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Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice

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ABSTRACT

Antiviral immune responses play as a double edged sword in resolution of infection and pathogenesis of acute lung injury caused by infection with highly pathogenic influenza A viruses. Here we show that type I interferons (IFNs) are important in protection against acute influenza A virus infection not only via their antiviral activity but also via their anti-inflammatory activity. IFN α receptor (IFNAR) knock-out (KO) mice exhibited increased mortality and morbidity with higher viral load after infection with influenza virus A/FM/1/47 (H1N1, a mouse-adapted strain) compared with wild-type (WT) mice, though the viruses were finally eliminated in both groups. The levels of proinflammatory cytokines in the lungs were significantly higher, while the level of IL-10 in the lungs was significantly lower in IFNAR KO mice than in WT mice during the course of infection. Restoration of IL-10 during an ongoing virus infection significantly reduced the levels of proinflammatory cytokines and improved mortality of IFNAR KO mice. These results suggest that type I IFNs are responsible not only for direct resolution of viral load but also for suppression of immunopathology caused by influenza A virus through IL-10 production.

1. Introduction

Type I interferons (IFNs) IFN-αs and IFN-β are critical effectors of the innate immune response to viral infection (Randall and Goodbourn, 2008). Type I IFNs bind with high affinity to a cell surface receptor complex, IFN α receptor (IFNAR) comprised of IFNAR1 and IFNAR2, thereby invoking a cascade of signaling events that lead to transcriptional activation of numerous IFN-stimulated genes (ISGs) (Platanias, 2005). ISG-encoded proteins exhibit antiviral activity by inhibiting viral replication (Sadler and Williams, 2008). Type I IFNs also contribute to immune responses by enhancing natural killer (NK) cell activity (Nguyen et al., 2002) and antiviral T cell responses to viral infection (Honda et al., 2005; Kolumam et al., 2005). Meanwhile, there are several lines of evidence that type I IFNs exert anti-inflammatory activity that inhibits immune response via inhibition of interleukin (IL)-1, IL-18, or IL-12 and/or enhancement of IL-10 production (Billiau, 2006; Gonzalez-Navajas et al., 2012; Guarda et al., 2011). The opposing roles of type I IFNs in protection against bacterial infection were confirmed *in vivo* for *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Manca et al., 2005; Stockinger and Decker, 2008). However, the dichotomous roles of type I IFNs in protection and regulation of immune responses during viral infection remains unknown.

Highly pathogenic influenza A viruses cause acute severe pneumonia that results in high morbidity and significant mortality (Gambotto et al., 2008; Gillim-Ross and Subbarao, 2006; Ng et al., 2006; Peiris et al., 2007). Elevated levels of serum proinflammatory cytokines and chemokines are known to contribute as "cytokine storm" to increased severity of disease caused by some strains of influenza A virus (Doherty et al., 2006; Maines et al., 2008; Taubenberger and Morens, 2008). Several proinflammatory cytokines including IL-1β and IL-6 have been reported to play an important role in influenza immunopathology (Imai et al., 2008; Schmitz et al., 2005). A unique environment such as the lungs develops a unique immunoregulatory system mediated by suppressive cytokines such as IL-10 that is responsible at least partly for limiting excessive inflammation and play an important role in homeostasis in the lungs (Akbari et al., 2001; Lloyd and Hawrylowicz, 2009; Wissinger et al., 2009). A balance in the levels of proinflammatory cytokines and suppressive cytokines may be crucial in host defense against highly pathogenic influenza virus infection.

In the present study, we show that IFNAR knock-out (KO) mice exhibited increased mortality and morbidity after infection with

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influenza virus A/FM/1/47 (H1N1, a mouse-adapted strain). The viral titers in the lungs at an early stage were significantly higher in IFNAR KO mice than in wild type (WT) mice. The levels of IFN-γ, IL-6 and IL-1β in the lungs were significantly higher while the IL-10 level was significantly lower in IFNAR KO mice than in WT mice during the course of infection. Restoration of recombinant IL-10 to IFNAR KO mice alleviated cytokine storm and improved mortality. Type I IFNs functioning as a double-edged sword by their antimicrobial effects and anti-inflammatory effects are important in protection against acute influenza A virus infection.

2. Materials and methods

2.1. Mice

IFN Alpha Receptor$^{-/-}$ 129/Sv (IFNAR KO) mice from B&K Universal Ltd. were backcrossed with C57BL/6J mice (The Jackson Laboratory) for more than seven generations. C57BL/6 Ly5.1-congenic mice were purchased from Charles River Japan (Hino, Japan). The mice were maintained in specific pathogen-free conditions and used at 7–12 weeks of age. The study design was approved by the Committee of Ethics on Animal Experiments of the Faculty of Medicine, Kyushu University. Experiments were carried out under the Guidelines for Animal Experiments. Laboratory animals were cared for and used in accordance with the experimental animal standards of Japan.

2.2. Reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD45.2 (Ly5.2) (104), anti-CD4 (RM4-5) monoclonal antibodies (mAbs); phycoerythrin (PE)-conjugated anti-F4/80 (BM8), anti-Ly-6G (Gr-1) (RB6-8C5), and anti-IL-10 (JES5-16E3) mAbs; allophycocyanin (APC)-conjugated anti-CD8α (53-6.7), anti-MHC class II (I-A/E) (M5/114.14.2), and anti-CD45.1 (Ly5.1) (A20) mAbs; and Biotin-conjugated CD31 (PECAM-1) (390) mAb were purchased from eBioscience (San Diego, CA). FITC-conjugated CD11b (M1/70), CD11c (HL3), Ly-6G (1A8), and Ly-6C (AL-21) mAbs; APC-conjugated IFN-γ (XMG1.2) mAb; and PerCP-Cy5.5-conjugated anti-CD3ε (145-2C11), anti-CD19 (1D3), and NK1.1 (PK136) mAbs were purchased from BD Biosciences (San Jose, CA).

2.3. Virus

Influenza virus A/FM1/47 (H1N1, a mouse-adapted strain) was provided by the Osaka Prefectural Institute of Public Health, Japan (Brown, 1990; Okuno et al., 1994). Influenza virus A/PR/8/34 (H1N1) was generously provided by Dr. Stephen Turner (the University of Melbourne, Australia). 50% mouse lethal dose (MLD_{50}) was determined in each group of mice by infecting with serial dilutions of virus in a volume of 20 μl. Approximately MLD_{50} of influenza virus A/FM/1/47 was 25 pfu to WT mice, 12 pfu to IFNAR KO mice. Approximately MLD_{50} of influenza virus A/PR/8/34 was 3×10^4 pfu to WT mice and 1×10^4 pfu to IFNAR KO mice. For intranasal infection, mice were fully anesthetized by intraperitoneal injection of 200 μl 10% pentobarbital and then were infected with 20 μl viral suspension (12–25 pfu of A/FM/1/47 or 3×10^4 pfu of A/PR/8/34 in PBS) dropped into one nostril of each mouse.

2.4. Viral titer

Madin–Darby canine kidney (MDCK) cells were plated at 1×10^6 cells in a flat-bottomed 6-well plate 24 h before infection. Supernatants from serially diluted lung homogenates were used at 37 °C for 2 h to infect the MDCK cells. The cells were subsequently overlaid with D-MEM (MP Biomedicals) mixed with 0.75% agarose (Lonza) in the presence of 1 μg/ml *N*-acetyltrypsin (Sigma). Plaques were counted 3 days after infection. The detection limit was 100 pfu.

2.5. Histology

Lung tissues were removed and fixed with 10% neutral buffered formalin and then embedded in paraffin. After the tissues were cut into round slices, the tissue sections were stained with hematoxylin and eosin (H&E) and examined microscopically. Ten fields of each slide were randomly selected, and inflammation was scored on a subjective scale of 0–4, or 5 (corresponding to no inflammation, very mild, mild, moderate, marked, and severe inflammation, respectively) by three independent readers.

2.6. Enzyme-linked immunosorbent assay

The levels of IFN-γ, IL-6, IL-1β, TNF-α, IL-12p70 and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) using a DuoSet ELISA kit (R&D Systems) according to the manufacturer's instructions.

2.7. Cell preparation

Lung tissues were minced and incubated in 1.5 h of stirring at 37 °C in RPMI 1640 with 10% fetal bovine serum (FBS), 150 units/ml collagenase (Invitrogen), and 60 units/ml DNase (Roche Applied Science). The resulting suspension was centrifuged to isolate cells, resuspended in RPMI 1640 with 10% FBS layered on 33% Percoll, and centrifuged at 600*g*. Cells at the bottom of the tube were harvested and washed with RPMI 1640 with 10% FBS two times before use.

2.8. Flow cytometric analysis

Cells were surface stained with various combinations of mAbs and then subjected to intracellular cytokine staining that followed the manufacturer's instructions (BD Biosciences). The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences). For the intracellular cytokine staining, lung cells were incubated with 10 μg/ml brefeldin A (Sigma–Aldrich) for 4 h at 37 °C in 96-well flat-bottom plates at a concentration of 1×10^6/well in a volume of 200 μl of RPMI 1640 containing 10% FCS. For detection of cytokine production from $CD4^+$ cells, the cells were incubated with 10 μg/ml NP-specific ARSALILRGSVAHK peptide 1 h before addition of brefeldin A. For detection of cytokine production from $CD8^+$ T cells, the cells were incubated with 10 μg/ml NP-specific ASNENMDTM peptide 1 h before addition of brefeldin A.

2.9. Generation of mixed bone marrow (BM) chimera

BM cells were prepared from WT (Ly5.1/5.1) mice and IFNAR KO (Ly5.2/5.2) mice by flushing of the femurs and tibias, and resuspended in PBS for injection. A mixture of 2×10^6 cells from WT (Ly5.1/5.1) and IFNAR KO (Ly5.2/5.2) mice was injected intravenously into lethally (10 Gy) irradiated WT recipient mice (Ly5.1/5.2).

2.10. Statistical analysis

Data were analyzed using GraphPad Prism 5 (GraphPad Prism Software). The difference in survival rates was evaluated by the log rank test (Mantel–Cox). Two sets of values were evaluated by the Student's *t*-test. Three sets of values were evaluated by ANOVA,

followed by the Tukey's Multiple Comparison Test. A P value of <0.05 was considered significant.

3. Results

3.1. Mortality, morbidity, and viral clearance in IFNAR KO mice inoculated intranasally with influenza virus A/FM/1/47

We first monitored the survival of IFNAR KO mice daily after influenza virus infection. We challenged WT and IFNAR KO mice with 25 pfu of influenza virus A/FM/1/47 (MLD_{50} of WT mice). All mice in the IFNAR KO group died within 10 days after intranasal infection with 25 pfu of influenza virus A/FM/1/47, while 67% of the WT mice survived beyond day 16 after infection (Fig. 1A). The IFNAR KO mice also had higher morbidity than the WT mice as assessed by body weight (Fig. 1A). We also infected these mice with 3×10^4 pfu of influenza virus A/PR/8/34 (MLD_{50} of WT mice), the standard strain typically used in the murine influenza infection model. As is the case with A/FM/1/47, all mice in the IFNAR KO group died while 71% of the WT mice survived beyond day 16 after infection (Fig. 1B). However, there was no significant difference in the body weight at an early stage of infection (Fig. 1B), and which was the same result as previously reported (Seo et al., 2011). Consistent with previous reports using other influenza strains (Koerner et al., 2007; Seo et al., 2011; Szretter et al., 2009), the IFNAR KO mice were highly susceptible to influenza virus A/FM/1/47 infection.

We next examined the kinetics of viral titers in the lungs following intranasal challenge of influenza virus A/FM/1/47. The viral titers in the lungs were significantly higher at an early stage (on days 3 and 6) in IFNAR KO mice than in WT mice. The viruses decreased to undetectable level (<100 pfu) in WT mice, and markedly decreased ($1.3 \times 10^3 \pm 71$ pfu) in IFNAR KO mice on day 9 (Fig. 1C).

We examined the morphological and histological changes in the lungs of IFNAR KO mice by day 9 after influenza A virus infection.

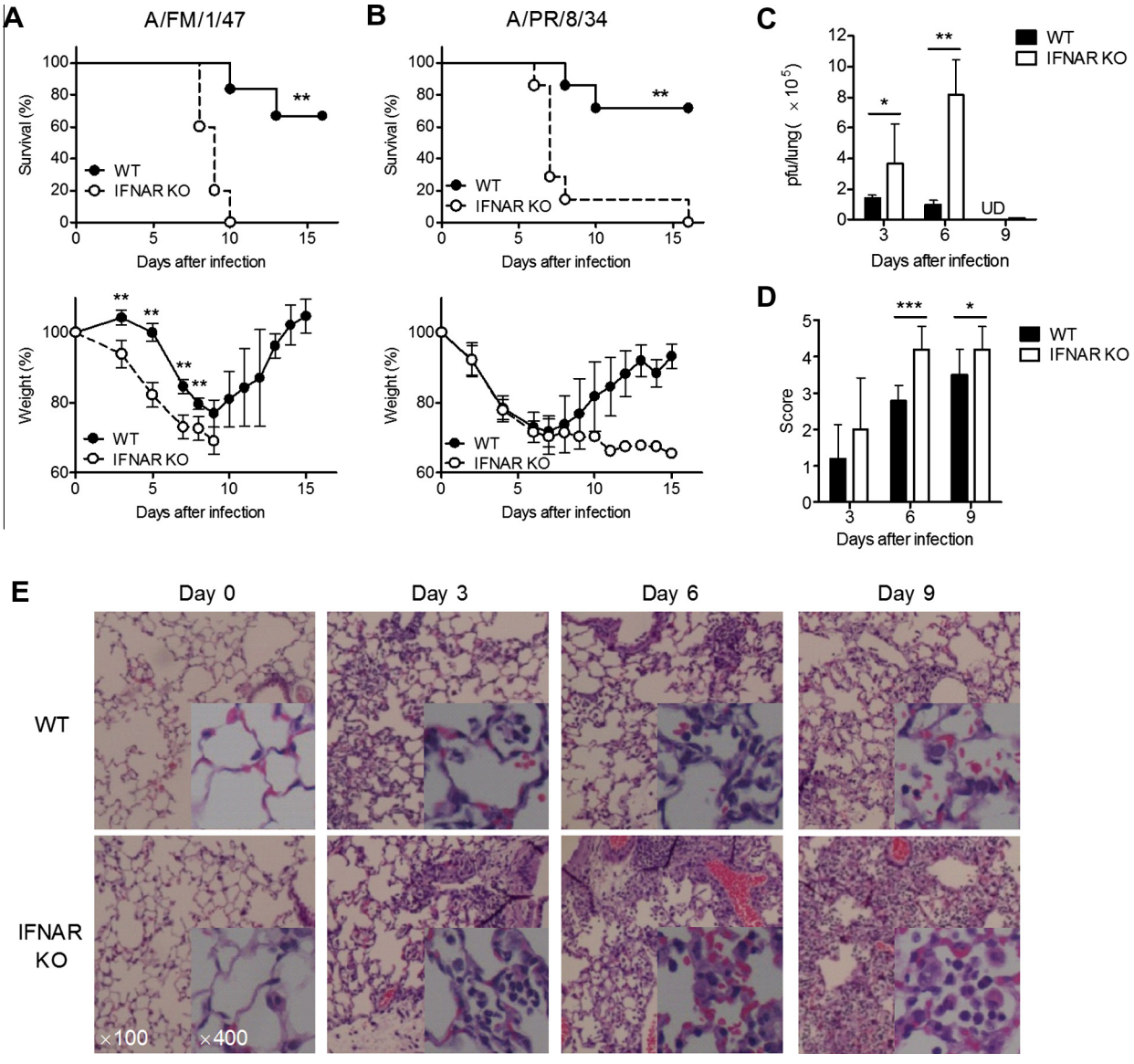

Fig. 1. Mortality, morbidity, and viral clearance in IFNAR KO mice after infection with influenza virus A/FM/1/47. (A) Survival rate and weight loss of WT and IFNAR KO mice after intranasal infection with 25 pfu of influenza virus A/FM/1/47. 6 mice in WT group and 5 mice in IFNAR KO group were used. (B) Survival rate and weight loss of WT and IFNAR KO mice after intranasal infection with 3×10^4 pfu of influenza virus A/PR/8/34. Each group consists of 7 mice. (C–E) WT and IFNAR KO mice were intranasally infected with 25 pfu of influenza virus A/FM/1/47. Each group consists of 3 mice. (C) Viral titers in the lungs on days 3, 6, and 9 after infection. UD: undetectable level. (D) Histological scores of lung tissues stained with H&E on days 3, 6, and 9 after infection. (E) Representative photos of lung tissues stained with H&E on days 3, 6, and 9 after infection. The data are representative of at least three independent experiments. Error bars represent mean ± SD. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

As shown in Fig. 1D and E, a typical morphological view of the lungs, the lungs of IFNAR KO mice were severely injured as assessed by massive and diffuse leukocyte accumulation, alveolar wall thickness, and alveolar hemorrhage in comparison with WT mice on days 6 and 9 after influenza virus infection. Various kinds of inflammatory cells including polymorphonuclear neutrophils, lymphocytes, and macrophages accumulated in the infected lungs. These results suggest that the lung injury induced by influenza A virus infection was exaggerated in the absence of type I IFN signaling.

3.2. Cytokine production in the lungs of IFNAR KO mice inoculated intranasally with influenza virus A/FM/1/47

To determine the proinflammatory cytokine levels following influenza virus A/FM/1/47 infection, we measured cytokine levels in the lung homogenates of IFNAR KO mice during the course of infection by using ELISA. The levels of IL-1β, IL-6, and IFN-γ were significantly higher in IFNAR KO mice than in WT mice on days 3 and 6 after infection (Fig. 2A). The levels of TNF-α and IL-12p70 showed no significant differences between the two groups after infection. Conversely, the IL-10 level in the lungs was significantly lower in IFNAR KO mice than in WT mice on days 5 and 8 after infection. The IL-10 level in the lungs of WT mice was sustained by the late phase of infection.

Next we compared the proinflammatory cytokine production between WT and IFNAR KO mice after infection with each MLD_{50} dose of viruses (25 pfu for WT mice and 12 pfu for IFNAR KO mice), which provides approximately the same viral load in both groups (Fig. 2B). Proinflammatory cytokines such as IL-1β, IL-6, and IFN-γ were significantly higher in IFNAR KO mice than in WT mice even in the same viral load (Fig. 2C), suggesting that excessive inflammation in IFNAR KO mice cannot be ascribed to different viral load alone.

3.3. IL-10-producing cell population infiltrating the lungs of IFNAR KO mice inoculated intranasally with influenza virus A/FM/1/47

To identify the IL-10-producing cell population infiltrating the lungs of mice inoculated intranasally with influenza virus A/FM/1/47, the lung cells were harvested on days 3, 6, and 9 after infection, and intracellular cytokine FACS analysis was carried out. The main producer of IL-10 during the course of infection was $CD11b^+$ cells (Fig. 3A). IL-10-producing $CD4^+$ cells especially $Foxp3^-$ cells increased in the late phase of infection, and though $CD8^+$ T cells also produced IL-10 on day 9, the

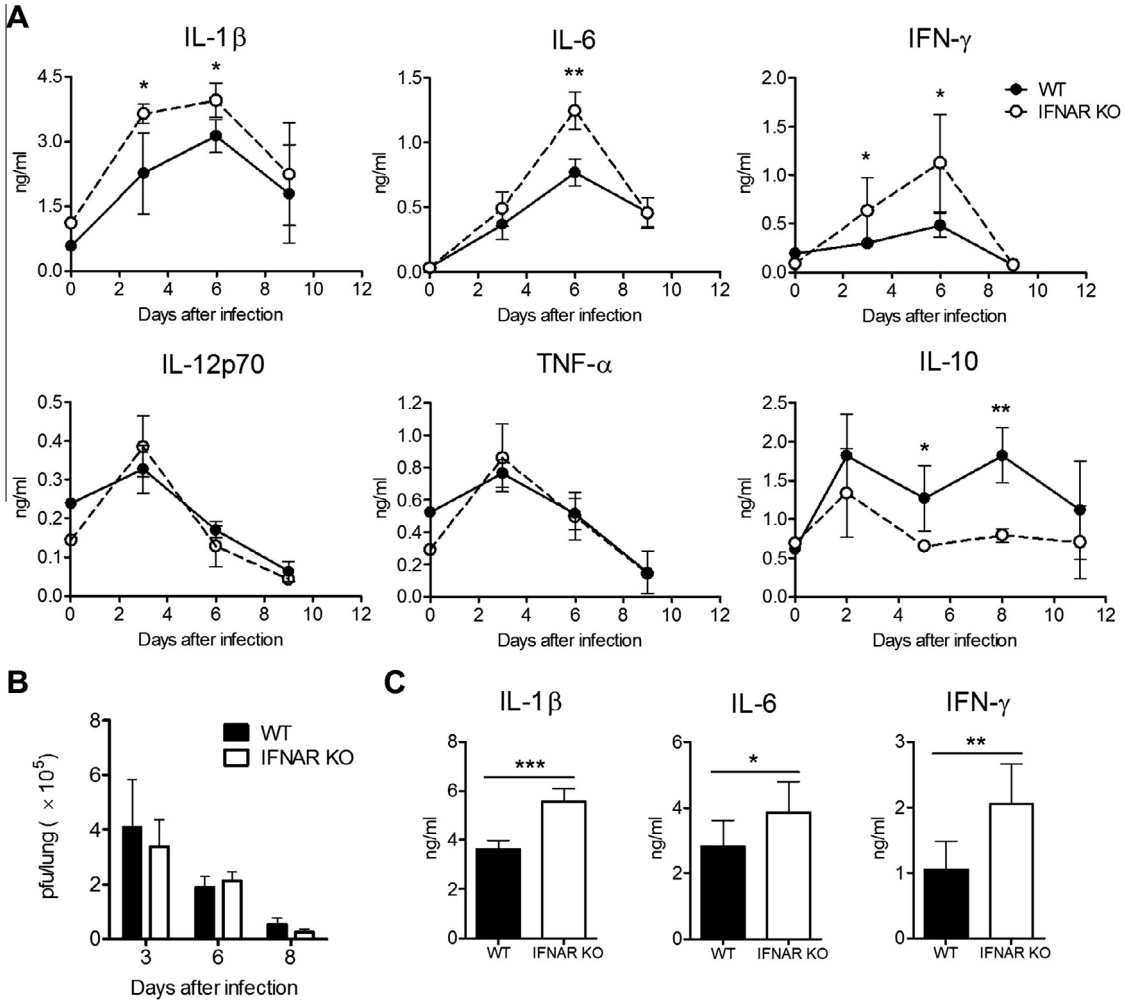

Fig. 2. Cytokine production in the lungs of IFNAR KO mice after infection with influenza virus A/FM/1/47. (A) The lungs were removed on the indicated days after infection with 25 pfu of influenza virus A/FM/1/47. Homogenized lungs were diluted with PBS, and cytokine levels of the supernatant were quantified by ELISA. Each group consists of 3–5 mice. (B and C) WT and IFNAR KO mice were intranasally infected with 25 pfu and 12 pfu of influenza virus A/FM/1/47, respectively. (B) Viral titers in the lungs on days 3, 6, and 8 after infection. Each group consists of 6 mice. (C) Cytokine levels in the lungs on day 6 after infection quantified by ELISA. Each group consists of 9 mice. The data are representative of at least three independent experiments. Error bars represent mean ± SD. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

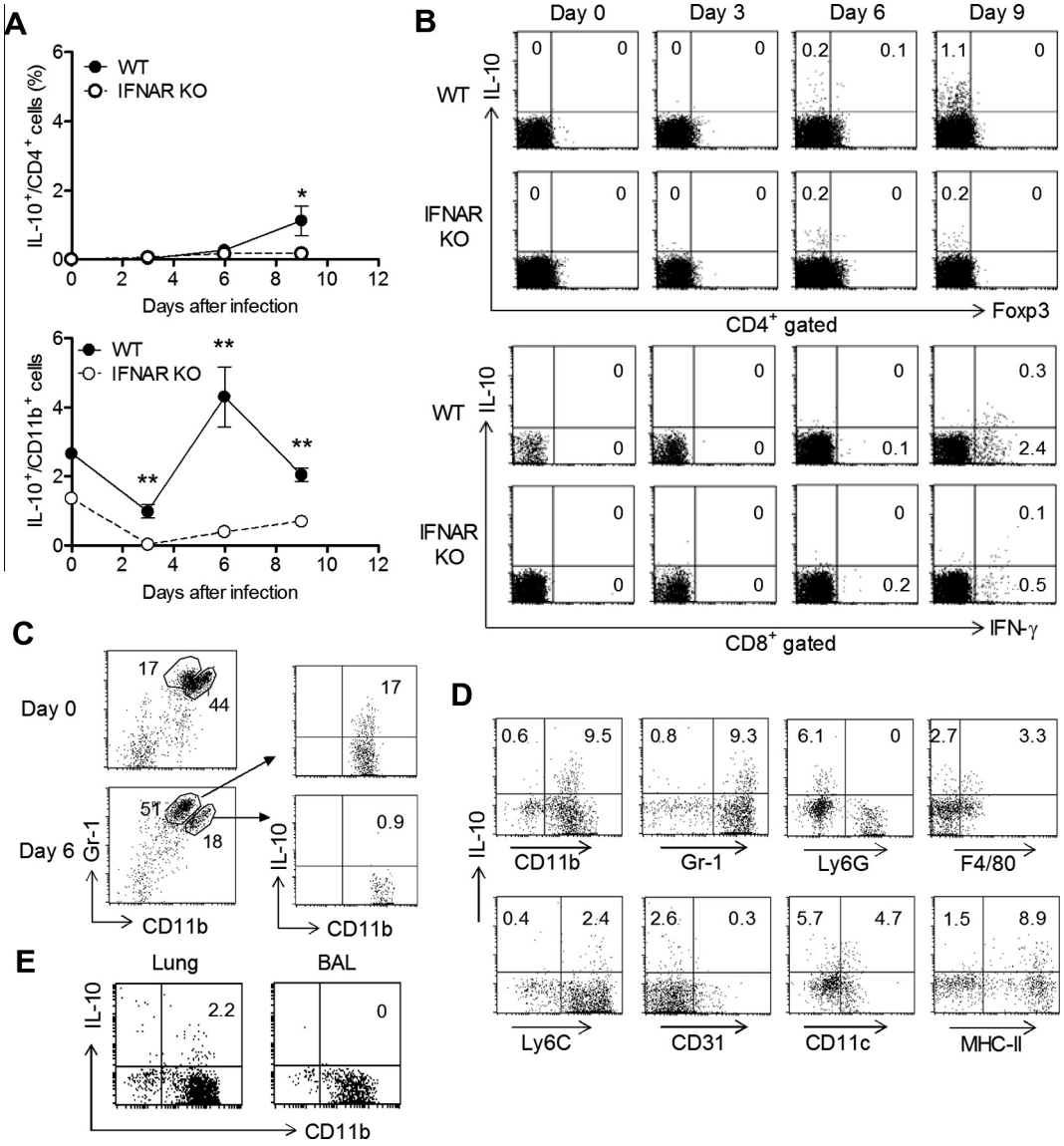

Fig. 3. IL-10-producing cells in the lungs after infection with influenza virus A/FM/1/47. (A) The lung cells were harvested on days 3, 6, and 9 after infection with 25 pfu of influenza virus A/FM/1/47. The samples were incubated with 10 μg/ml brefeldin A for 4 h at 37 °C. Expression of IL-10 was detected by intracellular staining and analyzed by flow cytometry. IL-10$^+$CD4$^+$ cells were analyzed after lymphocyte gating. IL-10$^+$CD11b$^+$ cells were analyzed after CD3$^-$CD19$^-$NK1.1^8 - cell gating. Each group consists of 3 mice. Error bars represent mean ± SD. $^*p < 0.05$; $^{**}p < 0.01$. (B) The lung cells were harvested on days 3, 6, and 9 after infection with 25 pfu of influenza virus A/FM/1/47. The samples were incubated with 10 μg/ml NP-derived ARSALILRGSVAHK peptide for CD4$^+$ cells and 10 μg/ml NP-derived ASNENMDTM peptide for CD8$^+$ cells for 1 h at 37 °C, then 10 μg/ml brefeldin A was added and incubated for 4 h at 37 °C. Expression of IL-10 was detected by intracellular staining and analyzed by flow cytometry. Dot plots are shown after CD4$^+$ (above) and CD8$^+$ (below) cell gating. (C) WT lung cells were harvested on day 6 after infection and incubated with 10 μg/ml brefeldin A for 4 h at 37 °C. After intracellular staining, IL-10-producing cells were analyzed by flow cytometry. The lung cells were analyzed after CD3$^-$CD19$^-$NK1.1$^-$ cell and CD11b^{high} or CD11b^{int} cell gating. (D) WT lung cells were harvested on day 6 after infection and incubated with 10 μg/ml brefeldin A for 4 h at 37 °C. After intracellular staining, IL-10-producing cells were analyzed by flow cytometry. The lung cells were analyzed after CD3$^-$CD19$^-$NK1.1$^-$ cell gating. (E) WT lung cells and airway cells (by broncho-alveolar lavage; BAL) were harvested on day 6 after infection and incubated with 10 μg/ml brefeldin A for 4 h at 37 °C. Those cells were analyzed after CD3$^-$CD19$^-$NK1.1$^-$ cell gating. The dot plots are representative of 3 mice. The numbers indicate the percentage of cells in the corresponding circles and quadrants.

amounts of IL-10 from these T cells were very small (Fig. 3A and B). The number of IL-10-producing CD11b$^+$ cells was significantly lower in the lungs of IFNAR KO mice than in WT mice. Most of the CD11b$^+$ cells expressed Gr-1$^+$, and one subset of these cells produced a high level of IL-10 (Fig. 3C). These cells also expressed CD11c^{int}, MHC-II^{high}, Ly6G^{neg}, F4/80^{int}, Ly6C^{high}, and CD31^{low} (Fig. 3D). There were no IL-10-producing cells in airways harvested by broncho-alveolar lavage (Fig. 3E). We stimulated those cells by poly I:C (the ligand of TLR3) as a positive control, which provided around 7% IL-10$^+$ cells in whole CD11b$^+$ cells (data not shown).

3.4. Direct effect of IFNAR signaling on IL-10 producing cells after infection with influenza virus A/FM/1/47

IFNAR signaling is known to enhance IL-10 production, although the precise signaling pathways remain elusive (Gonzalez-Navajas et al., 2012). To examine whether a direct or indirect effect of IFNAR signaling was critical to induce IL-10 production, we performed a mixed BM chimera experiment using Ly5.1-congenic C57BL/6 mice. Recipient WT mice (Ly5.1/5.2) were lethally irradiated and injected with a mixture of BM cells from WT (Ly5.1/5.1) and IFNAR KO (Ly5.2/5.2) mice (Fig. 4A). WT and IFNAR

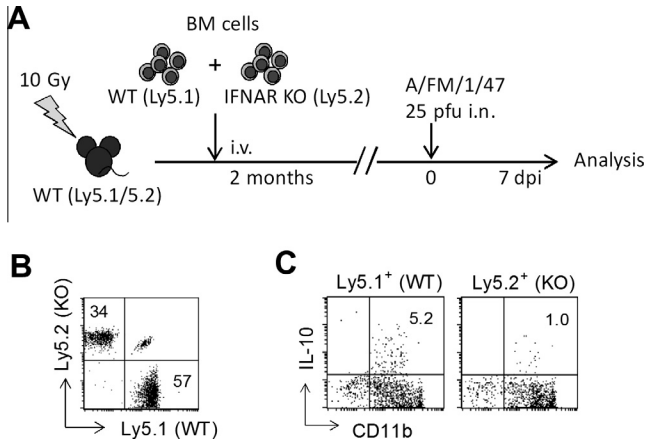

Fig. 4. IL-10-producing cells in the lungs of mixed bone marrow chimeric mice after infection with influenza virus A/FM/1/47. (A) Equal numbers (2×10^6) of BM cells from WT (Ly5.1/5.1) and IFNAR KO (Ly5.2/5.2) mice were mixed and injected intravenously into lethally (10 Gy) irradiated WT (Ly5.1/5.2) recipient mice. Two months after infection, the mice were infected with 25 pfu of influenza virus A/FM/1/47, and lung cells were analyzed on day 7 after infection. (B) Before influenza virus infection, WT (Ly5.1) and IFNAR KO (Ly5.2) cells in the peripheral blood of host (Ly5.1/5.2) mice were analyzed by flow cytometry. The numbers indicate the percentage of cells in the corresponding quadrants. (C) The lung cells were harvested on day 7 after infection and incubated with 10 μg/ml brefeldin A for 4 h at 37 °C. After intracellular staining, $Ly5.1^+$ or $Ly5.2^+$ gated IL-10-producing cells were analyzed by flow cytometry. The experiments were repeated two times, and 7 BM chimeric mice were used in each experiment. Dot plots are representative of 14 mice. The numbers indicate the percentage of $IL\text{-}10^+$ cells in $CD11b^+$ cells.

KO BM-derived cells were equally distributed in host mice 2 months after BM transplantation (Fig. 4B). On day 7 after infection, $CD11b^+$ cells from IFNAR KO mice produced a significantly lower level of IL-10 than that of WT cells in all BM chimeric mice (Fig. 4C). These results indicate that IFNAR on IL-10-producing cells is crucial to enhancing IL-10 production after influenza A virus infection.

3.5. Exogenous IL-10 protected IFNAR KO mice from lethality after intranasal infection with influenza virus A/FM/1/47

To elucidate the possibility that IL-10 induced by IFNAR signaling might suppress an excessive immune response resulting in lethal lung injury, we examined whether adding exogenous IL-10 can protect IFNAR KO mice from lethality after influenza virus infection. As shown in Fig. 5A, administration of recombinant IL-10 (rIL-10) after influenza virus infection improved survival rate in IFNAR KO mice, though it did not affect viral titers in the lungs (Fig. 5B). Lung inflammation assessed by H&E staining was less severe in rIL-10-treated IFNAR KO mice compared with PBS-treated KO mice (Fig. 5B and C). IL-6 and IFN-γ production in infected lungs also decreased in the rIL-10-treated IFNAR KO mice, though IL-1β level had no significant differences between PBS-treated and rIL-10-treated KO mice (Fig. 5D). These results suggest that impaired IL-10 production may have contributed to cytokine storm in IFNAR KO mice after influenza virus infection.

4. Discussion

Type I IFNs, especially IFN-αs and IFN-β, are critical effectors of antiviral activity directly through inhibition of protein translocation and suppression of viral duplication. IFNAR KO mice exhibited increased mortality with acute lung injury after influenza A virus infection. The viral titers in the lungs were significantly higher in IFNAR KO mice than in WT mice at an early stage after influenza A virus infection, though the viruses were equally eliminated afterward in both groups. These results indicate that although type I IFNs are important to eliminate influenza viruses at an early stage of infection, the lung injury worsened in IFNAR KO mice was caused not only by direct viral infection to lung epithelial cells. Increased levels of proinflammatory cytokines such as IL-1β, IL-6, and IFN-γ were also detected in the lungs during the course of infection. The invasion of viruses is initially sensed as pathogen-associated molecular patterns (PAMPs) by the host's innate immunity system, triggering rapid antiviral responses that involve the release of proinflammatory cytokines. IL-10 is also induced by TLR signaling for activation of NF-κB and ERK (Saraiva and O'Garra, 2010) and increased viral load induces higher level of IL-10 production in dose dependent manner (Sun et al., 2010). It is possible that increased viral load was responsible for the acute lung injury in IFNAR KO mice through both the direct cytopathic effect and cytokine storm mediated by increased proinflammatory cytokine production by innate immunity after influenza A virus infection. However, we also found that proinflammatory cytokines in IFNAR KO mice were still significantly higher than in WT mice even under the infection of the same viral load. Moreover, production of IL-10 was impaired in IFNAR KO mice in spite of increased viral load, especially during an ongoing infection, and restoration of IL-10 in IFNAR KO mice significantly suppressed proinflammatory cytokine production and improved lung pathology. These results raise a possibility that increased proinflammatory cytokine production in IFNAR KO mice during the course of infection with influenza A virus was due not only to increased viral load as PAMPs but also to decreased IL-10 production in the absence of IFNAR signaling. In our study, increased IL-1β in IFNAR KO mice was not suppressed by exogenous IL-10. Recently, Guarda et al. reported that type I IFNs inhibit IL-1 production not only via induction of IL-10 but also via repression of the activity of the NLRP1 and NLRP3 inflammasomes (Guarda et al., 2011). Therefore, it is also possible that defective IFNAR signaling increases IL-1β because IL-1β maturation machinery is not repressed.

The role of IL-10 during acute influenza virus infection appears to be contradictory. Sun et al. (2009) previously found that inhibition of IL-10 signaling during an ongoing influenza virus infection resulted in increased inflammation and decreased survival, whereas McKinstry et al (2009) reported that inhibition of IL-10 signaling before infection enhanced viral clearance and increased survival after influenza virus infection. Conclusions regarding the beneficial or detrimental role of IL-10 in these two studies were entirely dependent on the timing of IL-10 signaling. Defective IL-10 signaling at the initiation of acute infection may enhance viral clearance whereas defective IL-10 signaling later than the height of acute infection may accelerate acute lung injury by excessive immune responses.

In the present study, we found that main producer of IL-10 during influenza virus infection expressed $CD11b^+$. These cells also expressed $CD11c^{int}$, $MHC\text{-}II^{high}$, $Ly6G^{neg}$, $F4/80^{int}$, $Ly6C^{high}$, and $CD31^{low}$, and did not exist in airways. It has been reported that alveolar macrophages express $CD11b^{low}CD11c^{high}$, dendritic cells express $CD11b^{high}CD11c^{int\sim high}$, and monocytes/interstitial macrophages express $CD11b^{high}CD11c^{low\sim int}$ (Gonzalez-Juarrero et al., 2003; Janssen et al., 2011; Smith et al., 2009). Besides, myeloid-derived suppressor cells, recently reported heterogeneous cell population in chronic and acute inflammatory diseases, are immature cells expressing $CD31^{high}$ and $MHC\text{-}II^{low}$ (Cuenca et al., 2011). Considering all these data, we concluded that IL-10-producing cells during influenza virus infection are dendritic cells or monocytes/interstitial macrophages rather than alveolar macrophages. Single-stranded RNA viruses such as influenza A virus have been reported to trigger type I IFNs through recognition by TLR3 and retinoic acid-inducible gene I (RIG-I) in DCs, fibroblasts, or alveolar epithelial cells and through TLR7 in plasmacytoid DCs (pDCs) (Bowie and Unterholzner, 2008; Takeuchi and Akira, 2009), although

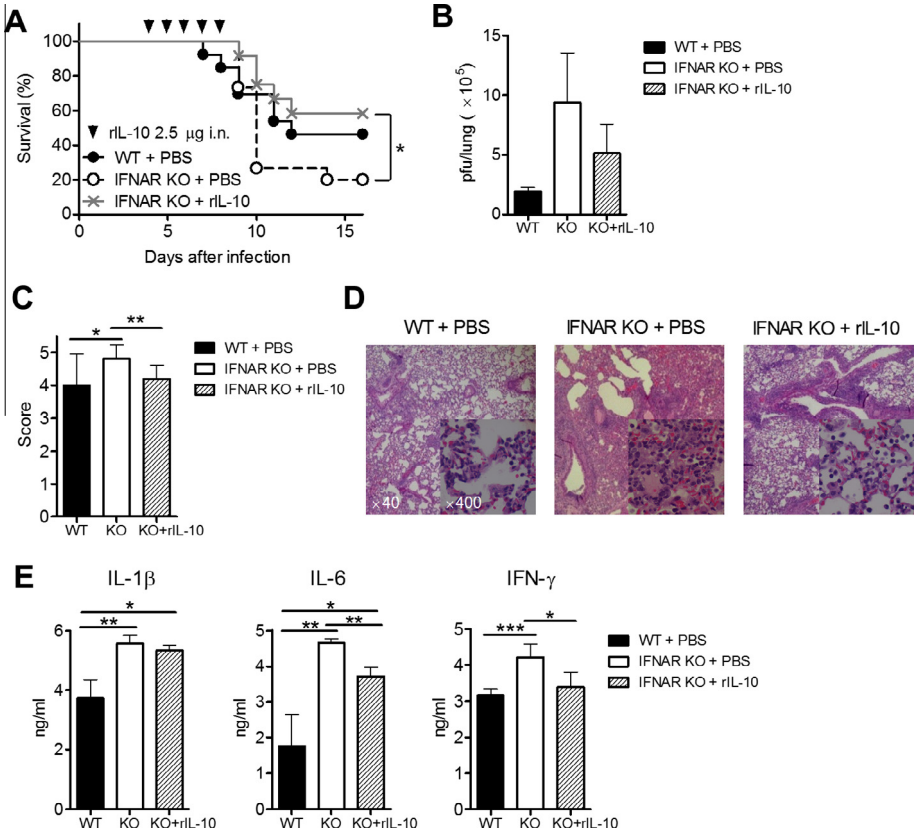

Fig. 5. *In vivo* administration of rIL-10 to IFNAR KO mice after infection with influenza virus A/FM/1/47. (A) Survival rate of rIL-10-treated IFNAR KO mice after infection with 25 pfu of influenza virus A/FM/1/47. 2.5 μg rIL-10 or PBS was intranasally administered to WT and IFNAR KO mice daily on days 4–8 after infection. Each group consists of 13–15 mice. Data were pooled from three independent studies. (B–E) rIL-10 or PBS was intranasally administered to IFNAR KO and WT mice daily on days 4–6 after infection. The lungs of each mouse were removed 12 h after administration of rIL-10 on day 6. Each group consists of 5 mice. (B) Viral titers in the lungs. (C) Histological scores of lung tissues stained with H&E. (D) Representative photos of lung tissues stained with H&E. (E) Homogenized lungs were diluted with PBS and the levels of IL-1β, IL-6 and IFN-γ in the supernatant were quantified by ELISA. The data are representative of at least three independent experiments. Error bars represent mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

NS1 protein of influenza A virus has been shown to inhibit type I IFNs production in infected cells (Hale et al., 2008). IFNAR signaling via local type I IFNs further amplifies type I IFNs synthesis in an IRF-7 dependent mechanism. We confirmed that IFNAR on IL-10 producers is essential to enhancing IL-10 production after influenza A virus infection, and IL-10 production was completely abolished in IFNAR KO mice especially at the late phase of infection. Taken together, we speculate that an amplification loop of type I IFNs may enhance IL-10 production during an acute infection and thereby suppress excessive production of proinflammatory cytokines, resulting in limiting of excessive inflammation and acute lung injury following influenza A virus infection. The IL-10 production by $CD11b^{+}$ cells transiently decreased at day 3 after infection. We speculate that IL-10 is produced constantly to keep lung homeostasis in a steady state of naïve mice, but $CD11b^{+}$ cells producing proinflammatory cytokines other than IL-10 migrated and increased at the early stage of infection, thereby IL-10-producing $CD11b^{+}$ cells may decrease at this stage.

In conclusion, in this study, we demonstrated the immunomodulatory function of type I IFNs during lethal influenza virus infection. Type I IFNs can be useful for antiviral therapy not only by suppressing viral replication but also by limiting excessive inflammation via IL-10 production.

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