Enhanced viral clearance in interleukin-18 gene-deficient mice after pulmonary infection with influenza A virus

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Summary

T helper 1 driven immune responses facilitate host defence during viral infections. Because interleukin-18 (IL-18) mediates T helper 1 driven immune responses, and since mature IL-18 is up-regulated in human macrophages after influenza virus infection *in vitro*, it has been suggested that IL-18 plays an important role in the immune response to influenza. To determine the role of IL-18 in respiratory tract infection with influenza, IL-18 gene-deficient (IL-18$^{-/-}$) and normal wildtype mice were intranasally inoculated with influenza A virus. Influenza resulted in an increase in constitutively expressed IL-18 in the lungs of wildtype mice. The clearance of influenza A was inhibited by IL-18, as indicated by reduced viral loads on day 8 and day 12 after infection in IL-18$^{-/-}$ mice. This enhanced viral clearance correlated with increased CD4^{+} T-cell activation in the lungs as reflected by CD69 expression on the cell surface. Surprisingly, interferon-γ (IFN-γ) levels were similar in the lungs of IL-18$^{-/-}$ mice and wildtype mice. Intracellular IFN-γ staining revealed similar expression levels in lung-derived natural killer cells, CD4^{+} and CD8^{+} T cells, indicating that IFN-γ production is IL-18-independent during influenza virus infection. Tumour necrosis factor-α production by CD4^{+} T cells was significantly lower in IL-18$^{-/-}$ mice than in wildtype mice. Our data indicate that endogenous IL-18 impairs viral clearance during influenza A infection.

Keywords: influenza; lung; cytokine; inflammation

Introduction

Respiratory tract infection with influenza A virus is associated with fever, chills, cough, sore throat, headache, general malaise and sometimes nausea and myalgia and may even lead to pneumonia.[1,2] Host defence against influenza virus infection is orchestrated by a complex interaction between immune-regulatory cells and soluble mediators such as cytokines and chemokines. Cellular immunity against respiratory tract infection with influenza virus is mediated by antigen presentation by macrophages and dendritic cells.[3] Cytokines and chemokines produced by virus-infected cells facilitate the immune response against influenza virus.[4]

Interleukin (IL)-18 is a monomeric cytokine, which is first synthesized as an inactive precursor protein, pro-IL-18, and released upon cleavage by caspase-1.[5,6] IL-18 has been shown to induce proliferation and differentiation of T cells towards a T helper 1 (Th1) response and is an important cofactor in IL-12 induced interferon-γ (IFN-γ) production by natural killer (NK) and T cells.[7] In addition, IL-18 has been shown to exert proinflammatory effects on macrophages and neutrophils, making this cytokine a pleiotropic pro-inflammatory mediator.[8,9] Whereas the central role of IL-18 in protective immunity against bacterial infections has been well established[5] the contribution of IL-18 in the host response to viral infection is less

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clear. Administration of IL-18 induced antiviral activity in mice infected with encephalomyocarditis[10] and vaccinia virus[11] and reduced mortality in mice with herpes simplex virus type 1 infection.[12] Recent studies have implicated IL-18 in the host response to influenza A infection. IL-18 has been shown to be up-regulated in human alveolar macrophages during influenza virus infection.[13,14] Furthermore, IL-18 gene deficient mice (IL-18$^{-/-}$ mice) displayed an impaired clearance of neurovirulent influenza A virus-infected neurons from the brain after intracerebral infection.[15] However, influenza A most commonly infects the airways. Therefore, in the present study we sought to determine the role of IL-18 respiratory tract infection with influenza A. Of note, while our studies were in progress Liu *et al.* reported that endogenous IL-18 improved early host defence against influenza A airway infection by augmenting NK cell mediated cytotoxicity.[16]

Materials and methods

Animals

All experiments were approved by the Institutional Animal Care and Use Committee of the Academic Medical Center of the University of Amsterdam. IL-18$^{-/-}$ mice were generated as described previously.[17] IL-18$^{-/-}$ mice were on the C57BL/6 background. Normal C57BL/6 mice, used as controls for IL-18$^{-/-}$ mice, were obtained from Harlan Sprague–Dawley (Horst, the Netherlands). Sex- and age-matched (8-week-old) mice were used in all experiments.

Experimental virus infection

Influenza A/PR/8/34 (ATCC no. VR-95; Rockville, MD) was grown on LLC-MK2 cells (RIVM, Bilthoven, Netherlands). Virus was harvested by a freeze/thaw cycle, followed by centrifugation at 680 ***g*** for 10 min. Supernatants were stored in aliquots at −80°. Titration was performed in LLC-MK2 cells to calculate the median tissue culture infective dose ($TCID_{50}$) of the viral stock.[18] A non-infected cell culture was used for preparation of the control inoculum. None of the stocks were contaminated by other respiratory viruses, i.e. influenza B, human parainfluenza type 1, 2, 3, 4 A and 4B, RSV A and B, rhinovirus, enterovirus, corona virus and adenovirus, as determined by polymerase chain reaction (PCR) or cell culture. Viral stock was diluted just before use in phosphate-buffered saline (PBS, pH 7·4). Mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Quenborough, UK) and intranasally inoculated with 10 $TCID_{50}$ influenza (1400 viral copies) or control in a final volume of 50 μl phosphate-buffered saline (PBS). Additionally, in a separate survival study mice were infected with 200 $TCID_{50}$ influenza A.

Determination of IL-18 and pro-IL-18 levels in the lung

Mice (six to eight mice per group) were anaesthetized using 0·3 ml FFM (fentanyl citrate 0·079 mg/ml, fluanisone 2·5 mg/ml, midazolam 1·25 mg/ml in H_2O; of this mixture 7·0 ml/kg intraperitoneally) and killed by bleeding out the inferior vena cava. Lungs were harvested and homogenized at 4° in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Lung homogenates were lysed with an equal volume of lysis buffer (300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% (v/v) Triton-X-100, 20 ng/ml pepstatin A, 20 ng/ml leupeptin, 20 ng/ml aprotinin, pH 7·4) and incubated for 30 min on ice, followed by centrifugation at 680 ***g*** for 10 min. Supernatants were stored at −80° until further use. IL-18 and pro-IL-18 levels were determined in these homogenates as described previously.[19,20] In brief, pooled samples of eight mice per timepoint were reduced with sodium dodecyl sulphate (SDS) sample buffer containing 20% (v/v) β-mercaptoethanol and denatured for 5 min at 95°, of which 20 μl was separated on a SDS–polyacrylamide gel (15%) and subsequently transferred to a polyvinylidene fluoride membrane. Non-specific binding sites on the membrane were blocked by incubation in PBST buffer (PBS with 0·05% (v/v) Tween-20 containing 2% (w/v) non-fat dry milk at 4° overnight followed by incubation with primary antibody, i.e. 3 μg of purified rat anti-mouse IL-18 monoclonal antibody (mAb; R & D Systems, Abingdon, UK) for 1 hr at room temperature. After three washes with PBST buffer containing 0·2% (w/v) non-fat dry milk, the membrane was incubated with peroxidase-conjugated rabbit anti-rat immunoglobulin G (IgG) antibodies (P0450; DAKO, Glostrup, Denmark) in a 1/2000 dilution in PBST buffer containing 0·2% (w/v) non-fat dry milk at room temperature. After washing, the IL-18 bands were visualized using Lumilight plus ECL substrate (Roche, Darmstadt, Germany) and a chemoluminescence detector with a cooled CCD camera (Genegnome, Cambridge, UK) from Syngene. Recombinant mouse (rm)pro-IL-18 and mature IL-18 (both 2 μg) were used as standards. rmIL-18 was obtained from R & D Systems; rmpro-IL-18 was kindly provided by Dr C. A. Dinarello (University of Colorado Health Sciences Center, Denver, CO).

IL-18 mRNA measurements in total lung homogenates

A volume of 100 μl of total lung homogenates were treated with 1 ml Trizol reagent to extract RNA. RNA was resuspended in 10 μl diethyl pyrocarbonate-treated water. cDNA synthesis was performed using 5 μl of the

RNA-suspension, Superscript reverse transcriptase and oligo dT15 primers. 2 μl out of 20 μl cDNA-suspension was used for amplification in a quantitative real-time PCR reaction (Lightcycler Sequence Detector System, Roche, Mannheim, Germany). A standard-curve was made using 10-fold dilutions of the amplification product. IL-18 mRNA expression levels were normalized for the amount of β-actin mRNA present in each sample. The following primers were used: 5′-ACGTGTTCCAGGA CACAACA-3′ (forward and 5′-ACAAACCCTCCCCACCTA AC-3′ (reverse) for IL-18, 5′-GCATTGCTGACAGGA TGCAG-3′ (forward) and 5′-CCTGCTTGCTGATCCACA TC-3′ (reverse) for β-actin.

Determination of viral outgrowth

Viral load was determined on days 2, 4, 8, and 12 after viral infection using real-time quantitative PCR as described.[21,22] cDNA synthesis was performed using 1 μl of the RNA-suspension and a random hexamer cDNA synthesis kit (Applera, Foster City, CA). Five μl out of 25 μl cDNA-suspension was used for amplification in a quantitative real-time PCR reaction (ABI PRISM 7700 Sequence Detector System). The viral load present in a sample was calculated using a standard curve of particle-coated influenza virus included in every assay run. The following primers were used: 5′-GGACTGCAGCGTAGACGCTT-3′ (forward); 5′-CATCCTGTTGTATATGAGGCCCAT-3′ (reverse), 5′-CTCAGTTATTCTGCTGGTGCACTTGCC-3′ (5′-FAM labelled probe).

Bronchoalveolar lavage lavage (BAL)

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). BAL was performed by instillation of two 0·5-ml aliquots of sterile saline into the right lung. The retrieved BAL fluid (approximately 0·8 ml) was spun at 260 ***g*** for 10 min at 4° and the pellet was resuspended in 0·5 ml sterile PBS. Total cell numbers were counted using a Z2 Coulter Particle Count and Size Analyzer (Beckman-Coulter Inc., Miami, FL). Differential cell counts were done on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, Newbury, UK).

Cytokine assay

Cytokine levels in total lung lysates were measured by cytometric bead assay (CBA; BD Biosciences, San Jose, CA). The mouse inflammation CBA kit includes IL-12 p70, tumour necrosis factor-α (TNF-α), IFN-γ, monocyte chemoattractant protein-1 (MCP-1), IL-10 and IL-6. The detection level for these cytokines is 2·5 pg/ml, i.e. 25 pg/g lung tissue.

Flow cytometry

Pulmonary cell suspensions were obtained by dispersing the lung tissue through nylon sieves. Cells (2×10^6) were collected in 96-well U-bottomed plates in fluorescence-activated cell sorting (FACS) staining buffer (PBS with 0·5% (w/v) bovine serum albumin). All samples were stained for 15 min at 4° with anti-CD4-phycoerythrin (PE; clone RM4-5, Pharmingen), anti-CD8-allophycocyanin (APC) (clone 53-6.7, Pharmingen) and anti-CD69-fluoroscein isothiocyanate (FITC) mAb (clone H1.2F3, Pharmingen). FACS analysis was done on a FACS Calibur with Cell Quest software (Becton Dickinson, San Jose, CA).

Intracellular cytokine staining

Pulmonary cell suspensions were used for intracellular cytokine staining. Cells (2×10^6) were stimulated for 4 hr with 1 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μM ionomycin in the presence of the protein-secretion inhibitor Brefeldin A (1 μg/ml, all from Sigma Chemical Co., St. Louis, MO). After cell-surface staining with PE-conjugated anti-CD4 mAb (clone RM4-5) and FITC-conjugated anti-CD8 mAb (clone 53-6.7, Pharmingen) in FACS staining buffer, cells were washed followed by fixation (5 min, 4% PFA (w/v) in (PBS) and permeabilization (30 min, 0·1% saponin in FACS staining buffer). Next, cells were incubated for 30 min in FACS staining buffer supplemented with 0·1% (v/v) saponin, 5% (v/v) normal mouse serum, and 2·5 μg/ml CD16/CD32 Fc Block. Cells were then incubated for 30 min with APC-conjugated anti-TNF-α mAb (clone MP6-XT22) or biotinylated anti-IFN-γ mAb (clone XMG1.2) (all from Pharmingen) in FACS staining buffer with 0·1% saponin. FACS analysis was done on a FACSCalibur with Cell Quest software (Becton Dickinson).

Statistical analysis

Data are expressed as mean ± SE. Comparison between the groups was conducted by using the Mann–Whitney *U*-test. Two-way ANOVA was used to analyse viral load in the lungs as a function of time. $P < 0{\cdot}05$ was considered to represent a statistical significant difference.

Results

Up-regulation of IL-18 during influenza virus infection in mice

To determine whether IL-18 is induced during influenza virus infection, mice were inoculated with influenza virus and killed on day 1, 4, 8 and 14 after infection. Western blot analysis of total lung lysates clearly showed that

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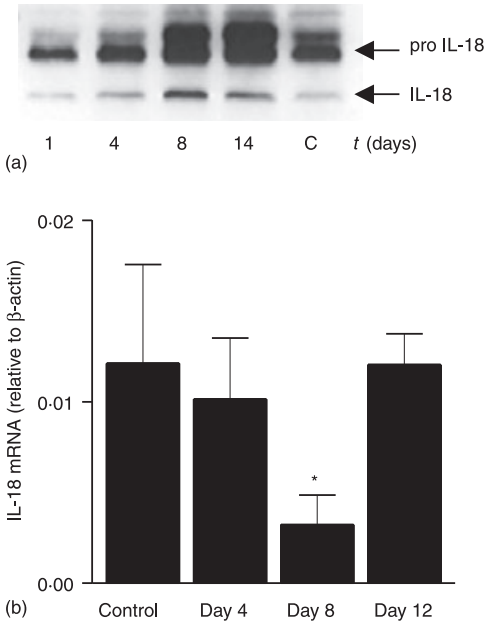

Figure 1. IL-18 expression in the lungs after influenza virus infection. Pulmonary IL-18 levels after infection with influenza virus infection were measured by Western blot (a). Each lane represents pooled total lung homogenates of eight mice. Samples were normalized for total protein content before they were pooled. Pro-IL-18 and IL-18 bands were identified by using recombinant mouse pro-IL-18 and mature IL-18. IL-18 mRNA expression (b) was measured by real-time quantitative PCR (eight mice per time-point, except control group (four mice)). Expression levels were normalized for β-actin m-RNA expression. Data are expressed as mean ± SE. $^*P < 0{\cdot}05$ versus control mice.

mature IL-18 was up-regulated on day 4 and 8 and was still elevated on 14 days after infection (Fig. 1a). Pro-IL-18 was not up-regulated during influenza virus infection in mice, suggesting that IL-18 levels increased by activation through caspase-1. Of note, a third band running at approximately 30 000 MW was clearly up-regulated on day 8 and day 14 after infection. Real-time quantitative PCR revealed that IL-18 mRNA was constitutively present in the lungs and did not increase during influenza infection (Fig. 1b). Remarkably, IL-18 mRNA is down-regulated on day 8 after influenza infection ($P < 0{\cdot}05$ versus control mice).

Recovery from influenza infection is enhanced in IL-18 gene-deficient mice

Loss of bodyweight can be used as a marker to follow the course of influenza infections in mice.[23] Bodyweight of wildtype and IL-18$^{-/-}$ mice was measured on day 0 and the day the mice were killed (eight mice per group per time-point). Bodyweight declined in all mice between day 4 and day 8 after infection. Both wildtype mice and IL-18$^{-/-}$ mice had regained their normal bodyweight on

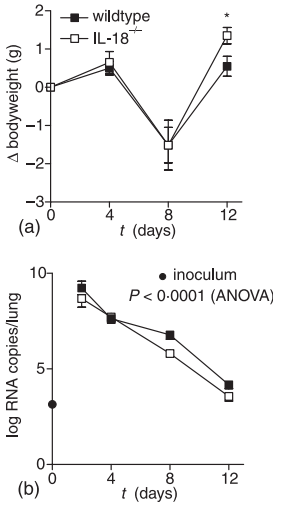

Figure 2. Bodyweight and viral load in lungs. Bodyweight was measured on days 0, 4, 8 and 12 after influenza virus infection. Data are expressed as change in bodyweight compared to day 0 (mean ± SE, six to eight mice per group). $^*P < 0{\cdot}05$ versus control mice. Viral load was determined on days 4, 8 and 12 after influenza virus infection (six to eight mice per group) in IL-18$^{-/-}$ mice (open squares) and wildtype mice (filled squares). Viral load is expressed as viral RNA copies per lung (mean ± SE). In control mice influenza was not detected (four mice per time-point, data not shown).

day 12 after infection. Remarkably, IL-18$^{-/-}$ mice, but not wildtype mice, showed a slight increase in bodyweight ($P = 0{\cdot}03$, Fig. 2a). These data indicate that IL-18$^{-/-}$ mice may have recovered more rapidly from influenza virus infection than wildtype mice. This observation was confirmed by enhanced viral clearance in IL-18$^{-/-}$ mice. Viral replication occurred between day 0 and day 2 after infection. Thereafter, influenza virus was effectively cleared from the lung (Fig. 2b). On day 2 and day 4 after infection, IL-18$^{-/-}$ mice and wildtype mice displayed similar viral loads. Thereafter, IL-18$^{-/-}$ mice showed a 90% reduction in viral load on day 8 and a 75% reduction in viral load on day 12 after infection ($P \leq 0{\cdot}0001$). Taken together, IL-18$^{-/-}$ mice recover more rapidly from influenza virus infection than wildtype mice which corresponded with an accelerated viral clearance.

Survival

To further assess the role of IL-18 during influenza virus infection, we inoculated mice with a lethal dose of influenza virus (200 $TCID_{50}$). Lethality was monitored at least twice a day after influenza infection in wildtype mice and IL-18$^{-/-}$mice (nine mice per group). No significant differences were observed between wildtype mice and IL-18$^{-/-}$mice (Fig. 3). These data indicate that, despite accelerated viral clearance, a lack of IL-18 does not improve survival after lethal infection with influenza virus.

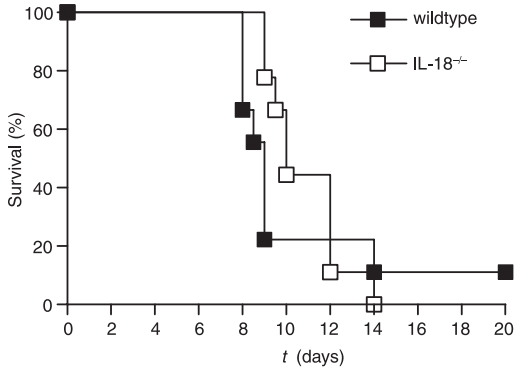

Figure 3. Survival after influenza virus infection in wildtype mice and IL-18$^{-/-}$ mice. Survival after influenza infection in wildtype mice (filled squares) and IL-18$^{-/-}$ mice (open squares). All mice (nine mice per group) received 200 $TCID_{50}$ influenza virus intranasally on day 0. Mice were monitored at least twice a day after infection.

Table 1. Leucocytes in bronchoalveolar lavage fluid on day 4 and day 8 after influenza virus infection

	Day 4		Day 8	
$\times 10^3$	wildtype	IL-18$^{-/-}$	wildtype	IL-18$^{-/-}$
Total cell count	204 ± 42	224 ± 37	1923 ± 445	843 ± 204
Granulocytes	52 ± 26	70 ± 26	223 ± 65	191 ± 29
Macrophages	143 ± 20	155 ± 24	1514 ± 353	676 ± 171
Lymphocytes	5·8 ± 1·8	2·9 ± 1·8	179 ± 50	47 ± 14*

Leucocyte counts (six mice per group for each time-point) are expressed as absolute numbers ($\times 10^3$). All data are mean ± SE.
*$P \leq 0{\cdot}05$ compared to wildtype mice.

Cells in BAL fluid

To address the role of IL-18 in recruitment of immune cells to the pulmonary compartment after influenza virus infection, BAL was performed on day 4 and day 8 after infection. No significant differences between wildtype mice and IL-18$^{-/-}$ mice were observed on day 4 after infection (Table 1). Total cells counts tended to be lower in IL-18$^{-/-}$ mice on day 8 after viral infection ($P = 0{\cdot}065$). Lymphocyte numbers were significantly lower in IL-18$^{-/-}$ mice than in wildtype mice. Neutrophil and macrophage numbers were also lower in IL-18$^{-/-}$ mice, but the differences were not significant ($P = 0{\cdot}093$ and $P = 0{\cdot}132$, respectively). These data indicate that reduced viral load in the lungs correlates with lower cell numbers in BAL fluid.

Pulmonary cytokine levels

To assess whether IL-18 is involved in influenza-induced cytokine expression, a cytometric bead assay was performed on total lung lysates on day 2, 4, 8 and 12 after

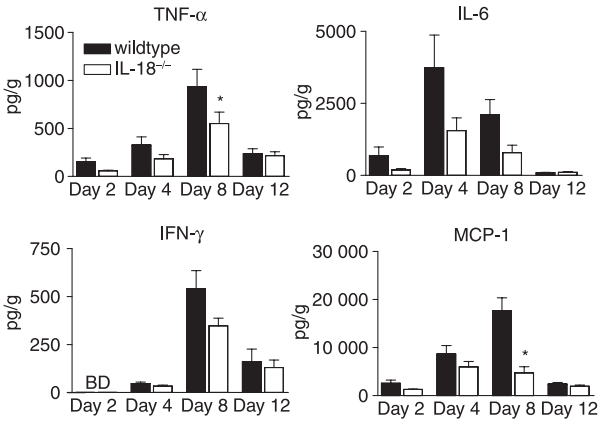

Figure 4. Cytokine expression in the lungs of IL-18$^{-/-}$ mice. TNF-α, IL-6, IFN-γ and MCP-1 levels in total-lung homogenates were measured in IL-18$^{-/-}$ mice (open bars) and wildtype mice (filled bars) on days 2, 4, 8 and 12 after influenza virus infection (four to eight mice per group). Data are expressed in pg/g lung tissue (mean ± SE). *$P \leq 0{\cdot}05$ versus wildtype mice. BD = below detection level, i.e. 25 pg/g lung tissue. Please note that IL-10 and IL-12 levels were below detection levels at all time-points.

infection. TNF-α and MCP-1 levels in the lungs of IL-18$^{-/-}$ mice were significantly lower on day 8 after influenza virus infection (Fig. 4). IL-6 levels on day 8 after infection were trendwise ($P \leq 0{\cdot}1$) lower in IL-18$^{-/-}$ mice than in wildtype mice. A trend towards lower production of TNF-α and MCP-1 by IL-18$^{-/-}$ mice was observed on day 2 after infection. Remarkably, IFN-γ levels were similar in wildtype mice and IL-18$^{-/-}$ mice at any timepoint after viral infection, while IFN-γ was not detectable on day 2 after infection. IL-10 and IL-12 were not detectable on any time-point included in this study.

Intracellular cytokine staining

Although IFN-γ levels were similar in wildtype mice and IL-18$^{-/-}$ mice, it cannot be excluded that IFN-γ was produced by different cell-types in these mouse strains. To assess differences in IFN-γ production in IL-18$^{-/-}$ mice and wildtype mice, lung-derived leukocytes were incubated with PMA/ionomycin and brefeldin A and subsequently stained for CD4, CD8 (T cells) or DX5 (NK cells) and IFN-γ. None of these cell-populations showed a significant difference in IFN-γ production between wildtype mice and IL-18$^{-/-}$ mice (Fig. 5). Because IFN-γ levels in total lung lysates were similar in wildtype and IL-18$^{-/-}$ mice and because IFN-γ was produced in equal amounts by T cells and NK cells, it can be concluded that IFN-γ production during influenza infection in mice is independent of IL-18. In contrast to IFN-γ production, TNF-α synthesis by $CD4^+$ T cells was significantly lower in IL-18$^{-/-}$ mice than in wildtype mice ($P = 0{\cdot}015$, Fig. 6). TNF-α production by $CD8^+$ T cells was similar in

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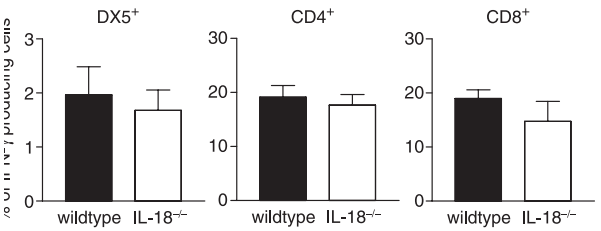

Figure 5. Cell-specific IFN-γ production. Cells (2×10^6), isolated from the lungs on day 8 after viral infection (seven mice per group), were incubated with PMA/ionomycin and brefeldin A for 4 hr at 37°. After incubation, cells were washed twice with PBS supplemented with 0·5% bovine serum albumin and subsequently stained with DX5-PE, CD4-FITC or CD8-PE. After fixation, the cells were left overnight for permeabilization and subsequent staining with IFN-γ-APC mAbs. Cell-specific IFN-γ production in leukocytes derived from the lungs of IL-18$^{-/-}$ mice (open bars) and wildtype mice (closed bars) was measured by FACS analysis and expressed as percentage (mean ± SE) of total DX5$^+$ cells, CD4$^+$ cells and CD8$^+$ cells.

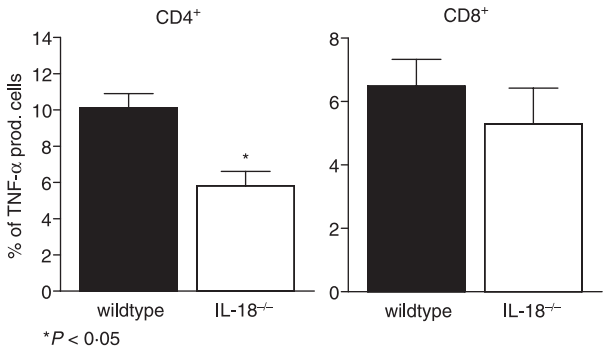

Figure 6. Cell-specific TNF-α production.2×10^6 cells, isolated from the lungs on day 8 after viral infection (seven mice per group), were incubated with PMA/ionomycin and Brefeldin A for 4 hr at 37°. Cells were stained with CD4-FITC, CD8-PE and TNF-α-APC mAbs. Cell-specific TNF-α production in leukocytes derived from the lungs of IL-18$^{-/-}$ mice (open bars) and wildtype mice (closed bars) was measured by FACS analysis and expressed as percentage (mean ± SE) of CD4$^+$ cells and CD8$^+$ cells.

both mouse-strains on day 8 after influenza virus infection. TNF-α levels in total lung lysates were significantly lower in IL-18$^{-/-}$ mice than in wildtype mice on day 8 after viral infection (Fig. 4).

IL-18 inhibits T cell activation

To further investigate the role of IL-18 during influenza virus infection in mice, the activation status of T lymphocytes was characterized by means of CD69 expression. On day 8 after viral infection, CD4$^+$ T lymphocytes of IL-18$^{-/-}$ mice demonstrated a significantly higher CD69 expression than CD4$^+$ T lymphocytes of wildtype mice (Fig. 7). CD69 expression on CD8$^+$ T lymphocytes was

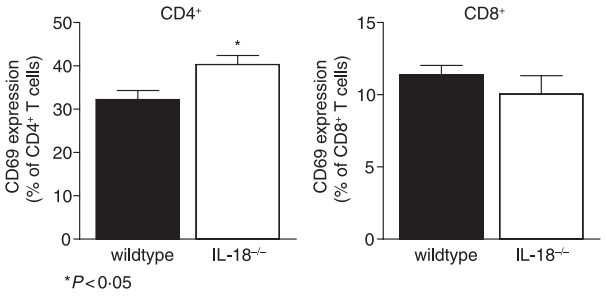

Figure 7. CD69 expression on T cells. CD69 expression on lung-derived CD4$^+$ and CD8$^+$ T cells in IL-18$^{-/-}$ mice (open bars) and wildtype mice (closed bars) on day 8 after influenza virus infection (six mice per group). Data are expressed as percentage of total number of CD4$^+$ and CD8$^+$ T cells in the lungs (mean ± SE).

similar in IL-18$^{-/-}$ and wildtype mice (Fig. 7). This suggests that IL-18, directly or indirectly, inhibits activation of CD4$^+$ T lymphocytes.

Discussion

Host defence against respiratory tract infections with influenza virus is orchestrated by a complex interaction between immune cells and regulatory cytokines. IL-18 was originally described as an important cofactor for IFN-γ release by NK cells and T cells, especially in combination with IL-12.[7] IL-18 has also been implicated in NK cell and T-cell proliferation[7,16,24] Fas ligand expression in T cells[25,26] and neutrophil recruitment and activation.[9] Considering these pleiotropic actions, endogenous IL-18 may influence the immune response to viral respiratory tract infections at multiple levels. In the present study, we investigated the role of IL-18 during influenza infection in mice. Western blot analysis revealed that virus-infected mice displayed an increase in mature IL-18 in their lungs. Because pro-IL-18 was not induced in the lungs of infected mice and since IL-18 mRNA even decreased after influenza virus infection, it can be concluded that IL-18 production after influenza virus infection in mice is dependent on proteolytic cleavage of pro-IL-18. In line, Pirhonen *et al.* showed that IL-18 release by human macrophages after influenza virus infection is regulated by proteolytic cleavage by caspase-1 and/or caspase 3.[13,14] Of note, IL-18 was constitutively expressed in lungs, which is in agreement with earlier studies.[21,27,28] Our data also indicate that a third band, running at approximately 30 000 MW, is up-regulated on day 8 and day 14 after viral infection. A similar expression pattern was observed in human macrophages after infection with influenza A.[29] It remains unclear whether pro-IL-18 exists as two separate isoforms or that sugar moieties attached to pro-IL-18 cause a shift on Western blot. Recently, a non-cleavable isoform of pro-IL-18 has been discovered in ovarian carcinoma cells.[30] However, this non-cleavable isoform has

been reported to run at 24 000 MW and therefore indistighuisable from pro-IL-18. Further research is required to identify the nature of this 30 000 MW band.

IL-18 has been shown to induce Th1-mediated immune responses.[7,24] Because Th1 responses are implicated in host defence against viral infections, IL-18 has been claimed to play an important role during respiratory tract infection with influenza virus.[4] Our data indicate that viral clearance is accelerated in IL-18$^{-/-}$ mice, suggesting that endogenous IL-18 hampers host defence against influenza virus rather than augments it. This enhanced viral clearance could be explained in part by enhanced CD4$^+$ T-cell activation in IL-18$^{-/-}$ mice as reflected by increased CD69 expression. Although these data indicate that endogenous IL-18 inhibits CD4$^+$ T-cell activation during influenza, we consider it unlikely that Th cell activation is directly diminished by IL-18. Interestingly, while our study was in progress, Liu *et al.* showed that early host defence against influenza virus was hampered in IL-18$^{-/-}$ mice as a consequence of reduced NK cell mediated cytotoxicity.[16] Although Liu *et al.* used the same mouse and influenza virus strain, some remarkable differences exist between their study and ours. First of all, Liu *et al.* found that virus titres in the lung on day 2 were significantly increased in IL-18$^{-/-}$ mice, while our data indicate that viral outgrowth is neither impaired nor enhanced in IL-18$^{-/-}$ mice on day 2 after infection. Moreover, viral clearance appears to be accelerated in IL-18$^{-/-}$ mice in our study, while Liu *et al.* found no differences between the two mouse strains from day 4–9. Of note, we determined viral load by real-time quantitative PCR, while Liu *et al.* used a standard plaque assay. The major difference between these two methods is that a standard plaque assay only detects viable virus, i.e. virus that has been shed by infected cells, whereas molecular techniques detect all viral particles. The differences between the two studies may be explained by different preparation of the viral stocks as well. Our influenza stock was grown in LLC-MK2 cells, while the viral stock used by Liu *et al.* was prepared in the allantoic cavity of chicken eggs. This difference may result in altered glycosylation patterns of the viral envelope and may affect the virulence as well. Besides, Liu *et al.* used a sublethal inoculum, at least in IL-18$^{-/-}$ mice, while our study describes a non-lethal model for both mouse strains. When we administered a lethal dose, no difference in lethality between IL-18$^{-/-}$ and wildtype mice was found, which is in accordance with the study by Liu *et al.* The study by Liu *et al.* indicated that IFN-γ levels were significantly reduced on day 2 after infection, while IFN-γ was not detectable in our study on day 2 after infection. IFN-γ levels were not significantly different on days 4, 8 and 12 after infection. Our data indicate that TNF-α and MCP-1 levels are significantly reduced on day 8 after viral infection. Because TNF-α and MCP-1 can be produced by several cell types, it remains unclear whether IL-18 targets macrophages or T cells. The fact that CD4$^+$ T cells showed lower production of TNF-α does not exclude a contribution of macrophages in this model for influenza virus infection. The reduced TNF-α production by CD4$^+$ T cells may have contributed to the enhanced CD4$^+$ T cell activation in IL-18$^{-/-}$ mice. Indeed, endogenously produced TNF-α has been shown to inhibit T cell activation. Cope *et al.* showed that treatment with anti-TNF-α alone enhanced hemagglutanin (HA) specific–T-cell receptor transgenic CD4$^+$ T-cell activation after stimulation with HA peptide, while exogenous TNF-α reduced HA-peptide-induced CD4$^+$ T-cell activation.[31] In line with our current finding, Puren *et al.* showed that IL-18 is able to induce TNF-α production in non-CD14$^+$ mononuclear cells.[26] Whether CD69 expression on CD4$^+$ T cells is directly affected by IL-18 or that endogenous TNF-α mediates the activation of CD4$^+$ T cells remains to be determined.

Besides reduced viral loads and reduced TNF-α and MCP-1 levels, IL-18$^{-/-}$ mice showed reduced numbers of lymphocytes. This reduced number of lymphocytes could be the consequence of the reduced viral load in the lungs. Alternatively and not mutually exclusive, lower T-cell numbers in BAL fluid may be due to impaired T-cell recruitment to the lungs. Although IL-18 has been shown to have chemotactic properties[9] it has never been implicated in T-cell recruitment. MCP-1, which was reduced in IL-18$^{-/-}$ mice, is primarily involved in monocyte recruitment.

IL-18 has been described to be an important cofactor in the induction IFN-γ production in NK cells and T cells.[7,8] Earlier *in vitro* investigations have established that IL-18 derived from influenza infected macrophages contributes to IFN-γ release by NK and T cells.[32] Our data indicate that IFN-γ production by NK-cells and CD4$^+$ and CD8$^+$ T cells was similar in wildtype and IL-18$^{-/-}$ mice during influenza A infection *in vivo*. Moreover, IFN-γ levels in total lung homogenates were also similar in both mouse strains. Taken together, we may conclude that IFN-γ production during influenza virus infection in mice is regulated in an IL-18-independent fashion. This finding is not unique; other conditions in which IL-18 does not contribute to IFN-γ production include staphylococcal enterotoxin B (SEB) induced systemic inflammation and whole blood stimulation with SEB and toxic shock syndrome toxin-1.[33,34]

IL-18 is a pluripotent proinflammatory cytokine that is an important component of the host response to infection. The functional diversity of this cytokine is illustrated by widespread expression of the IL-18 receptor, a member of the Toll/interleukin-1 receptor (TIR) family. Toll-like receptor-7 (TLR7), also a member of the Toll/interleukin-1 receptor (TIR) family, has recently been implicated in host response against influenza virus as well. Bone-marrow-derived plasmacytoid dendritic cells (DC) from TLR7$^{-/-}$ mice failed to produce IFN-α and IL-12 after stimulation with influenza virus and vesicular stomatitis

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virus.[35] These data indicate that IL-1/TIR family members play a critical role in response to single-stranded RNA viruses. Plasmacytoid DC have also been shown to express a functional IL-18R.[36] Whether IL-18-induced DC activation plays a critical role in host defence against influenza virus remains to be elucidated.

In conclusion, we demonstrate that constitutively present IL-18 is up-regulated in the lungs of mice infected with influenza A. Surprisingly, IL-18 deficiency was associated with an accelerated viral clearance accompanied by an enhanced activation of $CD4^+$ T cells. In light of our present findings, neutralizing antibodies against IL-18 may be useful as a treatment for influenza virus infection. Further research is required to determine the efficacy of anti-IL-18 antibodies. The IFN-γ-mediated antiviral response is likely not affected by anti-IL-18 treatment, as our data indicate that IFN-γ release is independent of IL-18 during influenza virus infection. The mechanism by which IL-18 inhibits viral clearance and/or T-cell activation remains to be determined.

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