and recovery of the host after influenza virus infection (*21,22*). As the two most important cytokines secreted by Th1 and CTLs, IFN-γ and TNF-α have attracted lots of attention in host anti-viral immune response. IFN-γ was first described in the year 1965 and has been tagged with an antiviral activity since then (*29*). Mounting evidence has revealed the link between IFN-γ and viral clearance (*26,30,31*). In the terrible outbreak of H7N9 in 2013, the critical role of IFN-γ was forcefully highlighted by the fact that the response of IFN-γ-producing $CD8^+$ T cells was enhanced in recovery patients compared to dead ones (*22*). TNF-α is traditionally regarded as a pro-inflammatory cytokine given its role in inflammatory disorders (*32,33*). However, TNF-α deficiency was reported to result in severe inflammatory infiltration and lung injury, together with enhanced inflammatory cytokine secretions like IL-1β, suggesting that TNF-α was required to control immunopathology rather than viral clearance (*34-36*). Thus we assume that the alleviated pneumonia in IL16-deficient mice might be correlated with increased TNF-α secreted from Th1 and CTLs. The mechanism by which TNF-α balances different effects on immunopathology under specific circumstances needs to be studied more in the future.

Differentiation of Th1 is mainly mediated by a T-box transcription factor, T-bet, which was first isolated from a Th1 cDNA library and considered as a Th1-specific transcription factor (*37*). It has been demonstrated that T-bet-deficient mice displayed severely impaired ability for IFN-γ secretion in $CD4^+$T cells and a marked reversion of Th1/Th2 balance, indicating that T-bet plays critical roles in IFN-γ secretion in Th1 (*38,39*). In the course of Th1 differentiation, IFN-γ/STAT1 and IL12/STAT4 are considered as the two main upstream pathways of T-bet. Upon T cell activation, IFN-γ/STAT1 signaling is supposed to initiate the first wave of T-bet expression, while IL12/STAT4 mainly participates in the second wave, which is critical in Th1 stabilization (*40,41*). Besides Th1, T-bet has also been reported to promote CTL activities by upregulating IFN-γ expression in $CD8^+$T cells, although the role of T-bet in $CD8^+$T cells was less dominant and clear than that in Th1(*40*). Since we observed elevated IFN-γ secretion both in Th1 and CTLs in IL16 KO mice, we assume that there might be some correlation between IL16 and T-bet signaling. Whether IL16 enhances Th1 polarization and CTL activity by directly interacting with T-bet signaling or by other unknown mechanisms requires further study.

To conclude, our data suggest a potential link between IL16 and IAV-specific cellular immunity, which might implicate IL16 as a supporting factor for IAV-related illness and propose new insight into the network of virus-related host factors. Moreover, understanding the mechanisms by which IL16 exerts an immunoregulatory effect on cellular immunity might bring new clues for possible therapeutic strategies against IAV-associated illness.

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