

Interleukin 12 is a primary cytokine responding to influenza virus infection in the respiratory tract of mice

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Summary. – We have reported previously that an increase in interleukin 12 (IL-12) production in the lungs of mice infected with Influenza A virus or an intranasal (i.n.) administration of IL-12 to the infected mice alleviated pneumonia (Tsurita *et al.*, *J. Pharmacol. Exp. Therapeut.* 298, 362–368, 2001). In this study, we found that in the bronchoalveolar lavage fluids (BALF) obtained from mice infected i.n. with Influenza A virus IL-12 was elevated on day 1 post infection (p.i.) and was followed by tumor necrosis factor α (TNF- α), IL-18, and interferons α , β , and γ (IFN- α , - β , and - γ) on day 2 p.i. Histochemical analyses of the infected lungs on day 1 p.i. showed the presence of IL-12 and IL-12 mRNA in mononuclear and macrophage-like cells and colocalization of macrophages with viral antigen, while other cytokines were absent. Thus, IL-12 was produced by macrophages infiltrating the infected epithelium as the first response cytokine and its production at the site of infection may direct an early immune defense to alleviate the severity of infection.

Keywords: Influenza A virus; interleukin 12; cytokines; macrophages; mice; pneumonia

Introduction

In the influenza infection cytokines are produced locally and systemically and their production is important in promoting host-immune defense to alleviate symptoms especially in the early stage of infection (Hayden *et al.*, 1998; Kaiser *et al.*, 2001; Cheung *et al.*, 2002; Seo *et al.*, 2002; Van Reeth, 2002). In a murine model, cytokines such as IL-12 and IL-18 as Th1 immune-response mediators, IFN- γ as a Th1 cytokine, IL-4 and IL-10 as Th2 cytokines, IL-1 α , IL-1 β , IL-6, and TNF- α as inflammatory cytokines, and IFN- α , - β have been shown to be produced in the respiratory tract during infection (Hennet *et al.*, 1992; Kurokawa *et al.*, 1996a,b, 2002; Tsurita *et al.*, 2001; Liu *et al.*, 2004).

The timely augmentation of IL-12 and IFN- γ levels in the airways of mice has been shown to alleviate pneumonia in the early phase of influenza infection (Tsurita *et al.*, 2001; Kurokawa *et al.*, 2002). IL-12 is a strong inducer of IFN- γ and is important in the development of Th1 cells followed by the generation of cell-mediated immunity contributing to the elimination of influenza virus (Monteiro *et al.*, 1998). IL-18, a novel cytokine with potent IFN- γ -inducing activity, plays an important role in NK cell activity and the Th1-mediated immune response in collaboration with IL-12 (Kaiser *et al.*, 2001; Tsurita *et al.*, 2001; Neff-LaFord *et al.*, 2003; Liu *et al.*, 2004). Kaiser *et al.* (2001) reported that the magnitude of the early decrease in viral titers correlated with initial levels of IFN- γ in nasopharyngeal lavage fluid of human volunteers. IFN- γ is suggested to be the critical cytokine in the cytokine cascade due to the influenza infection as well as to be responsible for viral clearance in humans. TNF- α , IFN- α , and IFN- β are also known to be produced locally in the early phase of influenza infection. Like IFN- γ they were reported to

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Abbreviations: BALF = bronchoalveolar lavage fluid; IFN = interferon; IL = interleukin; i.n. = intranasal; MAb(s) = monoclonal antibody(ies); p.i. = post infection; TNF = tumor necrosis factor

have potent antiviral properties for the influenza virus and responsibility for the pathogenesis of influenza infection through reduction of virus yield (Cheung *et al.*, 2002; Seo and Webster, 2002; Neff-LaFord *et al.*, 2003; Szretter *et al.*, 2007). Thus, IL-12, IL-18, TNF- α , and IFN- α , - β , - γ are produced in the early phase of influenza infection and may direct early host immunity against the influenza infection. However, an order which they are produced after influenza infection and how they contribute to early host-immune defense against influenza infection is unclear.

In this study, we pursued the production kinetics of IL-12, IL-18, TNF- α , and IFN- α , - β , - γ and viral antigen in BALF from the lungs of Influenza A virus-infected mice using ELISA, hybridization *in situ*, and immunohistochemical methods. We found that the production of IL-12 preceded that of other cytokines. Thus, the primacy of IL-12 production in the cytokine cascade was confirmed and as a result, it was suggested that IL-12 directed the early host immune defense against Influenza A virus infection.

Materials and Methods

Virus and cells. A mouse-adapted Influenza virus [A/PR/8/34 (H1N1)] was propagated in the lungs of mice and stored as described previously (Kurokawa *et al.*, 1996a,b, 1998, 2002; Furuta *et al.*, 2002). MDCK cells were grown and maintained in MEM supplemented with 5% or 2% heat-inactivated calf serum.

Mice. Female DBA/2 Cr mice (7-week-old, 19–21 g) were purchased from Japan SLC Inc. The mice were housed by 5 animals/cage in specific-pathogen-free conditions under a 12 hr light/dark cycle. The temperature in the room was kept at $23 \pm 2^\circ\text{C}$. The experimental protocols were approved by the Animal Experiment Committee of University of Toyama and Kyushu University of Health and Welfare, Japan, and their animal experimentation guidelines were followed in the animal studies.

Mice were infected i.n. with 10^3 PFU/25 μl of influenza virus or mock-infected with PBS under anesthesia (Kurokawa *et al.*, 1996b, 2002). The body weight of infected mice was measured daily.

BALF preparation. BALF was prepared from 4 to 6 mice on days 1 to 4 p.i. and the supernatant after centrifugation was stored at -80°C (Kurokawa *et al.*, 1996a,b, 1998, 2002).

Virus titration. Virus titers in BALF from influenza virus-infected mice were examined on days 1 to 4 p.i. Virus titers of the stored supernatant of BALF were determined by the plaque assay as described previously (Kurokawa *et al.*, 1996a,b, 2002).

ELISA of cytokines. The levels of cytokines were examined in BALF on days 1 to 4 p.i. IL-12, IL-18, IFN- γ , and IFN- α , - β levels in BALF were determined using ELISA kits (Amersham, Medical & Biological Laboratories, BioSource International, PBL Biomedical Laboratories, respectively) according to the manufacturers' instructions. The level of IL-12 was measured as the level of IL-12 p70 that was biologically active.

Immunohistochemical analyses. The lungs of infected mice were removed under anesthesia from at least 3 mice in each group

on days 1 and 2 p.i. and fixed for hematoxylin and eosin staining or rapidly frozen in liquid nitrogen for immunohistochemical examination as described previously (Imakita *et al.*, 2000; Kamiyama *et al.*, 2001; Fukuda *et al.*, 2003). Briefly, 6 μm thick serial sections of the frozen lungs were dried at room temperature, fixed with cold acetone, and then washed with TBS. Endogenous peroxidase, biotin, and alkaline phosphatase were blocked by treatment with 0.3% H_2O_2 in methanol, Biotin Blocking System or Levamisole (both Dako), respectively. After blocking with blocking solution (10% rabbit or goat normal serum, 2% BSA, 0.1% Triton-X 100 in TBS), the sections were incubated with the primary antibodies; rat anti-mouse MAbs to IL-12 (p40/70) (1:25, BD Bioscience Pharmingen), IFN- γ (1:25, BD Bioscience Pharmingen), F4/80 antigen of mature tissue macrophages (1:200, Serotec), and IL-18 (1:50, MBL), and MAb to influenza nucleoprotein (NP) (1:15, Fujita Health University). The sections treated with antibodies to IL-12, IFN- γ , IL-18, or F4/80 antigen were incubated with biotinylated rabbit anti-rat IgG (Dako), developed in streptavidin/alkaline phosphatase or a horseradish peroxidase complex (Dako) with the BCIP/NBT or DAB substrate-chromogen system (Dako), and counterstained with nuclear fast red or Mayer's hematoxylin. The sections with antibody to influenza NP were incubated with peroxidase-conjugated goat anti-rat IgG (Jackson ImmunoResearch Lab) at room temperature for 1 hr, and the DAB substrate-chromogen system (Dako) was used for visualization of the immune reaction.

In situ hybridization was performed to detect IL-12 mRNA using the digoxigenin-labeled murine IL-12 p40 DNA probe. Mice were perfused with 0.1 mol/l phosphate buffer (pH 7.4) containing 4% paraformaldehyde and the lungs were rapidly frozen in liquid nitrogen as described by Kamiyama *et al.* (2001). Briefly, the frozen sections (6 μm thick) were fixed with 4% paraformaldehyde in PBS and were treated with 0.2% Triton-X 100 and 10 $\mu\text{g}/\text{ml}$ proteinase K. They were again fixed with 4% paraformaldehyde and immersed in 2 mg/ml glycine sequentially dehydrated with ethanol and dried. The IL-12 probe was prepared from total RNA of murine spleen by reverse PCR (Kurokawa *et al.*, 2003). The digoxigenin-labeled murine IL-12 p40 DNA probe was generated as a 497-bp fragment by PCR using a DIG DNA Labeling Mix (Roche) with the forward primer 5'-TCTCTGTCTGCAGAGAAGGTCACTGGAC-3' and reverse primer 5'-CTAGGATCGGACCCTGCAGGGAACA ATGC-3'. The sections were pre-hybridized with 50% formamide in 5x SSC and then hybridized with denatured digoxigenin-labeled probes at 46°C overnight (Kamiyama *et al.*, 2001; Fukuda *et al.*, 2003). To detect bound digoxigenin-labeled probes, the sections were blocked with blocking solution [1% blocking buffer (Roche Applied Science), 0.1% Triton-X 100 in 0.1 mol/l Tris-HCl, 0.15 mol/l NaCl (pH 7.5)] at room temperature for 30 mins. They were incubated for 2 hrs with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche). After washing 3x with TBS, the reaction was developed with BCIP/NBT (Dako). Hybridization without DIG-labeled probes and with RNase-treated sections was used to confirm the absence of positively-stained IL-12 mRNA.

Statistical analysis. Student's *t*-test was used to evaluate the significance of the differences in mean cytokine levels on the days examined. $P < 0.05$ indicated statistical significance.

Results

Levels of cytokines in BALF from virus-infected mice

The kinetics of IL-12, IL-18, TNF- α , and IFN- α , - β , - γ production and virus titer were examined in BALF of infected mice on days 1–4 p.i. (Fig. 1, 2). Since the amount of BALF from a single mouse was not sufficient for simultaneous multiple cytokine assays, we could not examine the levels of 6 cytokines and a virus titer simultaneously. Therefore, the levels of IL-12, IL-18, IFN- γ , and virus titer (Fig. 1) and the levels of IL-12, TNF- α , and IFN- α , - β (Fig. 2) were obtained from two independent experiments using the same infection condition. On day 1 p.i. the virus titer in BALF was 197x higher than the inoculation titer and reached its maximal level on day 3 p.i. (Fig. 1). The body weight of infected mice decreased markedly from day 2 p.i. (data not shown). The productions of IL-12, IFN- γ , and IFN- β reached their maximal levels on day 2 p.i. and the maximal production of IL-18, TNF- α , and IFN- α was reached on day 3 p.i.. The production of all cytokines examined decreased on day

4 p.i. (Fig. 1, 2). The kinetics of production was similar to the profile of virus titer. However, on day 1 p.i. only IL-12 production was significantly increased as compared with the control and its increase on day 1 p.i. was confirmed in the two separate experiments. However, no significant increase of the other cytokine levels was observed on day 1 p.i. Thus, IL-12 production in the respiratory tract of infected mice preceded the production of IL-18, TNF- α , and IFN- α , - β , - γ .

Distribution of cytokines in the lungs of virus-infected mice

Because IL-12 was characterized as the first cytokine in response to influenza infection in the bronchial epithelium, the production and distribution of IL-12 was analyzed histochemically. Hematoxylin and eosin staining of lungs of influenza virus-infected mice on day 1 p.i. showed that mononuclear cells infiltrated mainly the fibrous connective tissues beneath the bronchiolar smooth muscle coat and partly into the alveolar septi (Fig. 3). Influenza antigen was observed focally in the bronchiolar epithelium and beneath

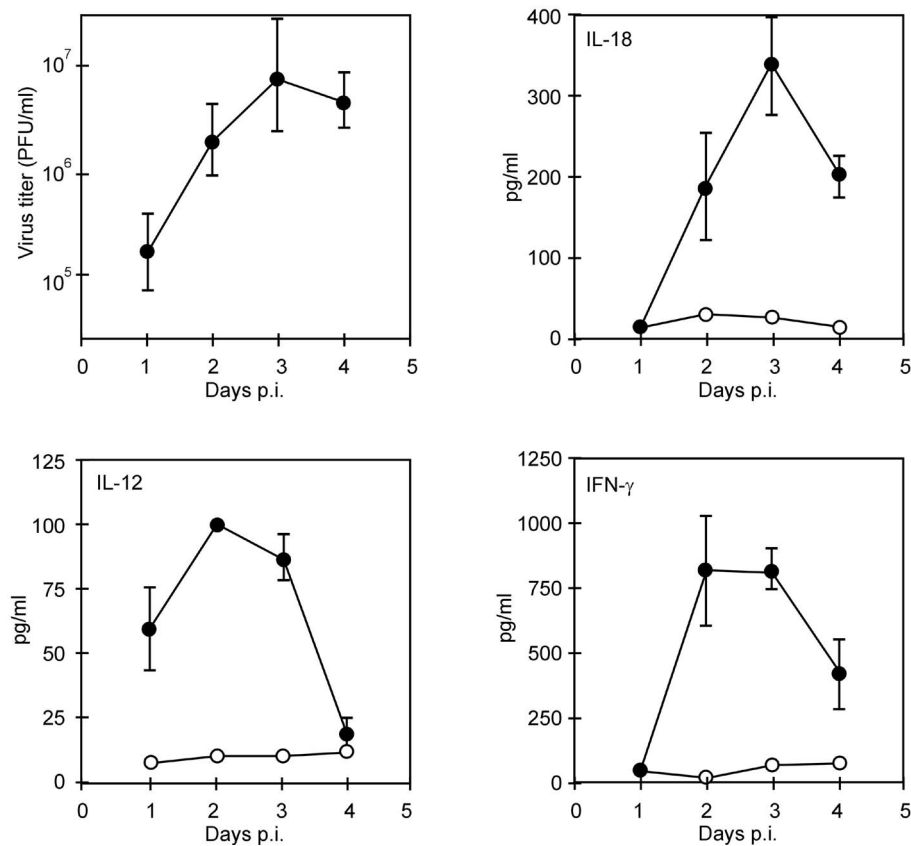


Fig. 1

Kinetics of virus titer and levels of IL-12, IL-18, and IFN- γ in BALF from infected (full circles) and mock-infected (empty circles) mice

Mean \pm SE values from 4–7 mice.

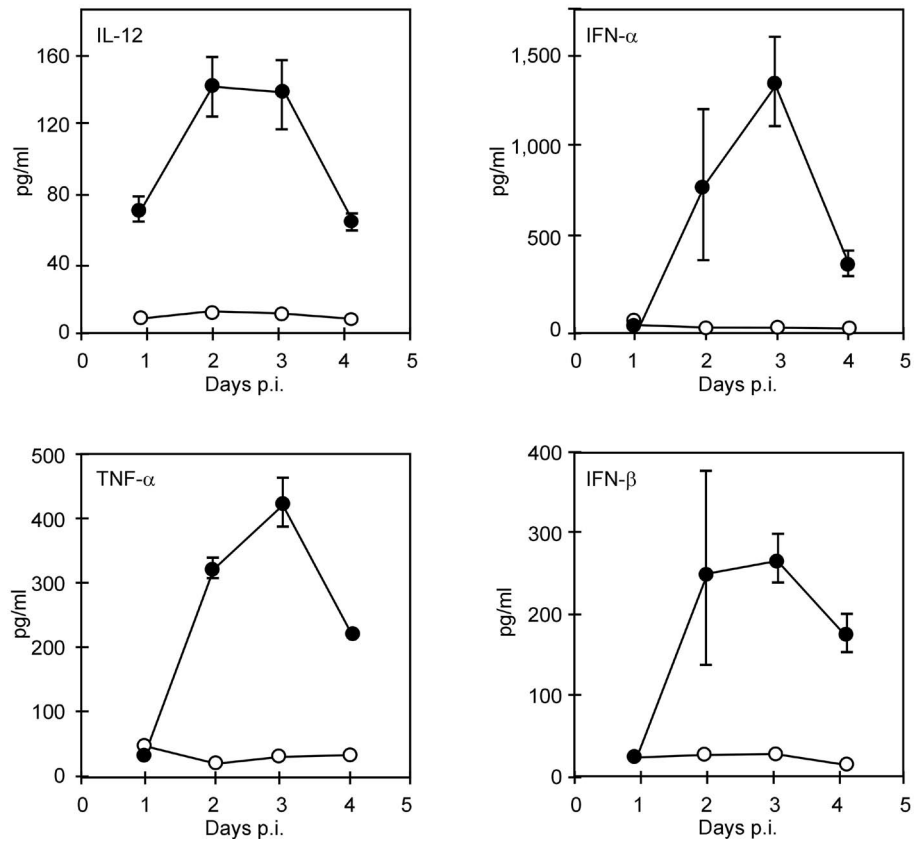


Fig. 2

Kinetics of levels of IL-12, TNF- α , IFN- α , and IFN- β in BALF from infected (full circles) and mock-infected (empty circles) mice
Mean \pm SE values from 4–6 mice.

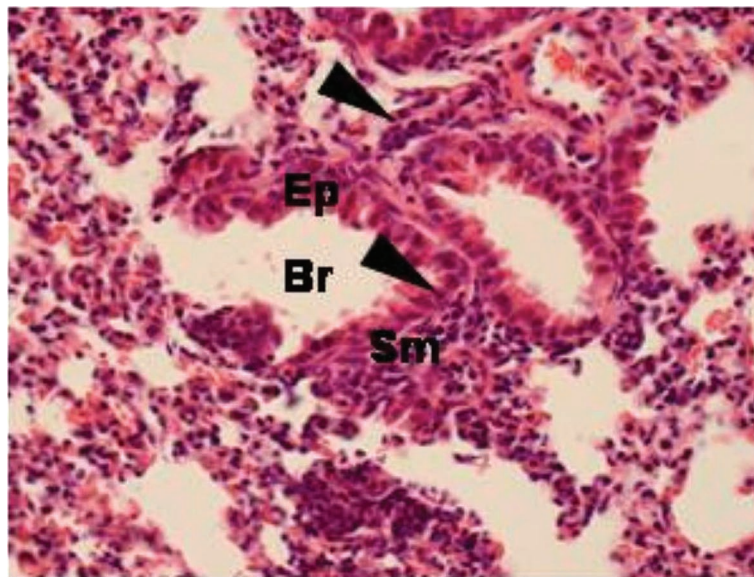


Fig. 3

Histology of the lungs of infected mice on day 1 p.i.

Hematoxylin and eosin staining. Macrophages (arrows), bronchioles (Br), epithelial cells (Ep), smooth muscle cells (Sm).

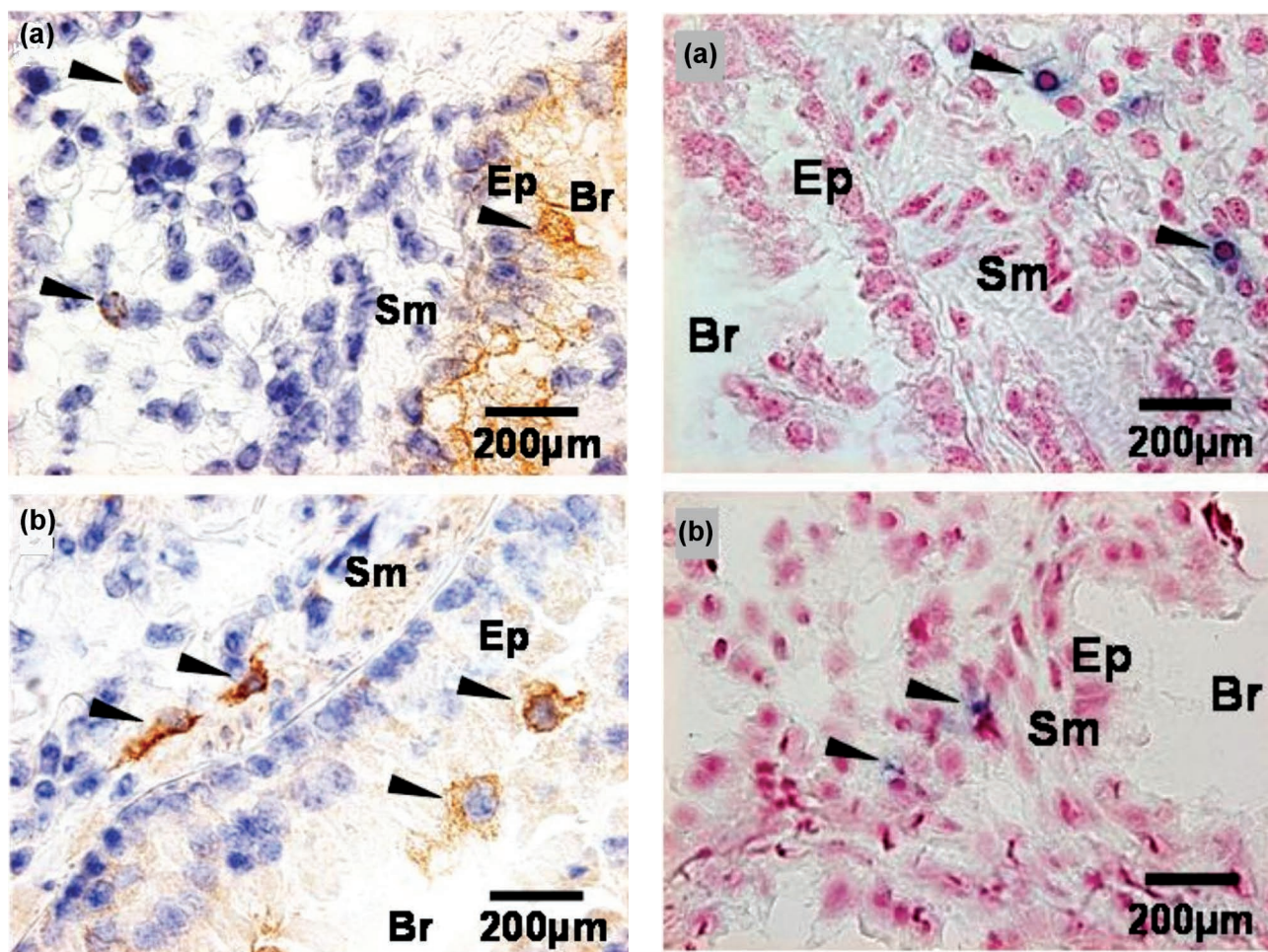


Fig. 4

Localization of viral antigen (a) and macrophages (b) in the lungs of infected mice on day 1 p.i.

Immunohistochemical staining. Viral antigen and macrophages (arrows), bronchioles (Br), epithelial cells (Ep), smooth muscle cells (Sm).

the bronchiolar smooth muscle coat (Fig. 4a). Macrophages were localized beneath the bronchiolar smooth muscle coat and partly in the bronchioles (Fig. 4b). IL-12 staining was observed on the mononuclear infiltrates beneath the smooth muscle coat in the lungs of infected mice (Fig. 5a), but not in the lungs of mock-infected mice (results not shown). We also detected IL-12 mRNA in the sub-epithelial area near the smooth muscle coat (Fig. 5b). IL-12 mRNA was identified on the macrophage-like cells by the absence of staining after RNase treatment (results not shown). IL-12 as well as influenza antigen and macrophages were localized around the smooth muscle coat near the infected epithelial cells. On day 2 p.i., diffuse IL-12 staining was observed in the sub-epithelial area of bronchioles that was the site of smooth muscle

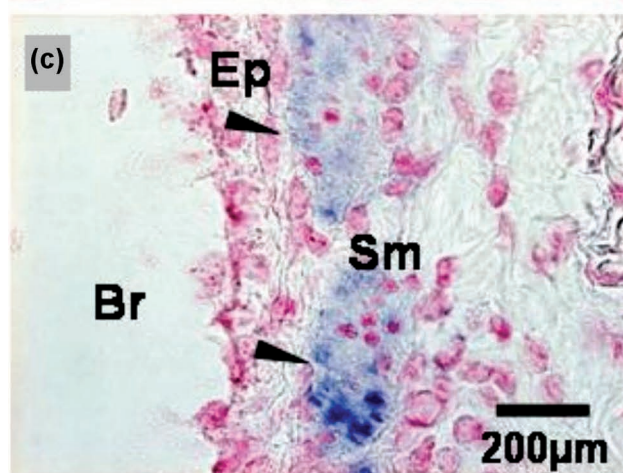


Fig. 5

Localization of IL-12 on days 1 (a) and 2 (c) p.i. and that of IL-12 mRNA on day 1 p.i. (b) in the lungs of infected mice

Immunohistochemical staining. IL-12 (arrows), bronchioles (Br), epithelial cells (Ep), smooth muscle cells (Sm).

coat (Fig. 5c). No IL-18 or IFN- γ staining was detected in the infected lungs on day 1 p.i. (results not shown). On day 2 p.i., IL-18 staining was diffusely observed on the epithelial cells and possibly on desquamated epithelial cells in the infected bronchioles (results not shown). IFN- γ staining was observed focally and/or densely in the interstitial area on day 2 p.i. and then on a wide range of pulmonary parenchyma including bronchioles on day 3 p.i. (results not shown). The dominance of IL-12 production in the infected lungs on day 1 p.i. was immunohistochemically confirmed.

Discussion

In the murine influenza infection model, the production of various kinds of cytokines in the respiratory tract has been shown (Hennet *et al.*, 1992; Kurokawa *et al.*, 1996a,b, 2002; Tsurita *et al.*, 2001; Liu *et al.*, 2004). IL-12, IL-18, TNF- α , and IFN- α , - β , - γ were reported to be produced in the early phase of influenza infection and suggested to direct an early host immunity against the influenza infection (Monteiro *et al.*, 1998; Tsurita *et al.*, 2001; Cheung *et al.*, 2002; Kurokawa *et al.*, 2002; Seo and Webster, 2002; Liu *et al.*, 2004; Neff-LaFord *et al.*, 2003; Szretter *et al.*, 2007). We previously showed that augmentation of IL-12 production or intranasal IL-12 administration in the early phase of influenza infection was effective in alleviation of influenza pneumonia in mice (Tsurita *et al.*, 2001; Kurokawa *et al.*, 2002). IL-12 is suggested to be an important immune mediator that contributes to the early host-defense immunity against influenza infection resulting in the alleviation of influenza pneumonia. Thus, in this study we focused on IL-12 production in the airways of influenza virus-infected mice and characterized the mode of IL-12 production in a series of cytokine production in response to the influenza infection.

The production of IL-12 on day 1 p.i. was confirmed in the sub-epithelial area near the bronchiolar smooth muscle coat of lungs. However, the kinetics of cytokine production in the early phase of influenza infection has not been demonstrated clearly. Thus, the finding in this study is probably crucial to understanding the interactions between influenza virus infection and the host immune system including timing and localization of the host defense in influenza infection.

In immunohistochemical analyses, we did not observe IL-18 and IFN- γ staining in the infected lungs on day 1 p.i., but did observe the presence of IL-12. Thus, IL-12 production was dominant in the BALF from influenza virus-infected mice on day 1 p.i. In mice, IL-12 contributes to early control of several virus infections including that of Influenza A virus (Monteiro *et al.*, 1998). However, some viruses such as measles virus and Human immunodeficiency virus have been reported to selectively inhibit IL-12 production and induce an immunosuppression (Karp *et al.*, 1996; Braun *et al.*, 2001). The increase of

IL-12 production prior to that of the other cytokines examined in this study suggested that IL-12 played a significant role in priming the cytokine cascade in the influenza infection. In our previous studies, clarithromycin and Kakkon-to, a medicinal herb, augmented IL-12 production on day 2 p.i. in BALF and reduced mortality by alleviating the pneumonia (Tsurita *et al.*, 2001; Kurokawa *et al.*, 2002). Nasal administration of IL-12 on day 2 p.i. only reduced the virus titer in BALF (Tsurita *et al.*, 2001). These results indicated that elevated production of IL-12 on day 2 p.i. might contribute to the reduction of virus titer and alleviation of pneumonia. Thus, elevated IL-12 production before it reached the maximal level observed on day 2 p.i. may be necessary in successful early immunity for the alleviation of influenza symptoms.

Influenza antigen was observed mainly in the bronchiolar epithelium and faintly beneath the bronchiolar smooth muscle coat in the lungs of influenza virus-infected mice on day 1 p.i. By this time, the macrophages were observed beneath the bronchiolar smooth muscle coat and in the bronchioles, and IL-12 staining was observed mostly beneath the bronchiolar smooth muscle coat. We could not exactly verify that macrophages produce IL-12 in the serial sections of the frozen lungs because of weak IL-12 staining, but the distribution of IL-12 staining and macrophages closely overlapped beneath the bronchiolar smooth muscle coat suggesting that macrophages activated by influenza virus antigens produced mainly IL-12. While IL-18, TNF- α , and IFN- α , - β are produced not only by macrophages but also by non-immune cells such as epithelial cells, IL-12 is mainly produced by macrophages, monocytes, and dendritic cells (D'Andrea *et al.*, 1992; Cameron *et al.*, 1999; Locksley *et al.*, 2001; Pirhonen *et al.*, 2001; Kashiwamura *et al.*, 2002; Pirhonen *et al.*, 2002; Seo and Webster, 2002). Thus, the macrophage may be a key immune cell to induce early defense-immunity against influenza infection and IL-12 is dominantly produced in macrophages activated by influenza virus antigens.

The distribution of IL-12 staining in the sub-epithelial areas on day 2 p.i. was diffuse along the smooth muscle coat of the bronchioles. In comparison to the distribution of IL-12 staining on day 1 p.i., the increased IL-12 production on day 2 p.i. detected in the kinetic analysis was immunohistochemically confirmed. The production of IL-18, TNF- α , and IFN- α , - β , - γ also markedly increased in BALF from infected mice on day 2 p.i. Thus, the cooperative and synergistic action of cytokines induced by influenza infection was probably augmented from day 2 p.i. in examined mice model. The present study showed that IL-12 was produced around the infected area of lungs and its production was important in priming of the early immune defense to combat influenza infection.

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