Cytokine 81 (2016) 23–27

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Interleukin-35 is upregulated in response to influenza virus infection and secondary bacterial pneumonia

Yi Chen [1], Chuan-jiang Wang [1], Shi-hui Lin, Mu Zhang, Sheng-yuan Li, Fang Xu *

Department of Emergency and Intensive Care Unit, The First Affiliated Hospital of Chongqing Medical University, Chongqing, China

ARTICLE INFO

Article history:
Received 10 November 2015
Received in revised form 24 January 2016
Accepted 25 January 2016
Available online 5 February 2016

Keywords:
IL-35
Influenza
Streptococcus pneumoniae
Pneumonia
Infection
Immune response

ABSTRACT

Postinfluenza pneumococcal pneumonia is an important cause of global morbidity and mortality. What causes this increased susceptibility is not well elucidated. IL-35 is a newly described cytokine in infectious tolerance. A murine model was established to study postinfluenza pneumococcal pneumonia and evaluate the role of IL-35 in host defense against postinfluenza pneumococcal pneumonia. Pulmonary IL-35 was rapidly up-regulated during murine influenza infection, which was partially mediated by type I IFN-α/β receptor signaling pathway. Secondary pneumococcal infection led to a synergistic IL-35 response in influenza-infected mice. Clinical analysis showed that IL-35 levels were significantly elevated in the patients with influenza infection compared with healthy individuals and influenza infection could induce IL-35 production from human peripheral blood mononuclear cells. These data suggest that IL-35 contributes to the increased susceptibility to secondary pneumococcal pneumonia at least in part by inhibiting the early immune response.

1. Introduction

Secondary bacterial pneumonia is an important complication responsible for illness and death during epidemic and pandemic influenza [1]. A number of causative bacteria have been described in patients, including *Staphylococcus aureus* and *Haemophilus influenza,* but *Streptococcus pneumoniae* is the most common pathogen, and over 50% of the deaths have been attributed to secondary pneumococcal pneumonia following primary influenza virus infection [2]. It is of clinical significance to understand the precise processes involved in the synergism between influenza virus and *S. pneumoniae*.

Several factors have been suggested to mediate the development of postinfluenza pneumococcal pneumonia. It has been commonly proposed that viral-induced epithelial cell damage could facilitate pneumococcal adherence and invasiveness [3]. Recent studies have also reported that influenza-induced dysfunctional innate immune responses played an indispensable role in the enhanced susceptibility to secondary pneumococcal infections [4]. In response to influenza virus infection, the host produces a variety of inflammatory mediators, including proinflammatory and immunoregulatory cytokines [5,6]. However, the role of these cytokines in the process of postinfluenza pneumococcal pneumonia is not ascertained, which hampers the development of effective prophylactic and therapeutic approaches.

IL-35 is a newly described anti-inflammatory cytokine, which is composed of the Epstein–Barr virus-induced gene 3 (EBI3) and the p35 subunit [7]. IL-35 plays regulatory roles in a variety of inflammatory diseases [8–16], however, its role in microbial infection is largely unknown. In this study, we have developed a murine model of postinfluenza pneumococcal pneumonia to investigate the expression of IL-35 upon secondary pneumococcal challenge after primary influenza infection. For the first time, we found elevated pulmonary IL-35 induced by influenza infection following secondary pneumococcal infection.

2. Materials and methods

2.1. Mice

C57BL/6 mice aged 6–8 weeks were obtained from and raised at Chongqing Medical University. IFNAR$^{-/-}$ mice raised on C57BL/6 background were purchased from The Jackson Laboratory. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the Chongqing Medical University.

* Corresponding author.
E-mail address: xufang828@126.com (F. Xu).
[1] Both Yi Chen and Chuan-jiang Wang contributed equally to this work.

http://dx.doi.org/10.1016/j.cyto.2016.01.016
1043-4666/

2.2. Influenza virus

Influenza virus strain A/PR/8/34 (H1N1, ATCC) was grown on MDCK cells. All viruses were harvested by a freeze/thaw cycle, followed by centrifugation at 680×*g* for 10 min. Supernatants were stored in aliquots at −80 °C. The viral titers of the stocks were characterized via median tissue culture-infective dose (TCID50) assay in MDCK cells.

2.3. Bacterial strains

Type 3 *S. pneumoniae* (ATCC 6303 clinical isolate with capsular serotype 3) were grown in Todd–Hewitt broth with yeast extract at 37 °C for 8 h or until log phase. The titers of the frozen stocks were quantitated on tryptic soy agar supplemented with 3% v/v sheep erythrocytes.

2.4. Experimental infection protocol

Mice were infected using an Inhalation Exposure System (Glass-Col, USA) for influenza virus infection, a dose of 200 PFU of influenza virus (in 100 μl sterile PBS) from a frozen stock or control PBS was given. Body weight and viral PFUs in lung homogenates of influenza-infected mice were then assessed. In secondary pneumococcal infection experiments, mice were anaesthetized with pentobarbital sodium intraperitoneally (i.p.) (30 mg/kg weight), and then 5000 CFU *S. pneumoniae* in 30 ml PBS was administered intranasally (i.n) into mice as described in previous studies [17], which mimicked the natural route of pneumococcal infection.

2.5. Clinical samples

Serum samples were collected from 30 healthy individuals and 33 patients who were confirmed influenza infection during 2009 H1N1 pandemic. This protocol was approved by the Clinical Research Ethics Committee of The First Affiliated Hospital of Chongqing Medical University, and informed consent was obtained from all participants according to the Declaration of Helsinki.

2.6. Cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat of healthy volunteers using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation as described in previous studies [18]. PBMCs were washed twice with PBS and resuspended in RPMI 1640 (Invitrogen).

2.7. Real-time PCR

Total cellular RNA was extracted from cells and organs with RNeasy columns (QIAGEN). Quantitative real-time PCR analysis for IL-35 EBI3 and IL-12 p35 was performed using specific Quantitect Primer/Probe assays (QIAGEN). HPRT or human β-actin was used as the reference gene.

2.8. ELISA

A murine IL-35 ELISA kit (BlueGene, Shanghai, China) was employed for analysis of IL-35 levels in the tissue supernatants, while a human IL-35 ELISA kits from eBioscience was used to determine IL-35 levels in the serum samples according to the manufacturer's instructions.

2.9. Statistical analysis

All data are expressed as the mean ± SD unless stated otherwise. Differences between groups of mice were analyzed using 2-tailed unpaired *t*-test, or 1-way ANOVA with Bonferroni's multiple comparison post hoc tests where appropriate. A p value of 0.05 or less was considered statistically significant.

3. Results

3.1. Influenza virus up-regulates early lung IL-35 expression

We investigated whether IL-35 transcripts are up-regulated in viral pulmonary infection. In our first attempt, we assessed the expression of EBI3 and p35 subunits of IL-35 in the lungs of mice infected with virus-associated molecular pattern, the synthetic double-stranded RNA poly(I:C). As expected, poly-ICLC treatment increased EBI3 and p35 mRNA levels in the lung of mice 6 h after infection (Fig. 1A). Further ELISA analysis confirmed that IL-35 protein production peaked on day 5 and persisted to day 12 (Fig. 1B).

Next, we examined whether infection with influenza virus has a similar effect on IL-35 expression as poly-ICLC treatment. We also found a significant increase in the relative mRNA expression of EBI3 and p35 in the virus-infected group, compared with non-infected control mice (Fig. 1C). IL-35-spcific ELISA also detected a strong up-regulation of IL-35 protein in the lungs (Fig. 1D).

Recently, it has been demonstrated that type I IFNs regulated anti-inflammatory cytokine IL-10 production [19]. Since early expression of type I IFNs is a molecular signature of influenza virus infection [20], we further investigated if influenza-induced IL-35 was regulated by type I IFNs. IFNAR-deficient mice after poly-ICLC or influenza infection had significantly less mRNA expression of EBI3 (Fig. 1E) and p35 (Fig. 1F), as well as decreased IL-35 protein production in the lungs compared with WT mice (Fig. 1G).

3.2. Secondary pneumococcal infection triggers synergistic production of IL-35 by influenza virus

Prior work has proposed that most secondary pneumococcal infection develops within the first 2 weeks after primary influenza infection, and maximal susceptibility to secondary pneumococcal challenge occurs between 5 and 7 days following primary influenza infection [21]. Thus, we established an infection model in which mice were challenged with *S. pneumoniae* at day 5 after influenza infection (Fig. 2A). In mice with prior influenza infection, a strikingly increase in pulmonary pneumococcal burden was detected at 48 h after secondary *S.pn challenge* (Fig. 2B). Similarly, markedly higher rates of bacteremia were noted in mice infected with combined influenza and *S.pn* compared with mice infected with *S.pn* alone (Fig. 2C). These differences in pulmonary and systemic pneumococcal loads were associated with increased mortality in virus/*S.pn*-infected mice when compared with *S.pn*-infected or virus-infected mice (Fig. 2D). Interestingly, in mice following *S.pn* challenge on day 5 after primary influenza infection, we found that secondary infected-mice had a synergistic increase in IL-35 production compared with mice infected with either *S.pn* or influenza virus alone (Fig. 2E).

3.3. Serum IL-35 levels are elevated in patients with influenza infection

To determine any clinical relevance translated from murine to human systems, we measured IL-35 levels in clinical samples. The serum levels of IL-35 in the patients with influenza infection were significantly elevated compared with healthy controls (Fig. 3A). In freshly isolated human PBMCs, both influenza infection