The pathological effects of CCR2+ inflammatory monocytes are amplified by an IFNAR1-triggered chemokine feedback loop in highly pathogenic influenza infection

Sue-Jane Lin1,2,3*, Ming Lo4, Rei-Lin Kuo1,2,3, Shin-Ru Shih1,2,3, David M Ojcius5, Jean Lu6, Chien-Kuo Lee7, Hui-Chen Chen8, Meei Yun Lin1, Chuen-Miin Leu9, Chia-Ni Lin3,10 and Ching-Hwa Tsai4*

Abstract

Background: Highly pathogenic influenza viruses cause high levels of morbidity, including excessive infiltration of leukocytes into the lungs, high viral loads and a cytokine storm. However, the details of how these pathological features unfold in severe influenza infections remain unclear. Accumulation of Gr1+ CD11b+ myeloid cells has been observed in highly pathogenic influenza infections but it is not clear how and why they accumulate in the severely inflamed lung. In this study, we selected this cell population as a target to investigate the extreme inflammatory response during severe influenza infection.

Results: We established H1N1 IAV-infected mouse models using three viruses of varying pathogenicity and noted the accumulation of a defined Gr1+ CD11b+ myeloid population correlating with the pathogenicity. Herein, we reported that CCR2+ inflammatory monocytes are the major cell compartments in this population. Of note, impaired clearance of the high pathogenicity virus prolonged IFN expression, leading to CCR2+ inflammatory monocytes amplifying their own recruitment via an interferon-α/β receptor 1 (IFNAR1)-triggered chemokine loop. Blockage of IFNAR1-triggered signaling or inhibition of viral replication by Oseltamivir significantly suppressed the expression of CCR2 ligands and reduced the influx of CCR2+ inflammatory monocytes. Furthermore, trafficking of CCR2+ inflammatory monocytes from the bone marrow to the lung was evidenced by a CCR2-dependent chemotaxis. Importantly, leukocyte infiltration, cytokine storm and expression of iNOS were significantly reduced in CCR2−/− mice lacking infiltrating CCR2+ inflammatory monocytes, enhancing the survival of the infected mice.

Conclusions: Our results indicated that uncontrolled viral replication leads to excessive production of inflammatory innate immune responses by accumulating CCR2+ inflammatory monocytes, which contribute to the fatal outcomes of high pathogenicity virus infections.

Keywords: Influenza A virus, CCR2+ inflammatory monocytes, IFNAR1, CCL2, CCL7 and CCL12

* Correspondence: suejane.lin@mail.cgu.edu.tw; chtsai@ntu.edu.tw
1 Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan
2 Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan
3 Full list of author information is available at the end of the article

© 2014 Lin et al; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background

Influenza A virus (IAV) is a common human respiratory virus and causes seasonal epidemic and pandemic infections. In the past 100 years, pandemics of influenza have been caused by the IAV strains H1N1 (1918), H2N2 (1957), H3N2 (1968) and H1N1 (2009) [1,2]. These pandemic strains vary in their virulence and pathogenicity. Compared to the 1968 and 2009 pandemics, the 1957 pandemic featured intermediate pathogenicity, while the virus causing the 1918 pandemic was relatively highly pathogenic in the human population [2]. Currently we are threatened by sporadic infections by emerging avian IAVs, including highly pathogenic avian H5N1 and H7N9 viruses [1,3]. Two well known, highly pathogenic IAVs, 1918 H1N1 and avian H5N1 cause high levels of morbidity including excessive infiltration of neutrophils and monocytes into the lungs, high viral loads and hypercytokinemia, with significant increases of IL-1, IL-6, IL-8, TNF, CXCL10 and CCL2 in the patients' plasma [4-6]. Thus, cytokines and chemokines induced at high levels by IAV infections have become targets for the development of IAV therapy. However, the results of experiments using knock-out mice indicate that none of them alone determines highly pathogenic virus-induced lethality [7,8]. Thus, we used another approach to identify the immune cell types that are recruited during infection and contribute to the excessive inflammatory responses during highly pathogenic virus infection.

H1N1 IAV circulate continuously in the human population, and the three H1N1 strains selected for this study display low, intermediate and high virulence in mice as follows: (1) seasonal H1N1 A/Taiwan/141/02 (141; low virulence); (2) pandemic H1N1 A/Taiwan/126/2009 (swine-origin influenza virus, SOIV; intermediate virulence) and (3) mouse adapted H1N1 A/Puerto Rico/8/34 (PR8; high virulence). Using these mouse models, we demonstrated that rate of viral clearance and disease severity is correlated with the numbers of a defined Gr1 + CD11b + myeloid population in the lung. Until now, it is not clear how and why they accumulate in the severely inflamed lung. In this study, we selected this cell population as a target to investigate the extreme inflammatory response during severe IAV infection.

In this paper, we report that CCR2+ inflammatory monocytes play a double edge sword in anti-viral responses and immunopathogenesis. Using established infection models with variable rates of viral clearance, which are accompanied by different levels of inflammatory infiltrates, we found that an amplified inflammatory chemokine feedback loop links the impaired clearance of highly pathogenic virus and a massive infiltration of CCR2+ inflammatory monocytes. So, we sought to investigate which cell types are responsible for the production of CCR2 ligands. Furthermore, we identified the inflammatory signals that are triggered by an impaired anti-viral response to induce expression of CCR2 ligands. Finally, the pathological effects of excessive accumulated CCR2+ inflammatory monocytes were explored during highly pathogenic IAV infection.

Overall, we provided a comprehensive study to address the detail mechanism why and how accumulated CCR2+ inflammatory monocytes involved in highly pathogenic IAV infections. Impaired clearance of virus led to spread of virus to newly arrived CCR2+ inflammatory monocytes and to sustain production of IFNAR1-induced CCR2 ligands, which attract BM-derived CCR2+ monocytes migrated to inflamed lung and amplify their own recruitment continuously through the IFNAR1-dependent chemokine feedback loop, resulting in an enhancement of CCR2+ inflammatory monocytes-mediated pathological effects.

Methods

Mouse strains

C57BL/6 and CCR2−/− mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). IFNAR1−/− mice were obtained from Dr. Chien-Kuo Lee (Graduate Institute of Immunology, National Taiwan University, Taipei, Taiwan). MyD88−/− mice were obtained from Hui-Chen Chen (Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan). Mice were maintained under specific pathogen free conditions in Chang Gung University. All animal experiments were performed according to the animal protocol approved by the Institutional Animal Care and User Committee of Chang Gung University and in accordance with the guidelines of Animal Care and Use of Laboratory Animals of the Taiwanese Council of Agriculture.

Virus preparation and inoculation

All segmented expression plasmids of IAV were kindly provided by Dr. Shin-Ru Shih of Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, Taiwan. Recombinant IAVs, seasonal 141, pandemic SOIV and mouse adapted PR8 were generated using a reverse genetics system, according to previous reports [16,17]. Briefly, 293 T cells were transfected by using 15 μl Trans IT-LT1 (Mirus Bio LLC) with 1 μg per each plasmid (pPolI-PB2, −PB1, −PA, −HA, −NP, −NA,
–M, –NS of 141, SOIV or PR8). Recombinant IAVs were harvested and propagated in 10 day-old embryonated chicken eggs. Harvested viruses were aliquoted and stored at –80°C until use. For IAV inoculation, mouse was infected intranasally with 200 PFU of virus.

Plaque assays
Lungs were harvested and grind tissue suspension were frozen in 600 μl aliquots. Viral supernatant was thawed and then 10 folds serially diluted. MDCK cells were cultured at a density of 1 × 10⁶ cells/well in a 6 well-plate. One hundred microliter of each serial dilution containing tryptsin was added to 90% confluent of MDCK cells. After 1 hour incubation, each well was overlaid with a ratio of 1:1 mixture of 0.8% agarose and 2× serum free DMEM to wells. Two days later, the plaques were visualized by addition of 1% crystal violet and plaque forming unit (units/lung) was calculated.

Preparation of lung leucocytes, mediastinal lymph node (MLN), bone marrow (BM) cells and PBMC
Harvested lungs were homogenized using a metal mesh and the suspension was treated with type I collagenase (Invitrogen) per lung for 30 mins at 37°C. Cells were recovered and washed once with complete PRMI medium containing 10% FBS, 1 mM glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin. The pelvic and femoral bones were harvested and BM cells were flushed out with complete RPMI medium by insertion of a 1 ml syringe with a 25G needle into one end of the bone. MLN cells were homogenized using glass slides with ground edges. Leukocytes were obtained from the lungs, peripheral blood, BM and MLN after RBC lysis buffer treatment.

Cytokine antibody array and ELISA
To obtain bronchoalveolar lavage fluid (BALF), airways were flushed three times with 0.5 ml sterile PBS and centrifuged to remove infiltrating cells. Pooled BALFs were assayed using the R & D mouse cytokine arrays (R & D Systems, Inc.) according to the manufacturer’s instructions. CCL2, CCL7 and CCL12 proteins were measured in serum using ELISA kits (eBioscience) according to the manufacturer’s instructions.

Immunofluorescent surface and intracellular staining
Two million cells were stained with fluorescently labeled mAbs, including Gr1, CD11b, Ly6C, Ly6G, CCR2 and CX3CR1 for 30 min at 4°C. All Abs were purchased from BD Biosciences, except for CCR2 mAb (R & D Systems). After staining, the cells were fixed with Cytofix (BD Biosciences) for 5 min at 4°C. For intracellular staining, cells were stained with fluorescently labeled anti-Gr1, –CD11b and -Ly6C mAbs and then fixed with Cytofix/cytoperm (BD Biosciences) for 20 mins at 4°C. Fixed cells were further stained with FITC-labeled anti-IAV nucleoprotein (NP) Ab (Abcam) for another 30 min at 4°C. Finally, the cells were washed and re-suspended in FACS buffer (PBS with 2% FBS) and analyzed by LSRII flow cytometry (BD Biosciences).

Cell sorting and Wright stain
Infiltrating leukocytes from the lungs were harvested and incubated with anti-Gr1, –CD11b and -Ly6G mAbs. Gr1 + CD11b+, Gr1-CD11b-, Gr1 + CD11b + Ly6G + (for granulocyte sorting) or Gr1 + CD11b + Ly6G- (for monocyte sorting) leukocytes were sorted by FACSAria (BD Biosciences). For morphological evaluation of Gr1 + CD11b + cells, sorted cells were spun onto glass slides at 250 rpm for 5 min using a Shandon Cytospin 3 Centrifuge (Global Medical Instrumentation Inc.) and stained with HemaTek stain Pak (Siemens Healthcare Diagnostic Inc.) using an automatic HemaTek hematology stainer (Bayer Healthcare, LLC.).

RNA extraction, reverse transcription and quantitative polymerase chain reaction (RT-QPCR)
Total RNA was extracted from isolated or sorted cells using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was used to synthesize cDNA with Superscript III reverse transcriptase (Invitrogen). TaqMan® Gene Expression Assays (Applied Biosystems) were performed to detect mouse CCL2, CCL7, CCL12, iNOS, IFNβ and GADPH mRNAs. Expression of the various genes was normalized with the GADPH level in each group. Relative gene expression was determined using △△Ct analysis.

Western blotting
Frozen lung tissues were lysed using lysis buffer (100 mM Tris, 250 mM NaCl, 0.5% sodium deoxycholate, 1 mM PMSF and 0.5% NP40). Tissue lysates were resolved by electrophoresis in SDS-polyacrylamide gels and electrotransferred onto Hybond-P PVDF membranes (GE Healthcare). Milk blocked blots were incubated with anti-actin and -NP antibodies at 4°C overnight and then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch) at room temperature for 1 hr. The proteins were revealed using the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Oseltamivir treatment
PR8-infected mouse was treated with 50 mg Oseltamivir daily according to a previous report [18].

Treatment with anti-IFNAR1 blocking antibody
Day 3 post-infected mice were anesthetized and then injected intranasally with either 50 μg of IgG isotype control antibody (Abcam) or 50 μg of anti-IFNAR1

Lin et al. Journal of Biomedical Science 2014, 21:99
http://www.jbiomedsci.com/content/21/1/99
Page 3 of 18
Figure 1 (See legend on next page.)
antibody (eBioscience). After 3 days, infiltrating cells were counted and then stained with specific Abs against with Gr1, CD11b, Ly6G, Ly6C and CCR2.

Adoptive transfer of BM enriched CCR2+ monocytes into mice
BM cells from naïve B6 mice were harvested and monocytes were enriched by negative selection using an EasySep™ Mouse Monocyte Enrichment Kit and EasySep™ magnet system (STEMCELL Technologies Inc.). Enriched monocytes were suspended in PBS at a concentration of 2.0 x 10^7 cells/ml and incubated with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) solution for 12 min at 37°C. One million CFSE-labeled cells were adoptively transferred via the tail vein into naïve or virus-infected mice. After 2 days, leukocytes were harvested from the lungs and stained with anti-Ly6C and anti-CCR2 antibodies. Finally, CCR2 + CFSE + transferred monocytes were traced using flow cytometric analysis.

Statistical analysis
Statistical significance of the data was analyzed by Student’s two-tailed t test.

Results
Excessive accumulation of CCR2+ inflammatory monocytes in severe IAV infection
We observed varying levels of body weight change and lung inflammation in the infected mice and investigated which infiltrating cell type was associated with severe inflammation. As shown in Figure 1A, mice infected with the mild 141 strain lost 5%-10% of their original body weight, while the moderate SOIV strain caused 15%-20% original body weight loss. Notably, severe PR8 infection caused progressive weight loss and led to 100% mortality in the infected mice at day 7-10 post-infection. In these infections, lung inflammation was dramatically correlated with body weight loss at day 7 post-infection (Figure 1B). Furthermore, we demonstrated that a defined Gr1 + CD11b + myeloid population is preferentially recruited to the infected lung, but only few to MLN (Figure 1C). Of interest, the total numbers of infiltrating leukocytes and Gr1 + CD11b + cells were significantly associated with the severity of inflammation (Figure 1D and E). Gr1 + CD11b + cells are a heterogeneous cell population, so the true identity of major infiltrating cells should be further characterized using the Wright stain and by the expression of Ly6G and Ly6C on cell surface. Gr1 + CD11b + sorted cells consisted mostly of mononuclear cells containing abundant cytoplasmic vacuoles and few segmented granulocytes (Figure 1F, upper panel). Furthermore, Gr1 + CD11b + cells are composed of approximately 68-81% monocytes (Ly6G-Ly6C^{high}) and 19-32% granulocytes (Ly6G + Ly6C^{intermediate}) (Figure 1F, lower panel).

Using specific Abs against surface CCR2 and CX3CR1, we further demonstrated that the infiltrating monocytes in the lungs were Ly6C^{high}CCR2+ inflammatory monocytes but not Ly6C^{low}CX3CR1+ patrolling monocytes (Figure 1G). Importantly, the numbers of infiltrating CCR2+ inflammatory monocytes were highly associated with the severity of inflammation (Figure 1H).

Cytokine and chemokine profiling of BALFs
To investigate the mechanism of extensive accumulation of CCR2+ inflammatory monocytes in severe inflammation, the cytokines and chemokines listed in Figure 2A were evaluated. According to the results of protein arrays, levels of G-CSF, CCL1, CCL2, CCL12, IL-10, CXCL9, IL-16 and CCL5 were correlated with the severity of lung inflammation (Figure 2A). Notably, both CCL2 and CCL12 are ligands of CCR2, in addition to CCL7. So, we speculated that the aggressive recruitment of CCR2+ inflammatory monocytes is linked to expression of CCR2 ligands. In Figure 1C, we found that Gr1 + CD11b + cells preferentially migrate to the lung but not to MLN. Therefore, we suggested that leukocytes infiltrating the lung may frequently induce CCR2 ligands to attract CCR2+ inflammatory monocytes. Indeed, all transcripts of CCR2 ligands
Figure 2 (See legend on next page.)
were over 4000 fold higher in the lung than in MLN (Figure 2B). In addition, the levels of CCR2 ligands in sera were clearly correlated with the numbers of infiltrating CCR2+ inflammatory monocytes (Figure 2C). These results suggested that robust expression of CCR2 ligands may contribute to the aggressive recruitment of CCR2+ inflammatory monocytes into the lungs.

**Induction of CCR2 ligands by CCR2+ inflammatory monocytes**

We sought to determine whether the infiltrating Gr1 + CD11b + cells are possible producers of CCR2 ligands. To test this possibility, total infiltrating leukocytes were separated into Gr1 + CD11b + cells and Gr1-CD11b- cells using a cell sorter (Figure 3A). Compared to leukocytes in the lungs of naïve mice, infiltrating leukocytes harvested from virus-infected mice had tens- to thousands-fold induction of CCR2 ligands (Figure 3B). The relative fold induction of CCR2 ligands was similar between total leukocytes and Gr1 + CD11b + sorted cells, suggesting that Gr1 + CD11b + cells are the main producers of CCR2 ligands. To confirm that CCR2+ inflammatory monocytes were producers of CCR2 ligands, granulocytes and monocytes were sorted from the Gr1 + CD11b + myeloid population (Figure 3C). As shown in Figure 3D, both cell types could express CCL2, CCL7 and CCL12, but more expression of these CCR2 ligands was seen in monocytes. Thus, our results suggested that infiltrating CCR2+ inflammatory monocytes act positively in a chemokine feedback loop to recruit more CCR2+ inflammatory monocytes.

**Induction of CCR2 ligands is dependent on IFNAR1-triggered signaling**

We next sought to determine which inflammatory signaling pathway was responsible for the induction of CCR2 ligands. Previous studies indicated that signaling pathways of MyD88 and type I IFN could modulate the recruitment of myeloid cells [19,20]. Therefore, MyD88−/− and IFNAR1−/− mice were used in this study. Compared to infected WT and MyD88−/− mice, the expression of CCR2 ligands by Gr1 + CD11b + cells was significantly reduced in infected IFNAR1−/− mice (Figure 4A). In addition, we found that the percentage of CCR2+ inflammatory monocytes was only reduced in IFNAR1−/− mice. In Figure 4B, CCR2+ inflammatory monocytes accounted for 81.8 ± 1.1% of total leukocytes in infected WT mice and 84.5 ± 4.5% in infected MyD88 deficient mice; however, CCR2+ inflammatory monocytes accounted for only 39.8 ± 0.35% in infected IFNAR1−/− mice. Thus, accumulation of CCR2+ inflammatory monocytes was suppressed when the IFNAR1-induced expression of CCR2 ligands was interrupted. Because aggressive recruitment of Gr1 + CD11b + cells was observed after day 3 post-infection (Figure 1E), we wondered whether intranasal treatment with an anti-IFNAR1 blocking antibody at day 3 post-infection could interrupt the influx of CCR2+ inflammatory monocytes. In Figure 4C and D, the recruitment of CCR2+ inflammatory monocytes was reduced significantly in anti-IFNAR1 blocking antibody-treated mice, but not in isotype control-treated mice. Overall, these data implied that excessive recruitment of CCR2+ inflammatory monocytes contributes to continuous activation of IFNAR1-induced expression of CCR2 ligands.

**Impaired anti-viral responses prolong IFNβ expression**

Type I IFNs (IFNa and IFNβ) are considered to bind the heterodimeric complexes of IFNAR1 and IFNAR2. Recent study has shown that induction of CCL2 and CCL7 is triggered by the IFNAR1-IFNβ signaling in IFNAR2−/− mice [21]. In addition, we also observed differential expression of CCR2 ligands among Gr1 + CD11b + sorted cells in 141, SOIV and PR8 infections (Figure 3B). Therefore, we examined the expression levels of IFNβ in all infected mice. As expected, expression of IFNβ as detected only in the Gr1 + CD11b + sorted cells harvested from PR8-infected mice at day 7 post-infection (Figure 5A). In addition, both granulocytes and monocytes in Gr1 + CD11b + population could express IFNβ (data not shown). Because detectable IFNβ production reflects activated viral replication, the anti-viral responses of the host were examined by measuring virus titers and detecting influenza NP expression in the infected lung. As shown in Figure 5B and C, 141-infected mice completely eliminated the virus at day 7. SOIV-infected mice still showed weak expression...
Figure 3 CCR2+ inflammatory monocytes produce high amounts of CCR2 ligand. Total leukocytes were harvested from naïve or virus-infected mice at day 7 post-infection. (A) Gr1 + CD11b + and Gr1-CD11b- cells were sorted from total leukocytes using a cell sorter. (B) RNAs were extracted from total leukocytes, Gr1 + CD11b + sorted cells and Gr1-CD11b- sorted cells from the virus-infected mice indicated. Experiment (n = 3-6 mice per group) was performed at least twice and one representative is shown. (C) Isolated leukocytes were stained with anti-Gr1, −CD11b and −Ly6G Abs. Gr1 + CD11b + Ly6G + and Gr1 + CD11b + Ly6G- cells were sorted. (D) RNA was extracted from total leukocytes, Gr1 + CD11b + Ly6G + sorted cells and Gr1 + CD11b + Ly6G- sorted cells from PR8-infected mice and expression of CCL2, CCL7 and CCL12 was measured by RT-QPCR. The mRNA relative folds were determined by normalizing the level of each group to the corresponding GAPDH level and then to total leukocytes from naïve mice (mean ± SEM; ns: no significant difference; * P < 0.05; ** P < 0.01; *** P < 0.001). Experiment (n = 4–5 mice per group) was performed twice and one representative is shown.
Figure 4 (See legend on next page.)
of NP at day 7 and the host completely cleared the virus at day 8 post-infection. Of note, PR8-infected lungs still showed strong NP expression and viral replication at day 7–8 post-infection. These data suggested that the duration of IFNβ production is a function of the rate of viral clearance. Next, we sought to explore why Gr1+CD11b+ cells produce abundant IFNβ in PR8-infected mice in the late phase of infection. We hypothesized that recruited CCR2+ inflammatory monocytes are infected by the PR8 virus, resulting in amplified production of IFNβ. Indeed, expression of influenza NP was detected in CCR2+ inflammatory monocytes in PR8-infected mice (Figure 5D). Thus, our results suggested that impaired clearance of PR8 virus prolonged expression of IFNβ, which led to infected CCR2+ inflammatory monocytes amplifying their own recruitment by an IFNAR1-triggered chemokine feedback loop. To determine whether high viral loads are potent inducers for CCR2+ monocyte infiltration, an anti-viral drug, Oseltamivir, was used to suppress virus replication in infected mice. In Figure 5E, body weight loss was attenuated when infected mice received Oseltamivir treatment, demonstrating the efficacy of Oseltamivir. Influx of CCR2+ inflammatory monocytes was dramatically reduced in Oseltamivir-treated mice, compared to PBS-treated mice (Figure 5F). Taken together, our results supported the concept that continuous recruitment of CCR2+ inflammatory monocytes by the IFNAR1-triggered chemokine feedback loop is attributable to the extended duration of IFNβ expression in the late phase of infection.

Pathological effects of CCR2+ inflammatory monocytes upon IAV infection

A previous study showed that monocytes are retained in the BM when they lack CCR2 expression [23]. To investigate the biological consequences of an excessive accumulation of CCR2+ inflammatory monocytes in the lungs, CCR2−/− mice were used to examine leukocyte infiltration, cytokine storm, expression of iNOS and the survival rate after a lethal dose challenge of PR8 virus. In the absence of infiltrating CCR2+ inflammatory monocytes, total leukocytes in the lung and expression of CCL1, sICAM-1, IFNy, IL-1ra, IL-16, M-CSF, CCL2, CCL12 and CXCL9 in BALF were decreased, suggesting that CCR2+ inflammatory monocytes contribute to the expression of these molecules (Figure 7A and B). Consistent with the results from
cytokine arrays, expression of CCR2 ligands was also significantly decreased in the infiltrating leukocytes of CCR2<sup>−/−</sup> mice, compared to WT mice (Figure 7C). A previous report has shown that iNOS is induced in activated myeloid cells and significantly involved in the development of IAV-induced pneumonitis [24]. As shown in Figure 7D, Gr1<sup>+</sup> CD11b<sup>+</sup> cells were the predominant producers of iNOS. Interestingly, expression of iNOS was correlated with the severity of inflammation. To demonstrate further the importance of CCR2+ inflammatory monocytes-mediated immunopathological effects, expression of iNOS and the survival rate were compared...
in PR8-infected WT and CCR2−/− mice. Expression of iNOS transcripts was dramatically reduced in infected CCR2−/− mice, (Figure 7E). Finally, 38.5% of infected CCR2−/− mice, but none of the WT mice, survived a lethal dose challenge of PR8 virus (Figure 7F). Thus, infiltrating CCR2+ inflammatory monocytes play a pivotal role in highly virulent IAV infection-mediated pathological effects.

**Discussion**

IAV not only infect pulmonary epithelial cells, endothelial cells and resident alveolar macrophages but also infiltrating granulocytes, monocytes and dendritic cells [18,25]. Furthermore, infected leukocytes are the main contributors to aggressive production of inflammatory innate immune responses. Before entering the inflated lung, these uninfected infiltrates are already primed with
Figure 7 Decreasing pathological effects in PR8-infected CCR2−/− mice. (A) Total leukocytes were harvested from the lungs and counted by trypan blue exclusion. These data are a composite of three independent experiments (n = 9 mice per group, mean ± SEM; ***P < 0.001). (B) Pooled BALFs were subjected to cytokine or chemokine expression analysis using cytokine protein arrays (n = 6 mice per group). (C) Relative expression of CCL2, CCL7 and CCL12 was measured by RT-QPCR. The mRNA relative folds were determined by normalizing the level of each group to the corresponding GADPH level and then to total leukocytes from WT mice (mean ± SEM). This is a representative result from two repeated experiments. (D) RNAs were extracted from total leukocytes, Gr1+ CD11b+ sorted cells and Gr1−CD11b− sorted cells from the virus-infected mice indicated. Relative expression of iNOS transcripts was measured by RT-QPCR. The mRNA relative folds were determined by normalizing the level of each group to the corresponding GAPDH level and then to total leukocytes from naïve mice (mean ± SEM). Experiment (n = 3–6 mice per group) was performed twice and one representative is shown. (E) RNAs were harvested from leukocytes isolated from the lungs of WT and CCR2−/− infected mice. Relative expression of iNOS was measured by RT-QPCR. The mRNA relative folds were determined by normalizing the level of each group to the corresponding GAPDH level and then to total leukocytes from WT mice (n = 3 mice per group; mean ± SEM). Experiment was performed twice and one representative is shown. (F) WT (n = 13) and CCR2−/− mice (n = 13) were infected with PR8 viruses. Survival rate was monitored daily until day 14 post-infection. These data are a composite of three independent experiments.
IFNAR1-dependent induction of CCR2 ligands

Bone marrow

IFNβ

Lung

Oseltamivir

141

SIV

PR8

5-10% weight loss

15-20% weight loss

Progressive weight loss

Recovery

Mild inflammation

Moderate inflammation

Severe inflammation

Accumulation of CCR2+ inflammatory monocytes

Viral load

Cytokine storm

iNOS expression

Influenza virus

CCR2+ inflammatory monocyte in lung

CCR2+ monocyte in bone marrow

IFNβ and IFNAR1

CCR2 ligands and CCR2

Anti-IFNAR1

Figure 8 (See legend on next page.)
type I IFN, which upregulates the levels of MAD5, RIG-I and IRF7 [26,27]. In addition, these IFN-stimulated molecules coupled with viral nucleic acids are responsible for amplified production of type I IFN [28]. Our findings revealed that the rate of virus clearance determines the duration of IFNβ expression in infiltrating Gr1 + CD11b + cells. Sustained expression of IFNβ was critical for aggressive recruitment of CCR2+ inflammatory monocytes in severe inflammation. When virus replication was suppressed by Oseltamivir, body weight and influx of CCR2+ inflammatory monocytes were significantly reduced. These results indicated that excessive accumulation of CCR2+ inflammatory monocytes plays a crucial role in the pathological outcomes of highly pathogenic H1N1 IAV infections. Recently, we are continuously threatened by sporadic infections by emerging avian influenza viruses, including highly pathogenic avian H5N1 and H7N9 viruses which rapidly develop acute respiratory distress syndrome, including excessive infiltration of neutrophils and monocytes into the lungs, high viral loads and hypercytokinemia [29,30]. Furthermore, it is worth to see whether the same phenomenon is observed in avian flu infections, such as H5N1 and H7N9. If accumulation of CCR2+ inflammatory monocytes is a common phenomenon in highly pathogenic influenza infection, CCR2+ inflammatory monocytes will be a good therapeutic target in infection.

The mechanism of accumulation of CCR2+ inflammatory monocytes in severe IAV infection remains largely unclear. Previous reports have shown that small numbers of neutrophils are recruited early in infection, followed by influx of large numbers of monocytes [4]. Based on our result, CCR2 ligands produced by neutrophils might play a key role in the early recruitment of monocytes. In Figure 1F, the ratio of neutrophils and monocytes was skewed according to degrees of inflammation at day 7 post-infection. This result indicated two things: (1) Monocyte attractive chemokines were not only provided by neutrophils but also by other inflammatory cells. Using cell sorting, we showed that accumulated CCR2+ inflammatory monocytes are the main contributors of CCR2 ligands and then amplify their own recruitment. (2) Infiltrating monocytes might interfere with the further influx of neutrophils in severe inflammation. A previous study has demonstrated that type I IFN suppresses neutrophil-mediated chemokine attraction, CXCL1 and CXCL2, leading to impaired recruitment of neutrophils [31]. Therefore, we suggested that sustained expression of IFNβ from CCR2 inflammatory monocytes interrupts the recruitment of neutrophils. Indeed, our data are consistent with previous reports that a doubling of neutrophil numbers is observed in CCR2/−/− and IFNAR1/−/− mice [20,32].

Production of type I IFN is a double edged sword in terms of viral clearance and virus-mediated pathogenesis. Previous studies have shown that prolonged induction of type I IFN was seen in highly virulent IAV infections, leading to severe consequences: (1) Type I IFN-induced apoptosis of alveolar epithelial cells by TRAIL is observed in severe IAV infections [33]. (2) Type I IFN-induced FasL expression in the epithelial cells of the lung contributes to the severity of infection [34]. (3) Type I IFN mediates the development of post-influenza bacterial infections [31]. In our study, CCR2+ inflammatory monocytes amplify their own recruitment by a prolonged IFNAR1-triggered chemokine feedback loop.

In our study, induction of CCR2 ligands in CCR2+ inflammatory monocytes was dependent on the IFNAR1-triggered signaling pathway. However, recruitment of CCR2+ inflammatory monocytes could not be completely abolished in IFNAR1/−/− mice, suggesting that induction of CCR2 ligands in other cell types by IFNAR1-independent pathways may not be excluded. Indeed, expression of CCL2 is regulated by Sphingosine-1-phosphate receptor-triggered signaling in pulmonary endothelial cells or by the Myd88-mediated pathway in pulmonary epithelial cells [35-37]. In addition, IL-1R signaling is also involved in CCL2 induction in undefined cell types during IAV infection [38]. A previous study has demonstrated that mice deficient in a single ligand, either CCL2 or CCL7, only can block 40-50% monocytes egressing from the BM [39]. Thus, it is not surprising that gene deficiency of CCL2 cannot protect mice against highly pathogenic virus-mediated...
death [7]. Our results indicated that CCL2, CCL7 and CCL12 were highly induced during IAV infections. Therefore, blocking of any single CCR2 ligand is not sufficient to block recruitment of CCR2+ inflammatory monocytes.

Recruited CCR2+ inflammatory monocytes play a critical role in innate and adaptive immune responses during IAV infections. In successful clearance of 141 and SOIV infections, CCR2+ inflammatory monocytes expressed high levels of IFNγ, MHC class I and MHC class II molecules than those molecules on monocytes isolated from PR8-infected mice (data not shown). Our and previous studies have demonstrated that monocytes are the mainly susceptible cell type to IAV infections [40,41]. Therefore, we suggested that the rate of viral clearance markedly determines the functional direction of infiltrating CCR2+ inflammatory monocytes toward in either protective or pathological role. In infections of MCMV and LCMV, CCR2+ inflammatory monocyte-produced large amount of iNOS and facilitate the production of nitric oxide (NO). NO plays a critical role to impair anti-CD8 T cell responses [14,15]. In our study, CCR2+ inflammatory monocytes expressed iNOS; and its expression was correlated with rate of viral clearance. Thus, these results implied that excessive accumulation of CCR2+ inflammatory monocytes might interfere effective anti-viral CD8 T-cell responses via excessive NO production in highly pathogenic IAV infections.

In summary, overabundant innate immune responses produced by monocytes contribute significantly to highly pathogenic virus-mediated fatal outcomes. Based on our findings, the proportion of CCR2+ inflammatory monocytes in the blood and concentration of CCR2 ligands in the serum have potential as translational biomarkers to predict IAV virulence and pathogenesis in an emerging pandemic infection and sporadic infections of avian IAVs. In addition, inhibition of recruitment of CCR2+ inflammatory monocytes or depletion of infiltrating CCR2+ inflammatory monocytes may provide an alternative immunotherapeutic way to reduce the damaging effects of accumulating CCR2+ inflammatory monocytes in highly pathogenic IAV infections.

Conclusion
The excessive accumulation of Gr1+CD11b+ cells is strongly associated with severe lung pathology in highly pathogenic 1918 H1N1 and avian H5N1 infections [42]. According to a detailed characterization of Gr1+CD11b+ cells, we found that CCR2+ inflammatory monocytes are a prominent cell type and that they contribute to overabundant inflammatory immune responses. In this study, we demonstrated that the accumulation of infiltrating CCR2+ inflammatory monocytes is determined by the efficiency of host in clearing the virus. Based on our findings, the CCR2+ inflammatory monocytes were one of determinants for pathogenicity of highly pathogenic IAV infection (Figure 8).

Abbreviations
IAV: Influenza A virus; IFNAR1: interferon-a/b receptor 1; SOIV: swine-origin influenza virus; MCMV: mouse cytomegalovirus; LCMV: lymphocytic choriomeningitis virus; BM: bone marrow; NP: nucleoprotein; MLN: mediastinal lymph node; BALF: bronchoalveolar lavage fluid; CFSE: carboxyfluorescein diacetate succinimidyl ester; NO: nitric oxide.

Competing interests
The authors declare that they have no competing interests.

Authors‘ contributions
SJL designed experiments, performed experiments, analyzed the data and co-wrote the manuscript; ML designed experiments, performed experiments and analyzed the data; RLK provided materials, SRS provided materials; DMO provided materials; JL provided materials; CKL provided materials; CML provided materials, CNL provided materials and CHT designed experiments and co-wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments
We thank Dr. Chen-Kung Chou of Department of Biomedical Science, College of Medicine, Chang Gung University, Taiwan, kindly provided the IVC system for breeding and management of mice. We thank Dr. Tim J. Harrison of UCL Medical School, London, UK, for reviewing the manuscript critically. We thank the Core Instrument Center of Chang Gung University for providing assistance with cell sorting. We thank Dr. Kuo-Feng Weng (Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, Tao-Yuan, Taiwan) for providing assistance with hypothesis cartoon. This work was supported by National Science Council, Chang Gung Memorial Hospital, National Health Research Institute and Excellent Translational Medicine Research Projects of National Taiwan University College of Medicine and National Taiwan University Hospital (grants: MOST-103-2320-B-182-028-MY3, NSC-102-2325-B-182-002, CMRPD1A0091, CMRPD1A0092 and CMRPD1A0093 to S.-J.L.; NSC-100-2320-B-002-100-MY3, NHRI-EX102-10031BI and 102R39012 to C.-H.T; NSC-100-2320-B-182-019-MY3 to R.-L.K; CMIU100-5-03 to H.-C.C).

Author details
1Research Center for Emerging Viral Infections, College of Medicine, Chang Gung University, Taoyuan, Taiwan. 2Graduate Institute of Medical Biotechnology, College of Medicine, Chang Gung University, Taoyuan, Taiwan. 3Department of Medical Biotechnology and Laboratory Science, College of Medicine, Chang Gung University, Taoyuan, Taiwan. 4Graduate Institute of Microbiology, College of Medicine, National Taiwan University Hospital, Taipei, Taiwan. 5Department of Molecular Cell Biology, Health Sciences Research Institute, University of California, Merced, CA, USA. 6Genomics Research Center, Academia Sinica, Taipei, Taiwan. 7Graduate Institute of Immunology, College of Medicine, National Taiwan University, Taipei, Taiwan. 8Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan. 9Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan. 10Department of Laboratory Medicine, Chang Gung Memorial Hospital, Linkou, Taiwan.

Received: 28 July 2014 Accepted: 15 October 2014 Published online: 18 November 2014

References
1. Graham-Rowe D: Epidemiology: Racing against the flu. Nature 2011, 480(S2):52–53.
2. Taubenberger JK, Kash JC: Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe 2010, 7(4):40–46.
3. Lam TT, Wang J, Shen Y, Zhou B, Duan L, Cheung CL, Ma C, Lytcott SJ, Leung CY, Chen X, Li L, Hong W, Chai Y, Zhou L, Liang H, Ou Z, Liu Y, Farooqui A, Kelvin DJ, Poon LL, Smith DK, Pybus OG, Leung GM, Shu Y, Webster RG, Webbby RJ, Peiris JS, Rambaut A, Zhu H, Guan Y: The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature 2013, 502(3):241–244.

The authors read and approved the final manuscript.
4. Perrone LA, Powlden JK, Garcia-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog. 2008, 4:e1000115.

5. Kobasa D, Jones SM, Shinua K, Kash JC, Copps. J, Elizaba H, Hatta Y, Kim JH, Halffman P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li YI, Karm MG, Feldmann H, Kawaoka Y. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007, 445:319–323.

6. Baskin CR, Bielefeldt-Ohmann H, Tumpey TM, Sabourin PJ, Long JP, Influenza a virus polymerase is an integral component of the central nervous system with a neurotropic coronavirus. J. Immunol. 2001, 167:4855–4892.

7. Lim JK, Obara CJ, Rivollier A, Pletnev AG, Kelsall BL, Murphy PM. Inflammation of myeloid suppressor cell numbers that inhibit viral-specific T cell immunity. Immunity 2012, 37:543–553.

8. Kawasaki T, Kaneko T, Nakahira K, Thomas P, Ploegh HL, Medzhitov R. Innate immune responses to influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 2007, 104:12470–12481.

9. Chen BP, Kuziel WA, Lane TE. Lack of CCR2 results in increased mortality and impaired leukocyte activation and trafficking following infection of the central nervous system with a neurotropic coronavirus. J. Immunol. 2011, 186:471–478.

10. Lin KL, Suzuki Y, Nakano H, Rambourg E, Gunn ML. Chemokine receptor CCR2 is critical for monocyte accumulation and survival in West Nile virus encephalitis. J Immunol. 2011, 186:9345–9352.

11. Dawson TC, Beck MA, Kuziel WA, Henderson F, Maeda N. Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus. J Pathol 2000, 191:156–1959.

12. Lin KL, Suzuki Y, Nakano H, Rambourg E, Gunn ML. Chemokine receptor CCR2+ monocyte-derived dendritic cells and exudate macrophages produce inflammation-induced pulmonary immune pathology and mortality. J Immunol 2008, 180:2562–2572.

13. Iijima N, Matthe LM, Iwasaki A. Recruited inflammatory monocytes stimulate antiviral TH1 immunity in infected tissue. Proc Natl Acad Sci U S A 2011, 108:284–289.

14. Daley-Bauer LP, Wynn GM, Mocarski ES. Cytopathology of influenza impairs antiviral CD8+ T cell immunity by recruiting inflammatory monocytes. Immunity 2012, 37:122–133.

15. Morris BA, Ubelhoer LS, Nakaya H, Price AA, Grakoui A, Pulendran B. Chronic but not acute virus infection induces sustained expansion of myeloid suppressor cell numbers that inhibit viral-specific T cell immunity. Immunity 2013, 38:309–321.

16. Kuo RL, Krug RM. Influenza a virus polymerase is an integral component of the CPSF50/N51a protein complex in infected cells. J Virol 2009, 83:1611–1616.

17. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y. Generation of influenza a viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A 1999, 96:9345–9350.

18. Manicasamy B, Manicasamy S, Belche-Villanueva A, Pisaniello G, Pulendran B, Garcia-Sastre A. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. Proc Natl Acad Sci U S A 2010, 107:15131–15136.

19. Babcock AA, Toft-Hansen H, Owens T. Signaling through MyD88 regulates leukocyte recruitment after brain injury. J Immunol 2008, 181:6481–6490.

20. See SJ, Kwon HJ, Ko HJ, Byun YH, Seong BL, Uematsu S, Akira S, Kweon MN. Type I interferon signaling regulates LysC (hi) monocytes and neutrophils during acute viral pneumonia in mice. PLoS Pathog 2011, 7:e1001304.

21. de Weerd NA, Vivian JP, Nguyen TK, Mangan NE, Gould JA, Branniff SJ, Zaker-Tabrizi L, Fung KY, Forster SC, Beddoo T, Reid HH, Rossjohn J, Hertzog PJ. Structural basis of a unique interferon-beta signaling axis mediated via the receptor IFNAR1. Nat Immunol 2013, 14:901–907.

22. Serbană NV, Parmer EG. Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2. Nat Immunol 2006, 7:311–317.

23. Tsou CL, Peters W, Si Y, Slaymaker S, Aislain AM, Weisberg SP, Mack M, Charo IF. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. J Clin Invest 2007, 117:562–570.

24. Karupiah G, Chen JH, Mahalingam S, Nathan CF, MacMicking JD. Rapid interferon gamma-dependent clearance of influenza A virus and protection from consolidating pneumonia in nitric oxide synthase 2-deficient mice. J Exp Med 1998, 188:1541–1546.

25. Hofmann B, Springer H, Kaufmann A, Bender A, Hasse C, Nain M, Germs D. Susceptibility of mononuclear phagocytes to influenza A virus infection and possible role in the antiviral response. J Leukoc Biol 1997, 61:408–414.

26. Hermesh T, Moltebo B, Moran TM, Lopez CB. Antiviral instruction of bone marrow leukocytes during respiratory viral infections. Cell Host Microbe 2010, 7:343–353.

27. Honda K, Takaoaka A, Taniguchi T. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. Immunity 2006, 25:349–360.

28. Gough DJ, Messina NL, Clarke CJ, Johnstone RW, Levy DE. Constitutive type I interferon modulates homeostatic balance through tonic signaling. Immunity 2012, 36:166–174.

29. Watanabe T, Kawaoka Y. Pathogenesis of the 1918 pandemic influenza virus. PLoS Pathog. 2011, 7:e1001218.

30. Mok CK, Lee HH, Chan MC, Sa SF, Lestra M, Nicholls JM, Zhu H, Guan Y, Peiris JM. Pathogenicity of the novel A/H7N9 influenza virus in mice. Mbio 2013, 4:e00362–13.

31. Shahangian A, Chow EK, Tian X, Kang JR, Gaffare A, Liu SY, Belperio JA, Cheng G, Deng JC. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest 2009, 119:1910–1920.

32. Wareing MD, Lyon A, Inglis C, Giannoni F, Charo J, Sarawar SR. Chemokine regulation of the inflammatory response to a low-dose influenza infection in CCR2−/− mice. J Leukoc Biol 2007, 81:793–801.

33. Hogner K, Wolff T, Pleschica S, Pog S, Gruber AD, Kinle J, Walmrath HD. Initial recruitment to lung endothelium is critical for monocyte responses to influenza A virus infection and possible role in the antiviral response. J Clin Invest 2008, 119:1047–1059.

34. Fujikura D, Chiba S, Muramatsu D, Kazumata M, Nakayama Y, Kawai T, Akira S, Kida H, Miyazaki T. Type-I interferon is critical for Fas-l expression on lungs cells to determine the severity of influenza virus pneumonia. PLoS Pathog 2013, 9:e1003188.

35. Tsou CL, Peters W, Si Y, Slaymaker S, Aislain AM, Weisberg SP, Mack M, Charo IF. Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites. J Clin Invest 2007, 117:562–570.

36. Tekis JC, Straub HH, Krueger R, Defrance C, Boons I, Focarete MA, Finkenstädt B, Kohler A, Lederer F, Lohr H, Wiesmayer S, Urban C, Hennig N, Lohmeyer J, Herold S. Macrophage-expressed IFN-beta contributes to apoptotic alveolar epithelial cell injury in severe influenza virus infection. Proc Natl Acad Sci USA 2012, 109:20901–20906.

37. Iijima N, Matthe LM, Iwasaki A. Recruited inflammatory monocytes stimulate antiviral TH1 immunity in infected tissue. Proc Natl Acad Sci U S A 2011, 108:284–289.

38. Duley LR, Wynn GM, Mocarski ES. Cytopathology of influenza impairs antiviral CD8+ T cell immunity by recruiting inflammatory monocytes. Immunity 2012, 37:122–133.

39. Morris BA, Ubelhoer LS, Nakaya H, Price AA, Grakoui A, Pulendran B. Chronic but not acute virus infection induces sustained expansion of myeloid suppressor cell numbers that inhibit viral-specific T cell immunity. Immunity 2013, 38:309–321.

40. Kuo RL, Krug RM. Influenza a virus polymerase is an integral component of the CPSF50/N51a protein complex in infected cells. J Virol 2009, 83:1611–1616.
42. Long JP, Kotur MS, Stark GV, Warren RL, Kasoji M, Craft JL, Albrecht RA, Garcia-Sastre A, Katze MG, Waters KM, Vasconcelos D, Sabourin PJ, Bresler HS, Sabourin CL: Accumulation of CD11b (+) Gr-1 (+) cells in the lung, blood and bone marrow of mice infected with highly pathogenic H5N1 and H1N1 influenza viruses. Arch Virol 2013, 158:1305–1322.
doi:10.1186/s12929-014-0099-6
Cite this article as: Lin et al: The pathological effects of CCR2+ inflammatory monocytes are amplified by an IFNAR1-triggered chemokine feedback loop in highly pathogenic influenza infection. Journal of Biomedical Science 2014 21:99.