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Interleukin-6 limits influenza-induced inflammation and protects against fatal lung pathology

Sarah N Lauder¹, Emma Jones¹, Kathryn Smart¹, Anja Bloom¹, Anwen S Williams¹, James P Hindley¹, Beatrice Ondondo¹, Philip R Taylor¹, Mathew Clement¹, Ceri Fielding¹, Andrew J Godkin¹, Simon A Jones¹, and Awen M Gallimore¹

¹Cardiff Institute of Infection and Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff, UK

SUMMARY

Balancing the generation of immune responses capable of controlling virus replication with those causing immunopathology is critical for the survival of the host and resolution of influenza-induced inflammation. Based on the capacity of interleukin-6 (IL-6) to govern both optimal T-cell responses and inflammatory resolution, we hypothesised that IL-6 plays an important role in maintaining this balance. Comparison of innate and adaptive immune responses in influenza-infected wild-type control and IL-6 deficient mice revealed striking differences in virus clearance, lung immunopathology and generation of heterosubtypic immunity. Mice lacking IL-6 displayed a profound defect in their ability to mount an anti-viral T-cell response. Failure to adequately control virus was further associated with an enhanced infiltration of inflammatory monocytes into the lung and an elevated production of the pro-inflammatory cytokines IFN- α and TNF- α . These events were associated with severe lung damage, characterised by profound vascular leakage, and death. Our data highlight an essential role for IL-6 in orchestrating anti-viral immunity through an ability to limit inflammation, promote protective adaptive immune responses and prevent fatal immunopathology.

Keywords

Adaptive Immunity; Heterosubtypic Immunity; IL-6; Innate Immunity; Pulmonary Damage

Introduction

Seasonal influenza infects 5–15% of the global population annually and results in significant morbidity and mortality [1]. Fatal infections are characterised by severe respiratory failure, which is associated with a profound early inflammatory infiltrate, lung haemorrhage and cytokine release [2–4]. Disease severity may therefore reflect the balance between immune-mediated control of virus replication and the extent of virus-driven immunopathology. The co-ordinate activities of the innate and adaptive immune systems are likely to contribute to this balance. In order to examine this relationship we studied the role that the cytokine, IL-6, plays in maintaining this balance. We considered this important, as IL-6 has been reported to correspond with the magnitude of strain-independent viral replication and disease severity both in animal models and in humans [5, 6]. Recent data using a mouse model of influenza

Corresponding author: Dr Sarah N Lauder, Cardiff Institute of Infection and Immunity, School of Medicine, Cardiff University, Heath Park, Cardiff, CF14 4XN, UK. LauderSN@cf.ac.uk Tel: +44 2920 68 7315 Fax: +44 2920 68 7303.

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infection indicate that mice lacking IL-6 are less likely to survive the infection thereby implying a beneficial effect of IL-6 in controlling the infection [7].

The impact of IL-6 on the host response to influenza infection could be wide-ranging. IL-6 regulates inflammation and orchestrates the transition from innate to adaptive immune responses ([8, 9, 10] and reviewed in [11]). In this context, IL-6 has a significant influence on the development of adaptive immunity. Indeed, mice lacking IL-6 are more susceptible to infection with a range of pathogens including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, vaccinia virus and vesicular stomatitis virus, where in each case compromised pathogen clearance was linked to generation of suboptimal T-cell responses in the knockout animals [12-14]. Furthermore, IL-6 deficient (IL-6^{-/-}) mice demonstrate resistance to antigen-induced arthritis and autoimmune encephalomyelitis [15, 16]. A role for IL-6 in promoting both CD8⁺ and CD4⁺ T-cell activities has been described and range from a requirement for the cytokine for optimal T-cell priming, differentiation, commitment, migration and effector function ([12-14, 17, 18] and reviewed in [19]). Using the human influenza virus E61-13-H17 (H17), which causes only a sub-clinical infection in mice, we previously reported diminished influenza-specific CD4⁺ T-cell memory responses in IL-6^{-/-} mice [20]. Overall, these data imply that the role of IL-6 in influenza infection is protective. We hypothesised that IL-6 would promote viral clearance, while offering protection against virus-induced lung pathology. Specifically, IL-6 control of innate immune responses would limit infection-associated tissue damage, whilst promoting optimal adaptive immunity resulting in viral clearance. To address these points, we examined the ability of wild type (WT) and IL-6^{-/-} mice to control primary infection with the mouse adapted influenza virus, A-PR8-34 (PR8) and subsequently secondary infection with A/X31 (X31). Unlike the human virus used in our previous study, PR8 replicates efficiently in mice and can cause fatal disease [21]. We compared viral clearance, development of innate and adaptive immune responses, and infection-associated lung pathology. We conclude that IL-6 plays a pivotal role in supporting an anti-viral host defence and helps to limit excessive tissue injury.

Results

IL-6^{-/-} mice succumb to sub-lethal doses of PR8

Both WT mice and mice lacking IL-6 demonstrate severe weight loss upon infection with 50 pfu of the pathogenic, mouse-adapted influenza strain, PR8 (Figure 1A) in agreement with a previous publication [7]. From day 8 post-infection, WT mice began to regain weight and all mice survived the infection. In contrast, a significant proportion of IL-6^{-/-} mice failed to recover (Figure 1B). As a further indicator of morbidity in the infected animals, body-temperature was measured at the same time-points. Following infection, all mice became hypothermic around five days post-infection and whilst there was no significant difference between the two groups, a trend towards greater reduction in core body temperature was observed in IL-6^{-/-} mice (Fig.1C). Whilst a hypothermic state is not typical during human influenza infection, reduced core body temperature during influenza infection is associated with increased morbidity and mortality in mice [22]. To determine whether the increased disease severity observed in the IL-6^{-/-} mice relates to a failure to control virus replication, lungs from infected animals were recovered and virus titres assessed at three time-points (days 2, 4 and 7) post-infection. Peak viral titres were observed at day 4 in infected WT animals after which time the infection was controlled. However, IL-6^{-/-} mice displayed a compromised ability to control influenza infection, as viral titres continued to increase up to day 7, post infection (Figure 1D).

Influenza infected IL-6^{-/-} mice exhibit increased inflammatory cytokines and monocytic cells

To confirm the presence of IL-6 during influenza infection, IL-6 levels in WT mice were measured. We found that IL-6 was significantly elevated following influenza virus infection (Supporting Information Figure 1A). In this regard, a robust acute phase response was elicited in WT mice, evidenced by an IL-6-dependent increase in systemic levels of serum amyloid-A (SAA) in these animals (Supporting Information Figure 1B). It was therefore predicted that impaired anti-viral immunity might also be associated with a reduced innate response to infection. Surprisingly, pro-inflammatory mediators associated with the activation of innate immune response were increased and BALF from infected IL-6^{-/-} mice (day-7 post infection) contained significantly elevated levels of TNF- α and IFN- α , and a trend for greater IFN- γ and IL-1 β as compared with that in WT controls (Figure 2).

To further compare the extent of the inflammatory response in infected IL-6^{-/-} and WT mice, BALF was recovered and the total number of leukocytes analysed by flow cytometry. NK cells, neutrophils and inflammatory monocytes were assessed in the BALF of WT and IL-6^{-/-} mice at days 2, 4 and 7 post-infection (Figure 3A – D, Supporting Information Figure 2). We observed no significant differences in the numbers of NK cells and neutrophils at any of the time-points tested (Figures 3B and C). However, there was a significant increase in the numbers of inflammatory monocytes in pulmonary exudates from IL-6^{-/-} mice as compared with that in WT controls (Figure 3D).

Phenotypic analysis of these cells revealed striking differences between WT and IL-6^{-/-} mice, with a substantial increase in the proportion of inflammatory monocytes producing TNF- α within the lungs of infected IL-6^{-/-} mice (Figure 4A) and an accompanying increase in BALF CCL2 levels (Figure 4B).

Influenza infection of IL-6^{-/-} mice is characterised by vascular leakage and severe lung damage

Such exaggeration of the pro-inflammatory environment in the lungs of infected IL-6^{-/-} mice might be expected to correlate with greater pulmonary pathology. When lung sections from influenza challenged mice were compared, both WT and IL-6^{-/-} displayed severe occlusion of the alveolar airspaces (Figure 5A, B, H, Supporting Information Table 1). However tissue damage was more pronounced in IL-6^{-/-} mice. Indeed, comparison of lungs at day 9 post-infection revealed an enormous disparity between IL-6^{-/-} mice (that had survived the initial infection) and WT mice (that controlled the viral infection). Extensive haemorrhage into the airway spaces (Figure 5G), leukocyte infiltration, and perivascular aggregation was observed in the lungs of IL-6^{-/-} mice. In contrast pulmonary pathology had almost completely resolved in WT animals (Figure 5D, E, H).

The greater degree of haemorrhage observed in the lungs of the IL-6^{-/-} mice implies enhanced vascular leakage in infected IL-6^{-/-} lungs compared with those of WT mice. This was confirmed by comparison of serum albumin levels in the lungs of both groups of infected mice (Figure 5I). Overall these data point towards a failure of the IL-6^{-/-} mice to control both viral load and to limit the excessive recruitment of damaging inflammatory monocytic cells to the lung. This inability to appropriately control the inflammatory response ultimately causes fatal damage.

IL-6 is required for optimal activation of influenza-specific T-cell responses

We next examined T-cell responses in WT and IL-6^{-/-} mice infected with 50 pfu of the influenza strain, PR8. We observed a trend for fewer CD4⁺ T cells and IFN- γ -producing CD4⁺ T cells in the BALF of IL-6^{-/-} compared with WT mice (Figure 6A). The total

number of CD8⁺ NP34-tetramer-positive and IFN- γ -producing CD8⁺ T cells were however markedly reduced in BALF recovered from IL-6^{-/-} mice (Figure 6 B, C). As IL-6 is known to facilitate migration of T cells to inflamed sites it was possible that the paucity of T cells in the lungs of IL-6^{-/-} mice reflected impaired T-cell migration [23]. Further analysis of tetramer-positive CD8⁺ T cells in the mediastinal lymph node however, also revealed lower numbers of these cells in mice lacking IL-6 compared with WT mice (Figure 6D). This implies a defect at the level of T-cell priming and / or expansion in the IL-6^{-/-} mice. When we examined the phenotype of dendritic cells purified from the lung draining lymph nodes, we found no difference either in number or expression levels of MHC class II, CD80 or CD86 between both groups of mice (Supporting Information Figure 5).

As IL-6 also controls T-cell proliferation and T-cell apoptosis, we assessed influenza-specific T cells in both IL-6^{-/-} and WT mice according to these parameters [24]. Specifically, we measured expression of the protein Ki-67, observed in proliferating cells, and the anti-apoptotic gene Bcl2, which protects cells from apoptosis (Figures 7 A and B). Whilst the total number and proportion of CD8⁺ T cells proliferating were comparable between both groups of mice (Figure 7C) the total number of proliferating influenza-specific CD8⁺ T cells were significantly reduced in the IL-6^{-/-} mice (Figure 7D). The total number and proportion of Ki67⁺ pulmonary infiltrating CD4⁺ T cells were also significantly diminished in the absence of IL-6 (Figure 7E). Further analysis of the CD8⁺, Tetramer⁺ CD8⁺ and CD4⁺ T cells indicated a trend towards reduced proportions of cells expressing Bcl2 in IL-6^{-/-} compared to WT mice (Figure 7 F, G and H).

IL-6 is required for establishing effective heterosubtypic immunity

As the memory response to influenza virus is thought to reflect the magnitude of the primary response in WT animals [25], we surmised that the generation of T-cell immunity against a different influenza A virus subtype (heterosubtypic immunity) would be impaired in the IL-6^{-/-} animals. To address this, we infected groups of WT and IL-6^{-/-} animals with 25pfu PR8 virus. This lower dose was chosen for this part of the study to guarantee recovery and survival of all experimental mice after primary infection (Supporting Information Figure 3). Mice were rechallenged with X31, a heterosubtypic strain of influenza virus, which shares a number of T-cell receptor epitopes to those displayed by PR8 virus, but not neutralising antibody determinants. The application of these heterosubtypic influenza strains therefore allows us to specifically examine expansion of influenza-specific memory T cells and their ability to confer protective immunity. Pulmonary viral load at day 4 post-secondary infection demonstrated a significant decrease in viral titres in WT memory mice compared with those in WT naïve mice, however, the same effect was not observed in the IL-6^{-/-} mice demonstrating a deficiency in their ability to control a second infection (Figure 8B). This finding was reflected in the weight loss measurements as IL-6^{-/-} but not WT mice, lost a comparable amount of weight upon X31 challenge as mice that had not previously been exposed to the PR8 virus (Figure 8A). Influenza-specific tetramer staining indicated a notable reduction in the number of antigen-experienced CD8⁺ T cells recalled to the lungs during the second infection in IL-6^{-/-} mice (Figure 8C). The impaired ability to control X31 in PR8-primed IL-6^{-/-} animals was also associated with lower numbers of CD4⁺ T cells in the BALF following challenge (Figures 8D). As a further measure of the antigen-specific memory CD4⁺ T-cell response, the influenza-specific proliferative capacity of CD4⁺ T cells, purified from mice 7-9 weeks following the resolution of the primary infection was significantly reduced in IL-6^{-/-} mice (Figure 8E). Overall, these data reflect early events during the primary phase of influenza infection confirming that the influenza-specific T-cell memory response is significantly compromised in the IL-6^{-/-} animals. A further striking feature of IL-6^{-/-} animals rechallenged with X31 was the extent of pulmonary damage observed during the second infection (Figure 8F). In particular, significant haemorrhage was

observed in the lungs of IL-6^{-/-} mice (Figure 8H, F), substantiating earlier observations that pulmonary damage during the primary infection results in lung leakiness that appears to be exacerbated by IL-6 deficiency during a subsequent infection.

Discussion

In this study we found that IL-6 has a significant impact on the severity of influenza virus infection rendering animals less able to control virus and more susceptible to lethal disease. Both innate and adaptive immune responses to influenza virus were affected by the absence of IL-6 reflecting the pleiotropic nature of the cytokine and also its role in co-ordinating the activities of these different arms of the immune system.

Both WT and IL-6 deficient mice showed a similar disease course in that all mice from both groups lost weight following influenza virus infection and, as reported previously, the mice experienced a hypothermic response rather than fever during the acute phase of infection [26]. Despite a previous report indicating that during the first five days post influenza infection WT mice experience a greater dip in body temperature compared to mice lacking IL-6, we observed no significant differences. The disparity in the findings of the two studies may reflect the doses of virus used to infect the mice: approximately three times more virus was used in our study, ensuring a greater extent of infection in both the WT and IL-6^{-/-} animals. In our study, the biggest difference between WT and IL-6^{-/-} mice occurred at day 8 post-infection; WT mice started to regain weight from day 9 whilst IL-6^{-/-} mice did not. Similar findings were reported in a recent study by Dienz and colleagues who demonstrated that whilst WT mice recovered from influenza virus infection, mice lacking IL-6 did not [27].

The severity of disease in the IL-6^{-/-} mice is associated with a failure to control influenza virus. The effect of this failure is profound, perpetuating virus-driven inflammatory responses and resulting in life-threatening immunopathology. This is characterised by an elevation in local pro-inflammatory cytokine production, most notably IFN- α and an associated increase in CCL2 and enhanced inflammatory monocytic cell recruitment to the lung. Several independent reports have confirmed a critical role for CCL2-mediated recruitment of inflammatory, TNF- α -producing macrophages to the lungs of influenza-infected animals resulting in pulmonary oedema, haemorrhaging of bronchial blood vessels and apoptosis of lung epithelial cells [28, 29, 30, 31]. Following caspase-3 staining of lung sections prepared from the mice, we failed to detect apoptotic cells thereby precluding a comparison of cell death in the lungs of WT and IL-6^{-/-} mice. The greater degree of inflammation observed in mice lacking IL-6 is however consistent with previous studies [27], and indicates that the pathology observed in the IL-6^{-/-} mice is caused by virus-driven inflammatory responses.

What is responsible for the failure to control virus? Previous data by Dienz et al. [27] report a role for IL-6 in promoting the survival of lung neutrophils, important for limiting virus replication during acute influenza infection. Significantly, infiltrating neutrophils from IL-6^{-/-} mice show impaired effector functions (phagocytosis and respiratory burst activity) [32]. During the innate phase of the influenza response we observed no difference in the numbers of neutrophils isolated from the BALF at any of the time points examined. However, it remains possible that these neutrophils do contribute to virus clearance and that this is more effective in WT compared to IL-6^{-/-} mice. Some differences between the two experimental systems preclude direct comparison. Most notably, in the Dienz study, mice were infected with higher doses of influenza and virus clearance from the pulmonary cavity was assessed by PCR to quantitate viral copy number and not by measuring the amount of infectious virus [27]. Also, by day 9 post-infection, it was reported that the numbers of

neutrophils were negligible in both WT and IL-6^{-/-} mice, indicating that neutrophils may not be critical for the resolution of infection.

The data presented herein indicate that IL-6 clearly promotes induction of the virus-specific T-cell response as the influenza-specific CD8⁺ T-cell, and to a lesser extent the CD4⁺ T-cell response, was diminished in both BALF and lung draining lymph nodes of IL-6^{-/-} mice. These data are in line with prior investigations, which have revealed an impact of IL-6 on the control of CD4⁺ T-cell responses [19, 33, 34], the differentiation of CD8⁺ T cells in vitro and, in certain infections, facilitating induction of optimal CD8⁺ T-cell responses [12, 13, 18, 35]. Several mechanisms may limit T-cell responses in the absence of IL-6. Studies have shown that IL-6 has a role in promoting dendritic cell (DC) maturation and effective antigen presentation in vitro [36, 37]. However, we observed no difference in either the total numbers or the maturation status of DCs isolated from the mediastinal lymph nodes following influenza infection (Supporting Information Figure 5) and we have been unable to detect any difference in the phenotype or function of WT or IL-6^{-/-} bone-marrow derived DCs infected with influenza virus in vitro (data not shown). It has been demonstrated in vivo that the presence of IL-6 promotes antigen-specific expansion of CD4⁺ T cells by protecting them from apoptosis [24]. In line with this, we found that fewer CD8⁺ and CD4⁺ T cells recruited to the infected lungs of IL-6^{-/-} mice expressed Bcl2, thus the lung-infiltrating T cells in these animals are likely to be more apoptosis-prone. Moreover, significantly fewer antigen-specific CD8⁺ T cells and CD4⁺ T cells proliferated in the pulmonary cavity and draining lymph nodes of influenza-infected IL-6^{-/-} compared with WT mice. Whether this influence of IL-6 is due to direct effects on the influenza-specific T cells is unclear. IL-6 could directly promote T-cell expansion and survival or act indirectly through effects on other cells. Indeed, in this study we observed that the ratio of CD4⁺Foxp3⁺ T cells to CD4⁺Foxp3⁻ T cells in BALF was significantly higher in IL-6^{-/-} mice compared with that in WT mice at day 7 post-infection (Supporting Information Figure 4). Such an alteration could explain the reduced T-cell response observed in the IL-6^{-/-} mice. Although no such difference was observed in the ratio of CD4⁺Foxp3⁺ T cells to influenza-specific CD8⁺ tetramer⁺ T cells, an elevated Treg-cell to CD4⁺Foxp3⁻ T-cell ratio could still account, through compromised CD4⁺ T-cell help, for the decreased influenza-specific CD8⁺ T-cell responses measured in the IL-6^{-/-} animals. Treg cells have previously been shown however to impact on influenza-specific CD4⁺ T-cell responses in IL-6^{-/-} mice. Using a different strain of influenza virus (E61-13-H17 (H17)), which replicates less efficiently in mice than the mouse-adapted PR8 virus adopted here, memory but not primary influenza-specific CD4⁺ T-cell responses were significantly impaired in IL-6^{-/-} animals; an effect that was due in part to the induction of influenza-specific Treg cells in the IL-6^{-/-} mice, but not wild-type mice [20]. Our current study implies that following infection with a pathogenic dose of influenza virus, IL-6-deficiency results in enhanced induction of Treg cells during the primary phase of infection, which may limit both the proliferation and survival of antiviral T cells.

We also observed an impairment of memory T-cell responses in the IL-6^{-/-} animals assessed in this study, which compromised the ability of the mice to clear infection following challenge with a heterosubtypic influenza virus. It has been reported previously that the size of the memory T-cell pool reflects the clonal burst size following initial stimulation with antigen [38]. Whilst we were unable to accurately quantitate, using tetramers, the frequencies of antigen-specific T cells in mice just prior to re-challenge, it is likely that the reduced recall response in the IL-6^{-/-} mice reflects both the compromised primary response as well as the ability of the memory T cells to expand optimally upon second encounter with antigen.

Curiously, we observed a significantly increased degree of lung damage in IL-6^{-/-} mice infected with a second influenza virus. It has been reported previously that IL-6 is required for regeneration of intestinal epithelial cells [39]. It is therefore possible that IL-6 plays an important role in repair of damaged lung epithelia following respiratory virus infection. These findings are of direct relevance to understanding the potential impact of drugs designed to interfere with IL-6 signalling e.g. tocilizumab, an IL-6R mAb, used to treat rheumatoid arthritis (reviewed in [40]). Indeed, due to the impact of IL-6 on intestinal epithelial regeneration, the drug is not recommended for individuals with diverticular disease. It is possible that IL-6 blockade may also exacerbate lung pathology during respiratory virus infections. Such experiments represent an important progression of this study, where the impact of IL-6 blockade is tested not in IL-6^{-/-} mice, but in mice given drugs to inhibit IL-6 signalling.

Collectively these data indicate that following infection with pathogenic titres of influenza virus, IL-6 is critical for the generation of robust influenza-specific T-cell responses in vivo, as demonstrated by a significant reduction in influenza-specific CD8⁺ and CD4⁺ T cells recruited to the lungs of mice following primary and secondary challenge with influenza virus. We hypothesise that as a result of this compromised T-cell response, IL-6^{-/-} mice struggle to control the infection resulting in virus-driven inflammatory innate responses and life-threatening immunopathology.

Whilst our data are consistent with these conclusions, we cannot rule out that IL-6 also modulates the immune response to influenza virus through its ability to direct anti-inflammatory activities. Xing et al. [35] previously showed, using a model of endotoxin-induced acute lung inflammation that IL-6 suppresses expression of pro-inflammatory cytokines, including TNF- α . A similar regulation of plasma TNF- α levels has also been reported in IL-6^{-/-} mice following *Streptococcus pneumoniae* infection [14]. Studies by Barton et al. [41] using a septic shock model demonstrated that IL-6 antagonized the effect of TNF- α in vivo, protecting against TNF- α induced mortality. In vivo blockade of TNF- α in murine influenza significantly reduces pulmonary immunopathology and cellular infiltrate without preventing virus clearance [42]. Thus, in our own study we cannot rule out the possibility that the absence of IL-6 exacerbates influenza-driven inflammatory responses in a manner that is independent of virus load.

Overall, the study presented herein underpins the importance of boosting the adaptive response to influenza virus whilst suppressing the inflammatory response. IL-6 plays an essential role in maintaining this balance.

Materials and methods

Mice

Female C57BL/6 wild type (WT) mice and IL-6^{-/-} mice were sourced from Charles River UK and used for experiments at 8 – 10 weeks of age. Mice were housed in Scantainers that were ventilated with HEPA-filtered air, and allowed access to standard mouse chow and water *ad libitum*. All experimental procedures conducted were in compliance with UK Home Office Regulations.

Murine Influenza Infection

Recombinant Influenza A virus strain A-PR8-34 (PR8, H1N1), and A/X31 (X31, H3N2) were obtained from the National Institute for Medical Research (London, UK) [43]. PR8 and X31 were amplified in embryonated chicken eggs and in vitro MDCK virus titration assays conducted to determine viral titre as described previously [44].

Prior to infection mice were weighed and ear coded. Mice were infected intra-nasally (i.n.) with 50 plaque forming units (pfu) of PR8 in 50 µl of sterile PBS, under light anaesthesia for primary acute influenza infection studies, days 0 – 7. For memory experiments, mice were infected with 25pfu of PR8 as above. Where stated, mice were rechallenged at 7 – 9 weeks post-primary infection with 200 pfu of X31 in 50 µl of sterile PBS under light anaesthesia. Body weight was recorded daily during the course of infection until mice were sacrificed or until day 26 for memory studies. Rectal temperatures were measured prior to the commencement of the study and until day 7 for acute studies.

In Vitro Virus Quantitation Assay

The right-hand lung lobes were extracted following perfusion and placed immediately into 1ml of serum-free IMDM (Invitrogen) on ice. Lungs were homogenised and stored at –80°C. Lung viral load was determined using the previously described virus titration assay [44]. Briefly, serial two-fold dilutions of lung homogenates were incubated with MDCK cells with a methylcellulose overlay in a 24-well plate for 48 hours. Cells were fixed, permeabilised and stained with a monoclonal antibody specific for the PR8 haemagglutinin, for X31 an anti-influenza nucleoprotein antibody (AbD Serotec) was used. Cells were incubated with a peroxidase-labelled secondary antibody (BioRad) and the plate was developed using AEC reagents (Sigma). Each stained cell was counted and the total pfu/lung was calculated.

Lung Immunohistochemistry

Lungs were perfused with 5mls of PBS and placed into 10% NBFS. Fixed lungs were embedded into paraffin and 5 µm sections cut. Sections were stained with haematoxylin and eosin and were scored blinded. Lungs were scored using a modified scoring criteria from Longhi et al. [45] and Ashcroft et al. [46], namely severity of interstitial leukocyte infiltration (0-3), and the magnitude of perivascular lymphoid aggregate (0-3) combined with the extent of perivascular lymphocytic aggregate within each section (0-10). The degree of haemorrhage (0-3) and extent of pulmonary fibrosis (0-3) was also scored. The mean score for each clinical parameter was then calculated and compared between groups.

Bronchoalveolar Lavage (BAL)

Mice were sacrificed and the lungs lavaged immediately with 1ml of PBS/EDTA. Approximately 90% of the total instilled volume was routinely recovered. BAL fluid (BALF) was kept on ice before centrifugation at 3000 rpm for 10 minutes at 4°C. Cell pellets were resuspended in FACS buffer and the cellular content quantified by flow cytometry. BALF was aliquoted and stored at –80°C for cytokine analysis.

Flow Cytometry

Cells isolated from the BALF were stained with fluorochrome-conjugated antibodies or MHC-I tetramers. Anti-NK1.1-FITC (BD), Anti-CD3-PerCP (BD), Anti-CD11c-PE-Cy7 (BD), Anti-GR-1-V450 (BD), Anti-CD11b-allophycocyanin-Cy7 (BD), Anti-CD4-Pacific Blue (BD), Anti-CD8-PerCP-Cy5.5 (BD), Anti-CD8-allophycocyanin-Cy7 (BD), Anti-IFN-γ-PerCP-Cy5.5 (BD), Anti-7/4-PE (AbD Serotec), Anti-F4/80-allophycocyanin (AbD Serotec), Anti-IL-10-allophycocyanin (eBioscience), Anti-TNF-α-FITC (eBioscience), Anti-Ki67-FITC (BD), Anti-Bcl₂-PE (BD) and Anti-FoxP3-PE-Cy7 (eBioscience) were used throughout these studies. The PE-labelled tetramer (D^b-ASNENMETM) used in this study was generated in-house using previously described methods [47]. Cells stained with directly conjugated antibodies were incubated with 1-2 µg/ml of antibody for 30 minutes at 4°C prior to washing and resuspending in FACS buffer (PBS, 2% FCS, 2 mM EDTA). Cells were fixed with FACS Fix buffer (PBS, 2% FCS, 2mM EDTA, 2% Formalin) prior to

analysis. Cells were acquired by flow cytometry (Cyan ADP, Beckman Coulter) and the data analysed using Summit Software (DAKO, Colorado, USA).

Intracellular cytokine staining of T cells was performed using single cell suspensions derived from BALF. Cultures were stimulated at 37°C in complete RPMI (Invitrogen, supplemented with 2 mM L-glutamine, 1mM sodium pyruvate, 50 µg/ml penicillin streptomycin and 10% foetal calf serum) with phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) (20 nM) and ionomycin (1 µg/ml; Sigma-Aldrich) for 4 hours; GolgiPlug (1 µl/ml; BD Biosciences) was added for the last 3 hours. After incubation, cells were incubated according to supplier instructions with Live/Dead Fixable Dead Stain Kit Aqua (Invitrogen, UK) to enable gating of live cells during analysis. Cells were washed and subsequently surface stained for CD4 and CD8 for 30 minutes at 4°C, washed, fixed and permeabilized (Foxp3 staining kit, eBioscience), blocked for 15 minutes with Fc Block (BD) and stained for TNF-α and IFN-γ for 30 minutes at 4 °C.

For intracellular cytokine staining of inflammatory monocytic cells, single cell suspensions derived from BALF were stimulated by culture at 37 °C with lipopolysaccharide (LPS; Sigma-Aldrich) (10mg/ml) for 5 hours; GolgiPlug (BD) was added for the last 4 hours. After incubation, cells were washed and subsequently surface stained for CD11c, CD11b, GR-1 and 7/4 for 30 minutes at 4 °C, washed, fixed and permeabilized (Foxp3 staining kit, eBioscience), blocked for 15 minutes with Fc Block (BD) and stained for TNF-α and IL-10 for 30 minutes at 4 °C.

Quantitation of Cytokines and Albumin in BALF

The level of IL-6 present in the BALF was determined using a Flow-Cytomix bead-based immunoassay (Bender Medsystems). Quantification of TNF-α, IFN-α, IFN-γ, IL-1β (all from R&D Systems), and CCL2 (eBioscience) in the BALF was performed using commercial ELISA. Circulating systemic levels of serum amyloid A (SAA) and the presence of serum albumin in the BALF were determined by ELISA (Invitrogen and Bethyl Laboratories, respectively).

CD4⁺ T-Cell Proliferation Assays

Spleens were harvested from mice previously infected with PR8 at 7-9 weeks post infection. The CD4⁺ T cells were isolated by positive selection using magnetic beads (Miltenyi Biotec). Purity of CD4⁺ T cells was > 95% as determined by flow cytometry. 2×10^5 CD4⁺ T cells were restimulated by the addition of 2×10^5 irradiated antigen presenting cells (APCs) isolated from a naïve spleen, in Iscove's Modified Dulbecco's Media (IMDM) (Invitrogen) supplemented with 10% (v:v) FCS. As previously described APCs were pulsed for 1 hour with increasing doses of UV-inactivated PR8 virus, washed and irradiated [48]. ³H-thymidine was added after 60 hours, cells were harvested after 72 hours and the counts per minute (CPM) determined.

Statistical Analyses

Statistical differences between WT and IL-6^{-/-} mice were calculated using Mann Whitney test. The statistical differences in figure 7E were assessed using one-way ANOVA with Tukey-Kramers multiple comparison post-hoc test, used to compare the means of WT and IL-6^{-/-} mice. p values of 0.05 were considered significant, with values of 0.01 considered highly significant. All experiments were performed at least twice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FasL	Fas Ligand
MDCK	Madin Darby Canine Kidney Cell
NA	Neuraminidase
NBFS	Neutral Buffered Formalin Saline
SAA	Serum Amyloid-A

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Figure 1. IL-6^{-/-} mice experience enhanced mortality and morbidity with delayed viral clearance during acute influenza infection

(A, B) WT and IL-6^{-/-} mice were infected i.n. with 50 pfu of PR8 and monitored daily for weight loss. The (A) percentage of initial weight and (B) Kaplan-Meier survival curves are shown and significance determined using the log-rank test (**p<0.01). (C) Core body temperature was determined on a daily basis as an indication of morbidity. (D) Lungs were harvested from the mice at the time-points indicated and the pulmonary viral load quantified. **p<0.01, Mann Whitney test. Results are expressed as the mean ± SEM, n = 6 mice/group from one experiment representative of three performed.

Figure 2. Acute influenza infection induces an enhanced proinflammatory cytokine response in IL-6^{-/-} mice

BALF was aspirated at day 2, 4 or 7 post infection from WT and IL-6^{-/-} mice infected with 50 pfu of PR8. (A) TNF- α , (B) IFN- α , (C) IL-1 β and (D) IFN- γ levels present in the BALF were determined by ELISA. Results are expressed as the mean $\pm$ SEM, n = 6 mice/group from one experiment representative of two performed. The significances of differences between WT and IL-6^{-/-} mice was assessed using Mann-Whitney test, * p < 0.05, ** p < 0.01.

Figure 3. IL-6^{-/-} mice exhibit exacerbated influenza induced airway inflammation

BALF was aspirated from WT and IL-6^{-/-} mice at days 2, 4 and 7 and the airway infiltrating cells were isolated. (A) The total numbers of cells and the numbers of (B) NK cells, (C) neutrophils, and (D) inflammatory monocytes, at the selected time points were determined by flow cytometry. The mean score $\pm$ SEM for each group is given, $n = 6$ mice/group, from one experiment representative of three performed. The significances of differences between WT and IL-6^{-/-} mice were determined using Mann-Whitney test, *** $p < 0.001$

Figure 4. Pulmonary inflammatory monocytic cells exhibit an altered phenotype in IL-6^{-/-} mice (A) BALF was aspirated from WT and IL-6^{-/-} mice at day 7 and the airway infiltrating cells isolated. BAL cells were stimulated for five hours in vitro with LPS, the numbers of inflammatory monocytic cells secreting TNF- α were determined by flow cytometry. (B) The presence of the chemokine CCL2 was determined in BALF at day 7 by ELISA. Results are expressed as the mean $\pm$ SEM, n = 6 mice/group from one experiment representative of two performed. The significances of differences between WT and IL-6^{-/-} mice was assessed using Mann-Whitney test, * p < 0.05, *** p < 0.001.

Figure 5. IL-6^{-/-} mice exhibit increased pulmonary immunopathology during acute influenza infection

Lungs were harvested from mice at the days indicated post infection and following perfusion were formalin fixed, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Representative sections from influenza-infected, (A, D) WT and (B, E) IL-6^{-/-} mice were compared with those from (C, F) uninfected WT mice at day 7 (top) and day 9 (bottom). Arrowheads highlight areas of lymphocytic perivascular aggregate, asterisks highlight areas of leukocyte infiltration into the airway spaces, and H indicates areas of haemorrhage (original magnification $\times 20$, scale bar 100 μm). (G) A representative section illustrating the extent of haemorrhage observed in IL-6^{-/-} mice at day 9 post infection is shown (original magnification $\times 20$). (H) The mean total histopathology score $\pm$ SEM for WT and IL-6^{-/-} mice is given, $n = 6$ mice/group from one experiment representative of three performed. (I) The level of serum albumin, a protein indicative of damage to the pulmonary epithelium, was determined in BALF at day 7 by ELISA. Results are expressed as the mean $\pm$ SEM, $n = 6$ mice/group from one experiment representative of two performed. The significances of differences between WT and IL-6^{-/-} mice was assessed using Mann-Whitney test. * $p < 0.05$.

Figure 6. Adaptive immune responses are diminished in IL-6^{-/-} mice

(A, B) BALF was aspirated from WT and IL-6^{-/-} mice at day 7 post infection and the airway infiltrating cells isolated. (A) The numbers of CD4⁺ (left axis), IFN- γ -producing CD4⁺ cells (right axis) and (B) CD8⁺ (left axis) and IFN- γ -producing CD8⁺ cells (right axis) were determined by flow cytometry. (C) Total numbers of CD8⁺ NP34-tetramer⁺ cells were determined from the BALF. (D) The total number of CD8⁺ NP34-tetramer⁺ cells was determined in the mediastinal lymph node that drains the pulmonary cavity. Results are expressed as the mean $\pm$ SEM, $n = 6$ mice/group from one experiment representative of three performed. The significances of differences between WT and IL-6^{-/-} mice was assessed using Mann-Whitney test, * $p < 0.05$.

Figure 7. T-cell proliferation and survival characteristics are altered in the absence of IL-6 BALF was aspirated from WT and IL6^{-/-} mice at day 7 post infection and the airway infiltrating cells isolated. Representative flow cytometry plots depict the expression of (A) Ki67 on CD8⁺ T Cells, and (B) Bcl2 on CD8⁺ T cells. (C) The numbers of CD8⁺, (D) NP-34⁺ tetramer⁺ CD8⁺ T cells and (E) CD4⁺ T cells expressing the proliferation marker Ki67 were determined by flow cytometry. Total numbers of (F) CD8⁺ T cells, (G) NP-34⁺ tetramer⁺ CD8⁺ T cells and (H) CD4⁺ T cells expressing the anti-apoptosis gene Bcl2 were also determined by flow cytometry. The significance of differences between WT and IL-6^{-/-} mice was assessed using the Mann-Whitney test, * p < 0.05, ** p < 0.01.

Figure 8. Protective immune responses were significantly reduced in IL-6^{-/-} mice

(A) At 7 – 9 weeks post infection all mice were rechallenged with 200 pfu of X31. Mice were weighed prior to secondary infection to provide a baseline 100% starting weight and for 4 days thereafter. Each data point represents the mean $\pm$ SEM for each group, $n = 3 - 6$ mice/group. (B) At day 4 post secondary infection mice were sacrificed and the lungs harvested following perfusion with PBS. Lungs were homogenized and the viral load determined. Plots represent the mean of each group $\pm$ SEM, $n = 6$ mice/group, 4 mice/group for PR8 naïve mice. (C, D) BALF was aspirated from WT and IL-6^{-/-} mice at 4 days post secondary infection and the airway infiltrating cells isolated. The numbers of (C) CD8⁺ Influenza-specific tetramer⁺ cells, and (D) CD4⁺ cells were determined by flow cytometry.

Plots represent the mean $\pm$ SEM, $n = 6$ mice/group, 4 mice/group for PR8 naïve mice. (E) To determine the proliferative capacity of antigen-specific CD4⁺ T cells, spleens were isolated from WT and IL-6^{-/-} mice between 7 – 9 weeks post infection. CD4⁺ T cells were isolated by magnetic separation and stimulated by the addition of splenocytes loaded with PR8. The antigen specific proliferation of WT and IL-6^{-/-} CD4⁺ T cells was determined by thymidine incorporation at 72 hours. Each data point represents the mean $\pm$ SEM, $n = 4$ mice/group. The significances of differences between WT and IL-6^{-/-} mice was assessed using one-way ANOVA with Tukey-Kramers post-hoc multicomparison test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (F-H) Lungs were harvested from mice at day 4 post-secondary infection and following perfusion were formalin fixed, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Representative sections from influenza infected, (G) WT and (H) IL-6^{-/-} mice are shown. (F) Sections were scored for the extent and severity of haemorrhage present within the lungs of WT and IL-6^{-/-} infected with PR8 and rechallenged with X31 4 days previously and PR8 naïve WT and IL-6^{-/-} mice infected with X31 4 days earlier. Plots represent the mean haemorrhage score $\pm$ SEM, $n = 4 - 6$ mice/group. All data shown are from one experiment representative of two performed. Statistical significance between WT and IL-6^{-/-} mice was determined using Mann-Whitney test, * $p < 0.05$.