Antiviral Research 92 (2011) 319–328

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

GM-CSF modulates pulmonary resistance to influenza A infection ☆

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ARTICLE INFO

Article history:
Received 21 April 2011
Revised 29 July 2011
Accepted 26 August 2011
Available online 8 September 2011

Keywords:
GM-CSF
Influenza
Epithelial cells
Alveolar macrophages
SP-A
Scavenger receptors

ABSTRACT

Alveolar type II epithelial or other pulmonary cells secrete GM-CSF that regulates surfactant catabolism and mucosal host defense through its capacity to modulate the maturation and activation of alveolar macrophages. GM-CSF enhances expression of scavenger receptors MARCO and SR-A. The alveolar macrophage SP-R210 receptor binds the surfactant collectin SP-A mediating clearance of respiratory pathogens. The current study determined the effects of epithelial-derived GM-CSF in host resistance to influenza A pneumonia. The results demonstrate that GM-CSF enhanced resistance to infection with 1.9×10^4 ffc of the mouse-adapted influenza A/Puerto Rico/8/34 (PR8) H1N1 strain, as indicated by significant differences in mortality and mean survival of GM-CSF-deficient ($GM^{-/-}$) mice compared to $GM^{-/-}$ mice in which GM-CSF is expressed at increased levels. Protective effects of GM-CSF were observed both in mice with constitutive and inducible GM-CSF expression under the control of the pulmonary-specific *SFTPC* or *SCGB1A1* promoters, respectively. Mice that continuously secrete high levels of GM-CSF developed desquamative interstitial pneumonia that impaired long-term recovery from influenza. Conditional expression of optimal GM-CSF levels at the time of infection, however, resulted in alveolar macrophage proliferation and focal lymphocytic inflammation of distal airways. GM-CSF enhanced alveolar macrophage activity as indicated by increased expression of SP-R210 and CD11c. Infection of mice lacking the GM-CSF-regulated SR-A and MARCO receptors revealed that MARCO decreases resistance to influenza in association with increased levels of SP-R210 in $MARCO^{-/-}$ alveolar macrophages. In conclusion, GM-CSF enhances early host resistance to influenza. Targeting of MARCO may reinforce GM-CSF-mediated host defense against pathogenic influenza.

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1. Introduction

Influenza A has caused recurrent pandemics resulting in the deaths of over 50 million people during the last century (Kilbourne, 2006). Seasonal influenza continues to cause epidemics with significant morbidity and mortality throughout the world every year. Recent outbreaks with the avian H5N1 and swine origin H1N1 strains re-emphasized the importance of influenza as a persistent threat to public health worldwide. Development of immunotherapies and vaccines that contain spreading of influenza infections hinge on research aimed at understanding the evolution, pathogenesis, and immune responses behind the rapid infection with different influenza strains (Peiris et al., 2010; Taubenberger and Morens, 2010; White et al., 2008). Evasion (Job et al., 2010; White et al., 2008) or dys-regulation (Baskin et al., 2009) of innate host defenses at early stage enables highly pathogenic influenza strains to avoid the ability of the host to limit viral replication resulting in

Abbreviations: *SFTPC* or *SP-C*, surfactant protein C gene promoter; *SCG1A1*, secretoglobin 1A1 gene promoter; *SR-A*, scavenger receptor class A; *MARCO*, macrophage receptor with collagenous structure; *tet-GM*$^{+/+}$, *GM-CSF*$^{-/-}$, *SCGB1A1-rtTA/(teto)*$_7$*CMV-GM-CSF*; GM, GM-CSF; SP-R210, surfactant protein receptor 210; pfu, plaque forming unit; ffc, fluorescent focus counts.

☆ This work was funded through the Office of the UTHSCT Director of Research to Z.C.C., a UTHSCT President's Council Grant to Z.S.C., and Public Health Service Grant ES11008 to L.K.

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doi:10.1016/j.antiviral.2011.08.022

excessive inflammation, lung injury, and death. Highly pathogenic influenza strains exhibit increased tropism for and ability to proliferate rapidly in alveolar type II epithelial cells (Zhang et al., 2010). While alveolar macrophages are crucial for innate host defense against influenza (Tate et al., 2010; Tumpey et al., 2005), highly pathogenic influenza viruses trigger deleterious inflammatory responses mediated by secretion of TNFα, compromising epithelial cell viability and the capacity of macrophages to clear the infection (Belisle et al., 2010; Herold et al., 2008).

While many cell types express GM-CSF, alveolar type II epithelial cells are an important source of GM-CSF in the lung (Burgess et al., 1977). GM-CSF expression facilitates surfactant homeostasis and immune functions of alveolar macrophages and type II epithelial cells (Carey and Trapnell, 2010; Shibata et al., 2001). GM-CSF regulates the differentiation and activation of alveolar macrophages (Shibata et al., 2001) and proliferation of alveolar macrophages and type II epithelial cells (Huffman Reed et al., 1997). *In vitro* studies indicate that GM-CSF causes rapid proliferation of alveolar type II epithelial cells thereby serving in repair and barrier protection of the respiratory epithelium during acute inflammation (Cakarova et al., 2009). Absence of GM-CSF disrupts terminal alveolar macrophage differentiation resulting in alveolar proteinosis (Carey and Trapnell, 2010), a disease caused by decreased surfactant catabolism by alveolar macrophages. Lack of GM-CSF impairs innate immune functions of alveolar macrophages (Shibata et al., 2001), enhancing susceptibility of the lung to infections (Ballinger et al., 2006). GM-CSF regulates expression of the transcription factors PU.1 (Shibata et al., 2001) and PPARγ (Baker et al., 2010), modulating diverse innate and metabolic functions of mature alveolar macrophages. Through PU.1, GM-CSF enhances the responsiveness of alveolar macrophages to bacterial LPS, increasing expression of the cell-surface pattern recognition receptors CD14 and TLR-4 (Carey and Trapnell, 2010). GM-CSF also enhances the antiviral responses of alveolar macrophages; GM-CSF and type I interferon act together to modulate macrophage polarization toward the M1 state of activation (Fleetwood et al., 2009). GM-CSF enhances expression of class A scavenger receptors SR-A and MARCO (Sulahian et al., 2008; Szeliga et al., 2008), which mediate clearance of viral and bacterial pathogens by macrophages (Arredouani et al., 2006; Haisma et al., 2009). SR-A and MARCO mediate internalization of microbial nucleic acids and activate endosomal TLR-3 and TLR-9 or cytosolic NOD-2 and NALP-3 sensors of viral and bacterial infection (DeWitte-Orr et al., 2010; Jozefowski et al., 2006; Mukhopadhyay et al., 2011). It is not known how diverse activities of GM-CSF impact host responses to influenza.

Recent studies have indicated that GM-CSF is useful as a therapy against influenza. GM-CSF enhances mucosal immune responses and the effectiveness of DNA vaccines (Herbert et al., 2009; Loudon et al., 2010). Direct administration of GM-CSF to the lung facilitates host resistance and survival from influenza infection (Huang et al., 2011, 2010). GM-CSF is expressed transiently 2 days after acute infection with influenza A in mice (Hennet et al., 1992). A recent humanized mouse model, in which the human GM-CSF gene replaced the endogenous murine GM-CSF, resulted in high levels of GM-CSF in the alveoli. Engraftment of human hematopoietic cells into humanized mice caused enhanced recruitment and differentiation of human alveolar macrophages in the mouse lung. Such mice exposed to influenza showed that GM-CSF potentiates antiviral responses of human alveolar macrophages (Willinger et al., 2011).

Here, *GM-CSF*$^{-/-}$ transgenic mice with elevated expression of GM-CSF in lung epithelial cells were used to determine the effect and timing of GM-CSF expression in pulmonary resistance to influenza. *GM-CSF*$^{-/-}$ mice are highly susceptible to influenza A infection. The findings indicate that GM-CSF modulates early host resistance by facilitating survival from acute infection with pathogenic influenza.

2. Materials and methods

2.1. Animal husbandry

The WT and transgenic mouse strains used in the present study were bred locally. Mice were used at 8–16 weeks of age. Breeding pairs of WT C57BL/6 mice were purchased from NCI (Frederisburg, MD) and maintained in a pathogen free facility in micro-isolator cages. Mice were provided standard chow and sterile water ad libitum. Transgenic *GM-CSF*$^{-/-}$ (*GM*$^{-/-}$) (Dranoff et al., 1994), bi-transgenic *SFTPC-GM-CSF*$^{+/+}$ (*SP-C-GM*$^{+/+}$) mice (Huffman et al., 1996; Szeliga et al., 2008), *SR-A*$^{-/-}$ and *MARCO*$^{-/-}$ (Zhou et al., 2008), all backcrossed for 10–12 generations to the C57BL/6 genetic background were used in the present study. The *SP-C-GM*$^{+/+}$ mice produce GM-CSF in pulmonary epithelial cells of *GM*$^{-/-}$ transgenic nice. To generate conditional GM-CSF-inducible mice, bi-transgenic *GM-CSF*$^{-/-}$, *SCGB1A1-rtTA* mice expressing the reverse tetracycline transactivator (rtTA) under the control of a rat epithelial Clara cell SCGB1A1 (previously designated as CCSP or CC10) promoter were mated with *GM-CSF*$^{-/-}$, *(teto)*$_7$*CMV-GM-CSF* mice to generate conditional tri-transgenic *GM-CSF*$^{-/-}$, *SCGB1A1-rtTA*/(*teto*)$_7$*CMV-GM-CSF*. Breeding pairs producing 75% tri-transgenic and 25% bi-transgenic littermate controls with 8–12 mice per litter were selected for further study. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at Tyler.

2.2. Generation of *GM*$^{-/-}$, *SCGB1A1-rtTA* and *(teto)*$_7$*-CMV-GM-CSF* mice

Transgenic *SCGB1A1-rtTA* mice bearing *rtTA* downstream from the 2.3 kb rat *SCGB1A1* promoter were generated as previously described (La Gruta et al., 2010). To generate (*teto*)$_7$*-CMV-GM-CSF* mice, the mouse GM-CSF cDNA was excised from the SP-C-GM-CSF plasmid (Huffman et al., 1996) with EcoRI and subcloned into the pUHD10-3 plasmid (Gossen and Bujard, 1992) downstream a promoter element containing the tet operator DNA binding sequence ((teto)$_7$) and a minimal CMV promoter. The plasmid was sequenced to ensure correct orientation and absence of mutations. The resulting (teto)$_7$-CMV-GM-CSF plasmid was used to generate transgenic mice using standard microinjection techniques at the University of Cincinnati transgenic core facility under IACUC approved protocols. Mice were initially generated on the FVB/N genetic background. Founder mice were subsequently backcrossed for 12 generations into the C57BL/6 genetic background and further cross-bred with C57BL/6 GM$^{-/-}$ mice (Dranoff et al., 1994). The resultant bi-transgenic *GM-CSF*$^{-/-}$, *SCGB1A1-rtTA* and *GM-CSF*$^{-/-}$, (*teto*)$_7$*-CMV-GM-CSF* mice were subsequently cross-mated to generate tri-transgenic *GM-CSF*$^{-/-}$, *SCGB1A1-rtTA*/(*teto*)$_7$*-CMV-GM-CSF* (hereafter abbreviated as *tet-GM*$^{+/+}$) mice and littermate controls as described above.

2.3. PCR genotyping

Mice were genotyped by PCR analysis of genomic DNA isolated from tail DNA biopsies from 14- to 25-day-old pups. The sense and antisense primers used to identify the *SCGB1A1-rtTA* transgene were 5′-ACTGCCCATTGCCCAAACAC-3′ and 5′-AAAATCTTGCCAGCTTTCCCC-3′, respectively. The primers used to identify the (teto)$_7$-CMV-GM-CSF transgene were 5′-GCCATCCACGCTGTTTTGAC-3′ and 5′-CCTGGGCTTCCTCATTTTTGG-3′. The PCR amplification product for *SCGB1A1-rtTA* was performed by denaturation at 95 °C for 5 min and then 30 cycles of amplification at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s, followed by a 7-min extension at 72 °C. The reaction conditions for the (*teto*)$_7$*CMV-GM-CSF*

were the same but the annealing temperature was increased to 59 °C. Both amplification products were 500 bp in size.

2.4. Administration of doxycycline

The *tet-GM*$^{+/+}$ mice and littermate controls obtained as described above were exposed to 1 mg/mL doxycycline in the drinking water. Water containing doxycycline was replaced every 2–3 days.

2.5. Influenza virus preparation and titration

Mouse adapted influenza A/Puerto Rico/8/34 (PR8) H1N1virus strain was grown in the chorio-allantoic cavity of embryonated hen eggs. Viral particles were purified by sucrose density gradient centrifugation (Arora et al., 1985; Hartshorn et al., 1988, 1997) and quantitated using a fluorescent focus assay of influenza A virus infection as previously described (van Eijk et al., 2003). Briefly, MDCK cell monolayers were prepared in 96 well plates and grown to confluency. These layers were then infected with dilutions of purified influenza A preparations for 45 min at 37 °C in PBS and tested for presence of infected cells after 7 h using a monoclonal antibody directed against the influenza A viral nucleoprotein (provided by Dr. Nancy Cox, CDC, Atlanta, GA). Aliquots of purified virus were stored frozen in PBS at −80 °C until use.

2.6. Mouse infections

For infections, 8- to 16-week-old male and female mice were anesthetized using a mixture of ketamine (80 mg/kg) and xylazine (10 mg/kg). Mice were infected intranasally with a total volume of 40 μl containing 1.9×10^4 or 1.9×10^3 ffc of influenza A virus PR8. Mice were weighed daily and observed at 12-h intervals for visual signs of clinical disease including labored breathing, huddling, and ruffled fur. Mice that developed symptoms of severe pneumonia were euthanized and recorded as dead. WT and SR-A$^{-/-}$ mice developed evidence of severe illness along with loss of 25–30% of body weight as previously reported for WT mice (Srivastava et al., 2009).

2.7. Lung GM-CSF levels

The concentration of GM-CSF was determined in lavage and post-lavage lung homogenates. Lung lavage was collected in a total of 4.5 mL of Tris-buffered saline composed of 25 mM Tris, pH 7.5, 0.15 M NaCl and 0.6 mM EDTA. Lavage was centrifuged to isolate cells, and supernatants stored frozen at −80 °C until use. The post-lavage lung tissue was homogenized in 2.0 mL of PBS using a polytron homogenizer, centrifuged to remove cell debris, and stored frozen at −80 °C until use. The levels of GM-CSF were determined by ELISA using a commercial kit (eBiosciences) according to directions provided by the manufacturer.

2.8. Flow cytometry

Alveolar macrophages isolated by lung lavage were processed, stained, and analyzed by flow cytometry as described in detail previously (Sever-Chroneos et al., 2011). Briefly, cells were stained with monoclonal PE/Cy5-conjugated CD11c antibody clone N418 (eBiosciences, San Diego, CA), rabbit polyclonal SP-R210/Myo18A antibody (Protein-Tech, Chicago, IL), and a goat polyclonal anti-SR-A/Msr1 antibody (R&D Systems, Minneapolis, MN). SP-R210 and SR-A were visualized using secondary Alexa647-conjugated goat anti-rabbit and FITC-conjugated donkey anti-goat antibodies (Molecular Probes Inc., Eugene, OR), respectively. Alveolar macrophages were gated according to forward and side-scatter properties collecting 15,000 total events per sample. Quadratic or linear gating determined the percentage of positive cells expressing each marker compared to background staining using isotype control antibodies.

2.9. Histology

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut 4.5 μm thick and tissue pathology was visualized by hematoxylin and eosin staining using standard procedures (Chroneos et al., 2009). Images were captured using an Olympus BX41 upright microscope equipped with an Olympus DP11 digital camera (Olympus, Melville, NY) at 10× magnification. Images were processed using Paint.Net software (http://www.getpaint.net) and imported into Microsoft® Word.

2.10. Immunohistochemistry

De-paraffinized tissue sections were processed for immunohisto-chemistry using the Ultravision LP alkaline phosphatase detection system according to the manufacturer's directions (Thermo Scientific, Fremont, CA). Tissues were stained with rabbit polyclonal antibodies raised against mature surfactant protein B (Seven Hills Bioreagents, Cincinnati, OH). Antibodies were used at a dilution of 1:500. Non-immune IgG was used as control. Stained sections were developed using Fast Red chromogen and counterstained for 10 s with hematoxylin.

2.11. Cytology

Alveolar cells isolated by lung lavage were deposited on glass slides using a Shandon cytospin centrifuge at a density of 25,000 cell per slides. Following spin at 1000 rpm for 5 min, cells were stained using the Diff-Quick differential staining kit and examined by light microscopy. Cell images were captured and processed as described in Section 2.9 above.

2.12. Statistical analysis

Statistical and graphical analysis of data was performed using Prism software (Graphpad Software, La Jolla, CA). Statistical comparisons were performed with the unpaired, non-parametric Student's *t*-test. Values of $p < 0.05$ were considered statistically significant. Survival curves were generated by the Kaplan–Meier method, and statistical analysis of survival curves was performed using the Greham–Breslow–Wilcoxon test. Analysis of flow cytometric data was accomplished using FlowJo software (TreeStar Inc., Ashland, OR).

3. Results

3.1. GM-CSF modulates survival to influenza A

To determine the effect of GM-CSF during influenza exposure, the survival of WT, GM$^{-/-}$, and SP-C-GM$^{+/+}$ mice was monitored following infection with 1.9×10^4 ffc of the mouse-adapted influenza A/Puerto Rico/8/34 (PR8) H1N1 virus strain delivered intranasally. WT mice were uniformly susceptible to as low as 600 ffc while *SP-C-GM*$^{+/+}$ mice were completely resistant at doses below 1.9×10^3 ffc (not shown). Similarly, all *SP-C-GM*$^{+/+}$ mice survived infection with 5 LD_{50} intranasal doses of PR8 (Huang et al., 2011). Here, *SP-C-GM*$^{+/+}$ mice remained partially resistant surviving significantly longer to lethal influenza infection with a dose in the range of 30–50 LD_{50} compared to WT and *GM*$^{-/-}$ mice. The mean survival of *SP-C-GM*$^{+/+}$ mice was 23 days compared to 8 days for

WT and 6 days for $GM^{-/-}$ mice (Fig. 1A and Table 1). These findings indicate that GM-CSF enhances the ability of the host to survive primary infection with highly virulent influenza.

Interestingly, $GM^{-/-}$ mice lost weight slowly compared to WT mice even though these mice were highly susceptible to the infection (Fig. 1B). Uninfected $GM^{-/-}$ and *SP-C-GM*$^{+/+}$ mice weigh significantly more that WT mice of the same age (Fig. 1C), in accordance with the neural role of GM-CSF in suppressing food intake (Reed et al., 2005). The *SP-C-GM*$^{+/+}$ mice, however, lost weight at a similar rate as WT mice while surviving *SP-C-GM*$^{+/+}$ mice regained only part of their body weight (Fig. 1B). These results suggest a role of lung GM-CSF in weight loss morbidity at higher doses of infection.

Influenza infection did not alter lung epithelial expression of GM-CSF via the *SFTPC* promoter as indicated by similar combined total amount of GM-CSF before and after infection (Fig. 1D). Interestingly, influenza infection changed the distribution of GM-CSF protein in alveolar lavage and tissue homogenates from 1.8:1 prior to infection to 4.1:1, 6.3:1, and 1:1 at 6, 8, and 11 days after infection, respectively (Fig. 1D). In this context, a recent study using isolated type II epithelial cells demonstrated that influenza PR8 strain did not alter mRNA expression but suppressed secretion of surfactant proteins SP-A and SP-D (Wang et al., 2011). The present results suggest that high levels of GM-CSF mitigate disruption of epithelial cell secretory function by influenza infection.

3.2. Interstitial lung disease after infection of *SP-C-GM*$^{+/+}$ mice with influenza

Although *SP-C-GM*$^{+/+}$ mice resisted early mortality they died by 30 days after influenza infection (Fig. 1B). Assessment of lung tissue sections revealed the histological features of degenerative desquamative interstitial pneumonia at day 7 (Fig. 2A) and day 29 after infection (Fig. 2B). Moderate interstitial thickening (open arrow) and alveolar spaces filled with macrophage aggregates (closed arrow) over large areas of the lungs were found 7 days after infection of SP-C-GM$^{+/+}$ mice (Fig. 2A). Degeneration of alveolar structure (closed arrow) and large spaces containing desquamated cells (open arrow) characterized the lungs of SP-C-GM$^{+/+}$ at 29 days after infection (Fig. 2B). The results indicate that high levels of GM-CSF impair appropriate tissue healing resulting in development of interstitial lung disease secondary to influenza pneumonia.

Table 1
Survival comparison of mice infected with influenza.

Mouse Genotype	Infection (pfu)	No. of mice	Mean survival (days)	Statistics
WT	1.9×10^4	13	8	[1]$p < 0.0002$
$GM^{-/-}$	1.9×10^4	18	6	
SP-C-GM$^{+/+}$	1.9×10^4	19	23	[1,2]$p < 0.0001$
SR-A$^{-/-}$	1.9×10^4	12	7	[1]$p < 0.0001$, [2]$p > 0.05$
MARCO$^{-/-}$	1.9×10^4	12	9	[1]$p < 0.0001$, [2]$p < 0.003$
WT	1.9×10^3	9	9	[2]$p > 0.05$
MARCO$^{-/-}$	1.9×10^3	9	12	[3]$p < 0.004$

[1] Compared to $GM^{-/-}$ mice.
[2] Compared to WT mice infected with 1.9×10^4 ffc.
[3] Compared to WT mice infected with 1.9×10^3 ffc.

3.3. Conditional expression of GM-CSF in lung

In order to determine at which point of influenza infection is GM-CSF expression in lung beneficial to survival, we used a novel mouse model where GM-CSF expression is induced by addition of doxycycline (1 mg/mL) in water. GM-CSF accumulated in the lavage between 2 and 3 days to a level of 1023.8 ± 98.3 pg after administration of doxycycline. Thereafter, the levels of GM-CSF in lung lavage decreased reaching a plateau at 135 ± 11.95 pg after 6 days on doxycycline suggesting clearance of GM-CSF from the alveoli. The total (lavage and tissue homogenate) levels of GM-CSF after 6 days on doxycycline equilibrate at 281 ± 28.7 pg in lung tissue (Fig. 3A) significantly lower than 862 ± 46.2 pg measured in lungs of *SP-C-GM*$^{+/+}$ mice above (Fig. 1D) suggesting inability of lung macrophages to successfully clear artificially high levels of

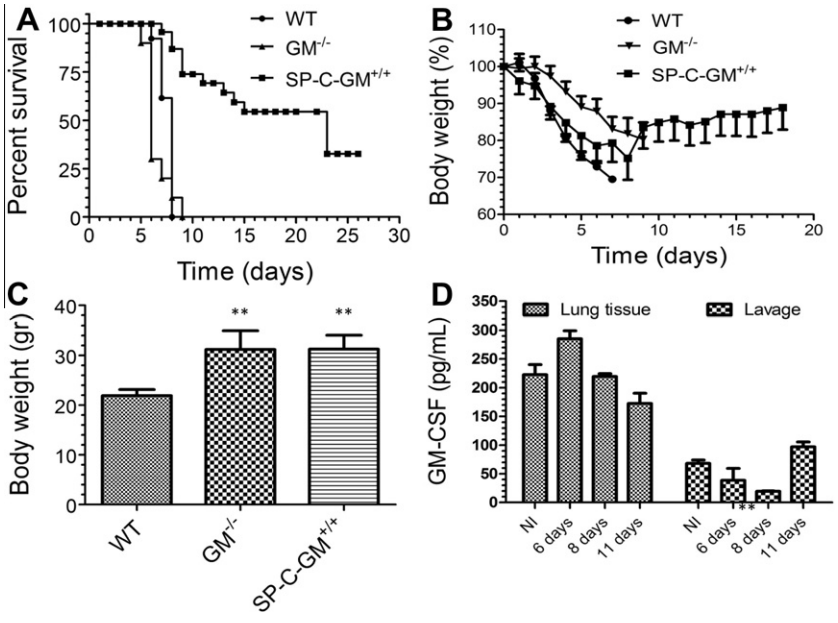

Fig. 1. GM-CSF enhances host survival from influenza infection. The survival profile (A), rate of body weight loss (B) of WT, *GM-CSF*$^{-/-}$ ($GM^{-/-}$), and *SFTPC-GM*$^{+/+}$ (SP-C-GM$^{+/+}$) mice, was determined after intranasal infection with 1×10^4 ffc of PR8. Infected mice were weighed daily and assessed visually every 12 h for the presence of clinical symptoms of the infection. Mice displaying severe illness were euthanized immediately and counted as dead. The number of mice used and statistical parameters derived from survival curves are listed on Table 1. (C) Comparison of body weight of uninfected WT ($n = 13$), $GM^{-/-}$ ($n = 19$), and SP-C-GM$^{+/+}$ (n = 18) mice. Data shown are mean ± SD. $^{**}p < 0.01$. (D) ELISA assays assessed the amount of GM-CSF in lavage and post-lavage lung homogenates before, and at indicated intervals after infection with PR8. Data shown are mean ± SEM. $N = 3$–4 mice per time point. $^{*}p < 0.05$, and $^{**}p < 0.001$ compared to un-infected control.

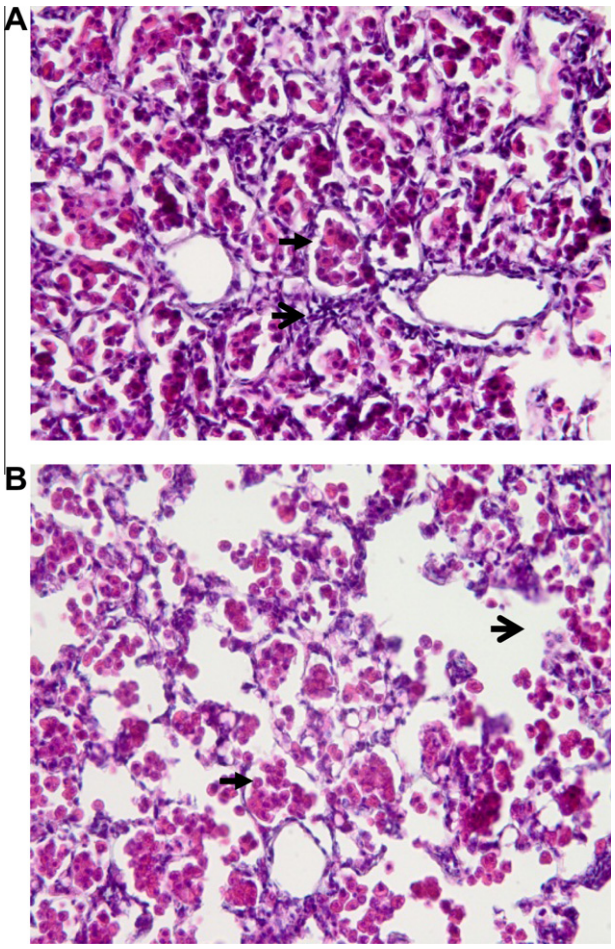

Fig. 2. Histopathology of infected *SP-C-GM*$^{+/+}$ mice. Lung histopathology was evaluated at 7 (A) and 29 (B) days after infection of *SP-C-GM*$^{+/+}$ mice with 1.9×10^3 ffc influenza PR8. Images were captured at 10× magnification. Interstitial thickening and macrophage aggregates at day 7 are indicated by closed and open arrows, respectively. At day 29, degeneration of alveolar structure (closed arrow) and large spaces containing desquamated cells (open arrow) are indicated.

GMCSF in *SP-C-GM*$^{+/+}$. Therefore, inducible *tet-GM*$^{+/+}$ mice model is much closer to the *in vivo* situation of GM-CSF lung expression levels.

Induction of GM-CSF enhances the functional phenotype of alveolar macrophages. The flow cytometry profile of alveolar cells from un-induced *tet-GM*$^{+/+}$ mice was marked by the presence of surfactant aggregates with low side and forward scatter properties and heterogeneous cells with low forward scatter properties on the left side of the macrophage gate (Fig. 3B). Six and 12 days after induction of GM-CSF, surfactant aggregates diminished whereas cells acquired more homogeneous side and forward scatter properties, consistent with the ability of GM-CSF to normalize surfactant levels and support terminal alveolar macrophage differentiation (Huffman et al., 1996; Shibata et al., 2001). Microscopic evaluation of cytospined alveolar macrophages before and after induction of GM-CSF confirmed the morphological differentiation of alveolar macrophages (Fig. 3C). The lavage of uninduced *tet-GM*$^{+/+}$ mice contained a heterogeneous population of small immature macrophages and large foamy macrophages engorged with lipid (Fig. 3C, left), consistent with the presence of alveolar proteinosis in GM$^{-/-}$ lungs. In contrast, the lavage of induced *tet-GM*$^{+/+}$ mice contained morphologically mature alveolar macrophages with ruffled membranes and membrane extensions (Fig. 3C, right). Further, alveolar macrophage differentiation was confirmed by phenotypic expression of CD11c (Fig. 4A). Expression of CD11c increased 4-fold to 30% 6 days after administration and nearly 9-fold to 88% of alveolar macrophages 6 and 12 days after administration of doxycycline in water (Fig. 4A). GM-CSF enhanced expression of the SP-A receptor SP-R210 earlier than CD11c (Fig. 4A). The percentage of cells expressing SP-R210 increased more than 5-fold to 72% on day 6 although it declined somewhat to 58% by 12 days (Fig. 4A). The number of cells in lavage of tet-GM$^{+/+}$ before induction of GM-CSF was $2.10 \pm 0.65 \times 10^6$, consistent with proliferation of immature macrophages and accumulation of foamy macrophages as reported previously in the lungs of *GM*$^{-/-}$ mice (Dranoff et al., 1994; Paine et al., 2001). The number of alveolar macrophages decreased to $0.91 \pm 0.27 \times 10^6$ cells 12 days after induction of GM-CSF, reflecting the disappearance of large foamy macrophages and local differentiation of immature macrophages. In the next three weeks, however, the number of cells increased significantly to $16.1 \pm 3.25 \times 10^6$ cells at 29 days after induction of GM-CSF by administration of doxycycline (Fig. 4B), indicating proliferation of differentiated alveolar macrophages only after prolonged exposure to high levels of GM-CSF. These results indicate that inducible expression of GM-CSF in lung epithelium restores surfactant homeostasis and alveolar macrophage function within 4 days after administration of doxycycline.

3.4. Conditional expression of GM-CSF confers protection against influenza in a time-dependent manner

In the absence of doxycycline, all *tet-GM*$^{+/+}$ mice succumbed to influenza infection by 6 days (Fig. 5A and Table 2), similar to the results in *GM-CSF*$^{-/-}$ mice (Fig. 1A and Table 1). Littermate control *GM-CSF*$^{-/-}$, *SCGB1A1-rtTA* mice had a mean survival of 6–6.5 days regardless of the absence or presence of doxycycline (Table 2), indicating that doxycycline does not affect influenza infection. The mean survival of *tet-GM*$^{+/+}$ mice increased significantly to 7 and 8 days when given doxycycline on the same day or even 3 days after infection (Table 2). Administration of doxycycline 2 and 6 days before infection also delayed mortality (Fig. 5A) and increased mean survival to 9 and 11 days (Table 2), respectively. Doxycycline administered 3 days before infection (Fig. 5A), so that levels of GM-CSF in the lung were the highest on the day of infection, resulted in 60% of mice surviving significantly longer than 11 days after infection similar to survival kinetics to *SP-C-GM*$^{+/+}$ mice (Fig. 1A). All induced mice lost weight rapidly between 2 and 7 days after infection (Fig. 5B). The body weight of mice that were induced before infection, however, stabilized or decreased slowly but these mice did not regain weight (Fig. 5B) even though they convalesced without signs of severe illness. Even removal of doxycycline 3 days after infection did not alter the survival profile of the −3 days doxycycline treated animals (not shown). These results indicate that there is a critical window of GM-CSF expression in the lung that enhances host survival from acute influenza pneumonia.

3.5. Induction of GM-CSF in tet-GM$^{+/+}$ mice restores focal peribronchiolar inflammation

The histological presentation of influenza infections was then evaluated after infection of mice with a lower dose of 1.9×10^3 ffc to address differences in survival. Fig. 6A and D show the alveolar architecture of uninfected WT and *GM*$^{-/-}$, respectively. Alveolar type II epithelial cells occupy alveolar corners as indicated by staining with antibodies against surfactant protein SP-B (Fig. 6A, open arrows). The lung histology of WT mice 6–7 days after infection revealed diffuse mononuclear cell infiltrates in peri-bronchial spaces that lacked contiguous alveolar structure (Fig. 6B), bronchiolar hemorrhage (Fig. 6C, open arrows), and hemorrhagic vacuolated alveolar epithelium (Fig. 6C, closed arrows). Lack of GM-CSF results in alveolar proteinosis, as indicated by the presence of

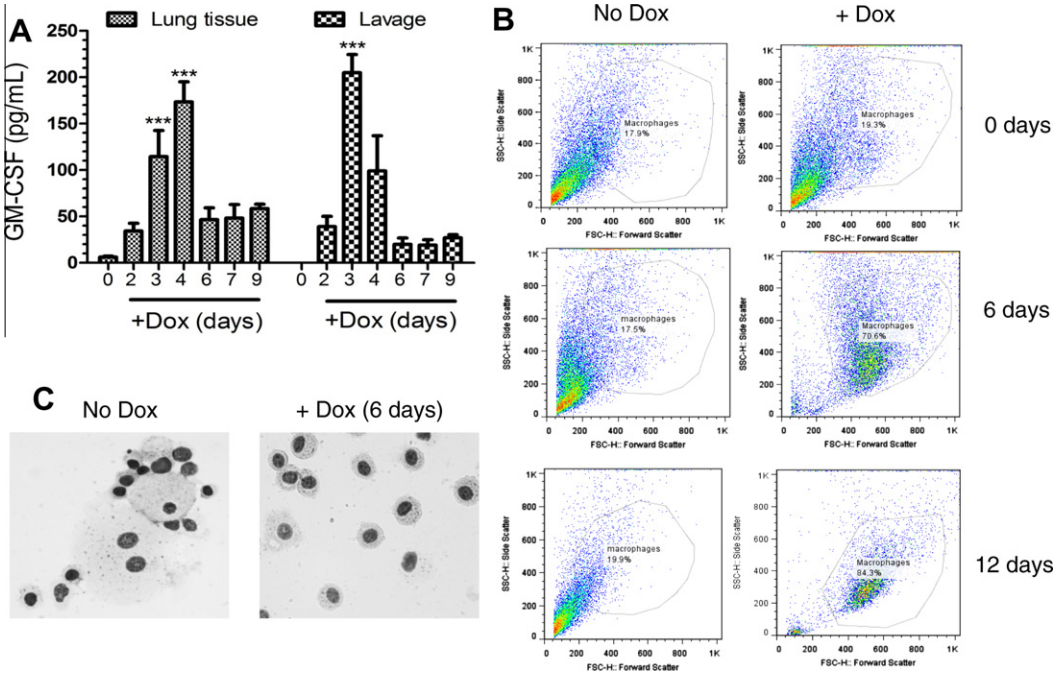

Fig. 3. Conditional expression of GM-CSF in the lung. *Tet-GM*$^{+/+}$ mice were provided sterile water supplemented with 1 mg/mL doxycycline to induce expression of GM-CSF. (A) ELISA assays assessed expression of GM-CSF in lavage and post-lavage tissue homogenate at indicated time intervals. Data shown are means ± SD, $n = 3$–5 mice per time point. $^{***}p < 0.001$ compared to the absence of doxycycline. (B) Flow cytometry reported the light scatter properties of cells in alveolar lavage before (0 days) or 6 and 12 days after addition of doxycycline in water. Gating distinguished the location of alveolar macrophages from surfactant debris at the bottom corner of the dot plots. Data shown are representative of 3–6 separate experiments per time point. (C) Representative cytospin evaluation of alveolar macrophages from *tet-GM*$^{+/+}$ mice before (left panel) and 6 days after administration of doxycycline (right panel).

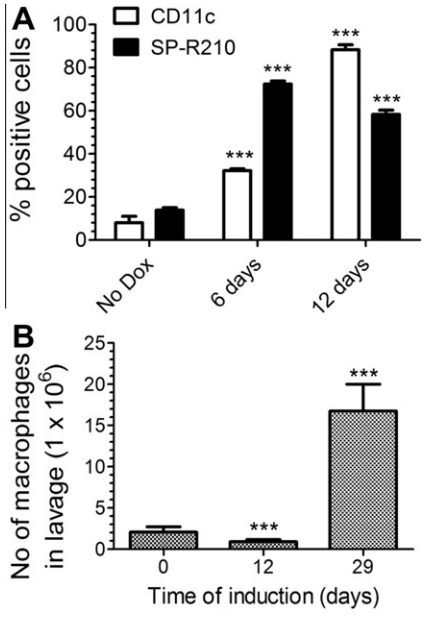

Fig. 4. Effects of conditional expression of GM-CSF on differentiation and number of alveolar macrophages. Alveolar macrophages were isolated by lung lavage before (0 day) or after induction of GM-CSF in *tet-GM*$^{+/+}$ mice. (A) Alveolar macrophage differentiation was assessed by dual flow cytometric cell surface staining using CD11c and SP-R210 antibodies. The percentage of gated alveolar cells that express CD11c and SP-R210 before or 6 and 12 days after induction of GM-CSF is shown for each marker. (B) Alveolar cells were counted using a hemacytometer before or at 12 and 29 days after administration of doxycycline. Data shown are means ± SD; $n = 3$ on day 0, and $n = 4$ on days 6, and $n = 3$ on day 29. $^{***}p < 0.001$.

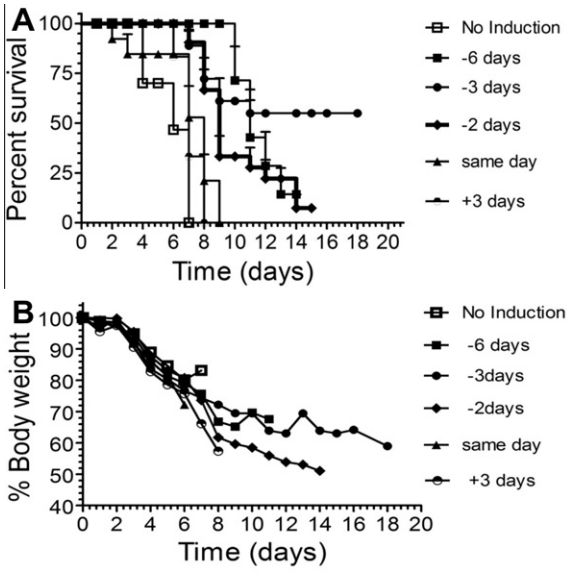

Fig. 5. Effect of conditional expression of GM-CSF on survival from influenza infection. Survival curves (A) and body weight (B) of *tet-GM*$^{+/+}$ mice was assessed after intranasal infection with 1.9×10^4 ffc of influenza PR8 of conditional mice placed on doxycycline 6, 3, or 2 days after, same day, and 3 days before infection. Mice displaying severe illness were euthanized immediately and counted as dead. Statistical parameters and number of mice used in this study are shown on Table 2.

amorphous surfactant aggregates staining with SP-B antibodies (Fig. 6D, arrows) in the alveolar lumen of uninfected *GM*$^{-/-}$ mice, consistent with previous results (Huffman et al., 1996). The alveolar proteinosis pathology in naive un-induced *tet-GM*$^{+/+}$ mice was similar to *GM*$^{-/-}$ mice (not shown). The lung histology of *GM*$^{-/-}$ mice 7 days after infection with influenza, however, showed many homogeneous eosinophilic surfactant globules filling alveolar and peri-bronchiolar spaces (Fig. 6E and F, open arrows). The morphology of the alveolar proteinosis material observed after

Table 2
Effect of GM-CSF induction on survival of *tet-GM*$^{+/+}$ mice from acute influenza infection.[1]

Doxycycline treatment relative to infection	No. of mice	Mean survival (days)	Statistics[2]
None	13	6	
−6 days	18	11	$p < 0.0009$
−3 days	23	>11	$p < 0.0001$
−2 days	15	9	$p < 0.0001$
Same day	12	8	$p < 0.0225$
+3 days	9	7.0	$p < 0.0368$

[1] The mean survival of littermate control *tet-GM*$^{+/+}$ mice was 6 and 6.5 days in the absence or presence of doxycycline. Mice were infected with 1.9×10^4 ffc of PR8.
[2] Compared to inducible mice that did not receive doxycycline.

infection of the 2-month-old *GM*$^{-/-}$ mice used here resembles the histology of uninfected 7-month-old *GM*$^{-/-}$ mice (Dranoff et al., 1994), suggesting that influenza infection exacerbated alveolar proteinosis in *GM*$^{-/-}$ mice. In contrast to the observations in WT mice (Fig. 6C), it is remarkable that the alveolar epithelium of *GM*$^{-/-}$ mice was not compromised by the influenza infection, suggesting that excess surfactant protected the alveolar structure of *GM*$^{-/-}$ mice. Under higher magnification, however, bronchi with flattened squamous epithelium contained light blue staining material suggesting accumulation of mucous in the airways of *GM*$^{-/-}$ mice (Fig. 6F, stars).

The histopathology of *tet-GM*$^{+/+}$ mice was then evaluated 3 days after induction of GM-CSF by administration of doxycycline that provides the best protection against influenza in these mice (Fig. 5). The histopathology of *tet-GM*$^{+/+}$ lungs showed focal well-organized peri-bronchiolar inflammation (Fig. 6G, H, and I, closed arrows) contiguous with largely intact alveolar epithelia containing many alveolar macrophages (Fig. 6G and I, open arrows). Alveolar proteinosis was not present. The abundance of alveolar macrophages in infected *tet-GM*$^{+/+}$ mice 3 days after induction of GM-CSF suggests rapid recruitment of macrophages. It is also possible, however, that GM-CSF stimulated local proliferation of macrophages in the presence of infection, even though GM-CSF did not enhance significant proliferation for several weeks in uninfected *tet-GM*$^{+/+}$ mice (Fig. 4 above). Formation of interstitial peri-bronchiolar lesions is consistent with coalescence of macrophages (Fig. 6G and I, closed arrows) with lymphocytes and other immune cells infiltrating the bronchial sub-mucosa (Fig. 6H, thick stealth arrows). Proteinaceous edema was limited in consolidated bronchiolar spaces at this stage of infection (Fig. 6H, stars). Bronchial epithelia displayed columnar epithelial cell morphology and bronchial spaces were largely clear of inflammatory exudates (Fig. 6H, thick stealth arrows). The histopathology of tet-GM$^{+/+}$ mice shown here is distinct from that observed in the constitutive *SP-C-GM*$^{+/+}$ mice in which a diffuse macrophage alveolitis was prominent 1–3 days after acute influenza infection (Huang et al., 2011). The present results indicate that induction of GM-CSF at the earliest stages of infection bolsters the ability of alveolar macrophages to organize innate and cell-mediated immunity against influenza.

3.6. The scavenger receptor MARCO modulates susceptibility to acute influenza infection

Next, we investigated how does GM-CSF-regulated, innate immune macrophage receptors affect recovery from acute influenza infection. GM-CSF enhances expression of scavenger receptors

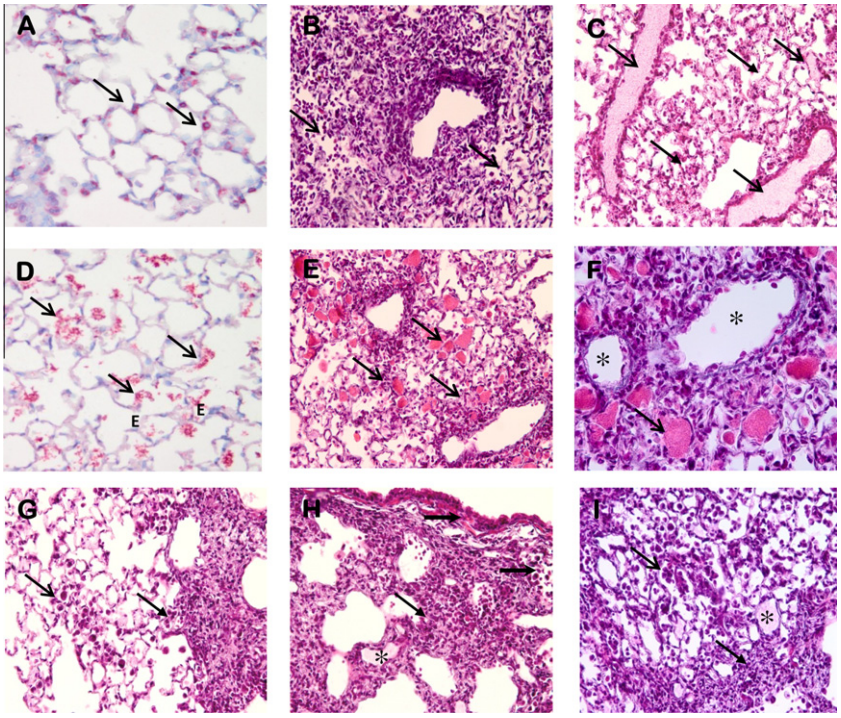

Fig. 6. Lung histopathology of WT and transgenic mice infected with influenza. The histology of naive WT (A) and *GM*$^{-/-}$ (D) mouse lungs was visualized after staining with anti-SP-B antibodies. Open arrows point to SP-B staining in alveolar type II epithelial cells in WT lungs (A) and surfactant aggregates accumulated in the alveolar lumen of GM$^{-/-}$ lungs (D). Lung histopathology after infection with 1.9×10^3 ffc of influenza PR8 was evaluated following H&E stained lung sections from WT (B and C), *GM*$^{-/-}$ (E and F), and *tet-GM*$^{+/+}$ mice infected 3 days after induction of GM-CSF with doxycycline. WT mice B and C; the open arrows on panel B show lymphocytic infiltrates in peribronchial spaces lacking defined alveolar structure in WT mice 6 days after infection. Open arrows on panel C show alveolar and bronchiolar hemorrhage and closed arrows on the same panel point to vacuolated alveolar epithelium. *GM*$^{-/-}$ mice E and F; open arrows on E and F show eosinophilic surfactant material filling alveolar spaces. The stars on panel F show bronchi with flattened epithelium with light blue eosinophilic material filling bronchial spaces. *tet-GM*$^{+/+}$ mice G–I; closed thin arrows on panels E, H, and I show organized lymphocytic inflammation in peribronchiolar interstitium. Thick stealth arrows on panel H point to sub mucosal lymphocytic infiltrates and bronchial epithelium with columnar morphology. The star on panel H indicates the presence of proteinaceous edema. Images H and I were captured at 40× magnifications. All other images are 20×. Representative images from 2 to 3 mice are shown.

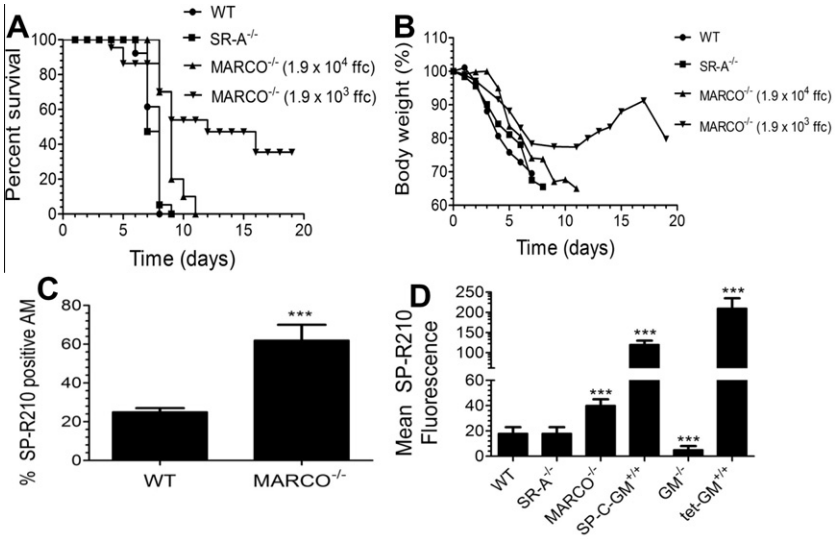

Fig. 7. Role of SR-A and MARCO on host survival from influenza infection. Survival (A) and body weight change (B) after intranasal infection of WT, SR-A$^{-/-}$, and MARCO$^{-/-}$ mice with PR8 influenza. WT and SR-A$^{-/-}$ mice were infected with 1.9×10^4 ffc of PR8. MARCO$^{-/-}$ mice were infected with either 1.9×10^4 or 1.9×10^3 ffc of PR8 as indicated. Infected mice were weighed daily and assessed every 12 h for the presence of clinical symptoms of the infection. Mice displaying severe illness were euthanized immediately and counted as dead. Statistical parameters and number of mice used in this study are shown on Table 1. (C) The percentage of SP-R210-positive alveolar macrophages from WT and *MARCO*$^{-/-}$ mice was assessed by flow cytometry. (D) The expression level of SP-R210 shown as mean fluorescence intensity in alveolar macrophages from WT and *SR-A*$^{-/-}$, *MARCO*$^{-/-}$, *SP-C-GM*$^{+/+}$, *GM*$^{-/-}$, and 6 day induced *tet-GM*$^{+/+}$ mice was assessed by flow cytometry. Data shown are means ± SD, $n = 3$–6 per mouse. $^{***}p \leqslant 0.001$ compared to WT mice.

SR-A and MARCO (Sulahian et al., 2008; Szeliga et al., 2008) which may modify host resistance to viral infection (DeWitte-Orr et al., 2010; Mukhopadhyay et al., 2011). SR-A modifies inflammatory responses via the SP-A receptor SP-R210 (Sever-Chroneos et al., 2011). Mean survival from influenza infection between *SR-A*$^{-/-}$ and WT mice were similar (Fig. 7A and Table 1). *MARCO*$^{-/-}$ mice, however, survived significantly longer (Fig. 7A) with a mean survival of 9 days (Table 1). At 10-fold lower infection dose, the mean survival of *MARCO*$^{-/-}$ mice increased to 12 days (Fig. 7A, Table 1) and also regained body weight more rapidly. The WT mice remained susceptible even to the low dose infection (Table 1). Interestingly, the percentage of macrophages expressing SP-R210 was 2-fold higher in uninfected *MARCO*$^{-/-}$ alveolar macrophages (Fig. 7C). Furthermore, the expression level of SP-R210 increased significantly as indicated by 2-fold increase in mean fluorescence compared to WT mice (Fig. 7D), indicating that MARCO is a negative regulator of SP-R210 expression. Comparatively, alveolar macrophages from the more resistant *SP-C-GM*$^{+/+}$ mice expressed 5-fold more SP-R210, while expression of SP-R210 in *tet-GM*$^{+/+}$ alveolar macrophages 6 days after induction was even higher, 13-fold more than WT mice (Fig. 7D). In contrast, SP-R210 expression decreased significantly in *GM*$^{-/-}$ alveolar macrophages. These results support the hypothesis that GM-CSF promotes survival from influenza infection by stimulating alveolar macrophage expression of the SP-A receptor SP-R210, whereas MARCO expression limits the resistance of mice to primary infection with influenza.

4. Discussion

The present findings show not only that GM-CSF enhances resistance to influenza but also pin-point the exact time window from 6 days before to 3 days after infection where GM-CSF is needed to reduce mortality from acute infection of highly pathogenic influenza A virus. Endogenous GM-CSF expression is crucial for initial survival as indicated by increased susceptibility of *GM*$^{-/-}$ mice compared to WT mice against influenza A virus. Expression of high levels of GM-CSF generated by constitutive or inducible promoters in lung epithelial cells bolstered the ability of *GM*$^{-/-}$ mice to survive influenza better than WT mice at doses as high as 50 LD_{50}. These studies are in agreement with and extend recent findings in which the constitutive *SP-C-GM*$^{+/+}$ mice were resistant to infection at doses up to 5 LD_{50} (Huang et al., 2011). The best protection of *tet-GM*$^{+/+}$ mice was observed when infection coincided with peak levels of GM-CSF 3 days after administration of doxycycline but GM-CSF was no longer needed if induced 3 days after infection. Albeit the increased resistance of both *SP-C-GM*$^{+/+}$ and *tet-GM*$^{+/+}$ mice to influenza, histological responses were different. The *SP-C-GM*$^{+/+}$ lungs displayed diffuse alveolar macrophage pneumonia 1–3 days after infection (Huang et al., 2011), where alveolar macrophage activation alone was sufficient to arrest replication of influenza. The early histology of *tet-GM*$^{+/+}$ mice, however, showed macrophage and focal lymphocytic infiltration of the distal airways, indicating that controlled GM-CSF expression results in activation of both innate and cell-mediated immunity against influenza. The present results support the concept that alveolar macrophage maturation, as initiated by GM-CSF expression, reinforces the development of mucosal immunity in the lung.

GM-CSF alters the activation state of alveolar macrophages through increased expression of innate immune receptors (Berclaz et al., 2007; Shibata et al., 2001). The protective effect of GM-CSF in *tet-GM*$^{+/+}$ and *SP-C-GM*$^{+/+}$ mice was associated with 5- to 13-fold higher levels of the SP-A receptor SP-R210 compared to WT mice. SP-R210 is responsible for clearance of SP-A-opsonized bacteria in *in vivo* infection (Sever-Chroneos et al., 2011). Studies in SP-A-deficient mice showed that the LD_{50} of an SP-D-resistant influenza A strain decreased 40-fold compared to WT mice (Hawgood et al., 2004). SP-A enhances uptake of influenza A by macrophages (Benne et al., 1997). Therefore, it is reasonable to speculate that GM-CSF increased the capacity of SP-A to protect mice against influenza via SP-R210. In addition to SP-R210, GM-CSF enhances expression of the class A scavenger receptors SR-A and MARCO (Szeliga et al., 2008; Winkler et al., 2008). These scavenger receptors have been implicated in anti-viral host defense and regulation of inflammatory responses in the lung (Dewitte-Orr et al., 2010; Jozefowski et al., 2006; Mukhopadhyay et al., 2011). Importantly,

SR-A acts as a co-receptor for SP-R210 regulating the timing and magnitude of inflammatory responses during clearance of SP-A-opsonized *Staphylococcus aureus* (Sever-Chroneos et al., 2011) *in vivo*. Lack of SR-A, however, did not alter survival from infection with PR8, suggesting that SR-A does not function in the same capacity during viral influenza as it does in the bacterial infection. In contrast, MARCO-deficient mice were significantly more resistant than WT mice. Interestingly, alveolar macrophages from *MARCO*$^{-/-}$ mice expresses significantly higher levels of SP-R210 compared to WT mice, indicating that MARCO suppresses expression of SP-R210 in WT mouse alveolar macrophages. In a parallel fashion, SP-R210 serves as a negative regulator of SR-A expression under normal conditions (Sever-Chroneos et al., 2011). Cross-talk between innate immune receptors has emerged as an important mechanism though which macrophages regulate their activation pathways (Seimon et al., 2006). It is possible that influenza disrupts signaling networks that govern activation of clearance receptors in macrophages. The present findings support the concept that high levels of GM-CSF surpass normal receptor cross-regulation directing expression and function of ancillary clearance mechanisms against influenza.

Consistent with the present findings in C57BL/6 *MARCO*$^{-/-}$ mice, a recent study showed enhanced resistance to influenza in *MARCO*$^{-/-}$ mice backcrossed to the Balb/c genetic background (Ghosh et al., 2011). These results emphasize that MARCO suppresses resistance to influenza independent of genetic background. Lack of MARCO resulted in increased levels of oxidized lipoprotein in connection with early recruitment of neutrophils, macrophages, and chemokines driving the increased early resistance of *MARCO*$^{-/-}$ mice to influenza A infection (Ghosh et al., 2011). Under normal circumstances, MARCO mediates clearance of oxidized lipids in the lung but in the presence of infection MARCO suppressed inflammatory mediators against influenza. Additional studies are needed to determine whether oxidized LDL induced expression of GM-CSF through the scavenger receptor SR-A as reported in earlier studies (Biwa et al., 1998).

Even though high levels of GM-CSF drive early resistance to influenza, uncontrolled expression of GM-CSF can lead to immunopathology disabling complete recovery from the infection in the long-term. The *SP-C-GM*$^{+/+}$ mice exhibit early but not long-term recovery from the infection. Histological analysis revealed desquamative interstitial pneumonia (DIP) as the likely cause of death secondary to infection with PR8 influenza. Persistent elevation of GM-CSF contributes to the development of chronic inflammatory diseases in the lung (Hamilton, 2008). DIP reflects accumulation of activated macrophages (Tazelaar et al., 2011). Earlier clinical reports documented a spectrum of interstitial lung diseases, including DIP, following influenza pneumonia in humans (Pinsker et al., 1981). Development of DIP was also observed after acute infection of SP-C-GM$^{+/+}$ mice with *Mycobacterium bovis* BCG (Szeliga et al., 2008) and *S. aureus* (Chroneos, unpublished data). These findings suggest GM-CSF as an etiology of interstitial lung disorders secondary to acute infectious pneumonia. Interestingly, oxidized phosphatidylcholine accumulates in alveolar macrophages of DIP patients (Yoshimi et al., 2005). Given that MARCO mediates uptake of oxidized lipoproteins as indicated above (Ghosh et al., 2011), it remains to be determined whether high levels of GM-CSF contribute to immunopathology through MARCO-mediated accumulation of modified lipids in infected lungs.

Influenza did not alter expression of GM-CSF via the inducible SCGB1A1 promoter driving expression of GM-CSF in bronchiolar Clara cells in *tet-GM*$^{+/+}$ mice. In *SP-C-GM*$^{+/+}$ mice, however, GM-CSF decreased significantly in the alveolar lavage relative to lung tissue in the initial phase of the infection, indicating that influenza attenuates secretion of GM-CSF by alveolar type II epithelial cells. Alveolar epithelial cells are highly vulnerable to influenza infection (Fukushi et al., 2011). In this context, influenza suppressed secretion but not mRNA levels of SP-A and SP-D by cultured alveolar type II epithelial cells (Wang et al., 2011) but did not affect secretion of inflammatory chemokines. More recently, knock-in replacement of the murine IL-3/GM-CSF gene locus with the human genes resulted in high levels of human GM-CSF in the lungs and development of alveolar proteinosis; the endogenous murine GM-CSF receptor responds to human GM-CSF poorly (Willinger et al., 2011). Engraftment of these mice with human macrophages reversed alveolar proteinosis. Human GM-CSF enhanced antiviral responses by engrafted human macrophages, but did not arrest proliferation of influenza virus, indicating that alveolar macrophages facilitate but are not sufficient for GM-CSF-mediated protection against influenza. Thus in addition to macrophage activation, high levels of GM-CSF at the time of infection are needed to protect normal secretory functions of alveolar type II epithelial cells during influenza infection.

In summary, high levels of GM-CSF enhance host resistance against influenza. Regulated expression of GM-CSF in epithelial cells of conditional *GM*$^{-/-}$ mice represents an important new strategy to discern protective functions of GM-CSF and identify therapeutic targets against influenza infection the future. Histological responses in *tet-GM*$^{+/+}$ mice following optimal control of high GM-CSF levels at the time of infection indicate a clinically relevant immune response consisting of alveolar macrophage proliferation and focal lymphocytic inflammation of the airways, reinforcing resistance to acute influenza pneumonia. The role of GM-CSF in enhancing the function of SP-R210 and its interaction with MARCO in long-term immunity against influenza and their respective roles in lung immunopathology requires further investigation. In the latter case, targeting of the scavenger receptor MARCO may enhance the efficacy of GM-CSF in treatment against highly pathogenic influenza strains.

Acknowledgements

We thank Kris Stewart and Woody Bradly in the UTHSCT vivarium for help with animal husbandry and genomic screening of transgenic mice.

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