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Anti-viral instruction of bone marrow leukocytes during respiratory viral infections

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Summary

Respiratory viral infections are the cause of severe diseases in humans. The outcome of the infection depends on the interaction of the pathogen with the immune system. The bone marrow is the primary site of hematopoiesis and releases large numbers of leukocytes in response to inflammation. Here we show that during infection with influenza or Sendai virus the lung communicates with the sterile bone marrow through type I interferons. While in the bone marrow, leukocytes exposed to type I interferons activate an anti-viral transcriptional program and become resistant to infection with different viruses. The protected bone marrow leukocytes are capable of migrating to the infected lung and contribute to virus clearance. These findings show that appropriate instruction of cells during their development in the bone marrow is needed for the effective innate control of infection.

Introduction

Infections with viruses that replicate in the respiratory tract trigger a robust inflammation of the lung. The inflamed lung produces cytokines, chemokines, and growth factors that promote a massive infiltration of cells. This infiltrate is composed by a large number of granulocytes, monocytes (Aldridge et al., 2009; Lin et al., 2008), natural killer cells (NK) (Gazit et al., 2006), plasmacytoid dendritic cells (pDCs), conventional DCs (cDCs) (Grayson et al., 2007; Wolf et al., 2009) and other cell types critical for the clearance of the infection and for the initiation of adaptive immunity (GeurtsvanKessel et al., 2008; Kim and Braciale, 2009; Moltedo et al., 2009). Much work has been performed to identify the chemokines that act to promote lung leukocytes infiltration during infection, to sub-classify these cells, and to study their role during anti-viral immunity. However, the effect that the lung cytokine milieu has on leukocytes in distal hematopoietic and lymphoid organs has been overlooked and whether such effect has implications in immunity to virus infection is unknown.

Among the cytokines produced in the infected lung are the type I interferons (IFNs) which have been extensively studied due to their potent anti-viral properties. Type I IFNs are produced by infected epithelial cells (Jewell et al., 2007), pDCs, alveolar macrophages, and other cells (Kumagai et al., 2007). Binding of type I IFNs to their receptor induces the transcription of a

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Author contributions

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number of IFN stimulated genes (ISGs) establishing an anti-viral state (Samuel, 2001). ISGs include genes coding for sensors of viral RNA like retinoic acid inducible gene (RIGI) and melanoma differentiation-associated gene-5 (MDA5), signaling proteins such as the interferon regulatory factor 7 (IRF7) and proteins involved in general resistance to viral infection such as myxovirus resistance 1 (Mx1) and ISG15. The importance of type I IFNs as a major anti-viral mechanism is evident as virtually all pathogenic viruses encode for antagonists of type I IFN production and/or function (Randall and Goodbourn, 2008; Weber et al., 2004). Additionally, type I IFNs affect the development of the immune response by promoting DC maturation (Luft et al., 1998), NK cell activation (Biron et al., 1999), $CD4^+$ Th1 bias (Murphy et al., 2000), and antigen specific T cell proliferation (Kolumam et al., 2005).

Studies conducted in vitro have shown that treatment of leukocytes such as monocytes (Gerlini et al., 2008) and monocyte derived DCs (Osterlund et al., 2005; Phipps-Yonas et al., 2008) with type I IFNs prior to virus infection limits the ability of the virus to replicate in these cells while improving their ability to stimulate T cells (Gerlini et al., 2008). We hypothesized that leukocyte priming by type I IFNs also occurs in vivo, and that leukocytes educated in the periphery by type I IFN signaling are recruited to the lung during infection to improve the efficiency of virus clearance.

The bone marrow (BM) is the major site of hematopoiesis in the adult. In the steady state, millions of leukocytes, erythrocytes and hematopoietic stem cells (HSCs) are released daily from the BM in order to replenish the hematopoietic compartment throughout the organism. Inflammation in any organ of the body significantly changes the environment and cell composition of the BM, leading to the release of monocytes (Serbina and Pamer, 2006; Tsou, 2007), neutrophils (Navarini et al., 2009; Wengner et al., 2008), NK cells (Bernardini et al., 2008) and HSCs (Elena Nardini, 2005; Ping Zhang, 2008) to the blood. The BM is therefore the most likely main supplier of cells to inflamed tissues. Studies using non-infectious inflammatory models, bacterial infections, or models of systemic infection have helped to elucidate some of the events occurring in the BM during inflammation. However, very limited research has been done with regard to the events occurring in the BM during a distal viral infection and the function of released BM cells in the resolution of a respiratory infection has not been investigated.

Infection with Sendai virus (SeV) or with most strains of influenza virus in mice does not lead to viremia since these viruses replicate exclusively in the respiratory tract (Scheid and Choppin, 1974; Tashiro et al., 1992). We chose SeV and influenza virus to study the systemic effect of cytokines produced in the lung during infection without the complicating contribution of direct viral infection of cells outside the respiratory tract. Here we demonstrate that infection of the lung is sensed systemically and that cells in distal organs including the BM are instructed to generate an improved response to the pathogen when they are recruited to the infection site. Overall our findings suggest that the communication between the infected lung and leukocytes developing in the BM is an integral part of the immune response to respiratory virus infections. This previously overlooked mechanism may provide useful insight for the development of novel medical strategies for the treatment of lethal virus infections and other immune disorders that involve BM leukocytes.

Results

SeV infection induces a systemic anti-viral response

To characterize the inflammatory response induced by SeV infection, we quantified the levels of multiple cytokines and chemokines in the lung and blood of mice at different times post infection (Figures 1A and 1B). Similar to the kinetics of inflammation reported for influenza virus infection (Moltedo et al., 2009), cytokines and chemokines began to be detected in the

lung on the second day after infection, reaching high levels at days 3–5 of infection. The same kinetics was closely followed in the blood (Figure 1B). While type I IFNs (IFNα and IFNβ) were detected in the lung, we were not able to detect them in the serum using conventional ELISA. As expected, the induction of chemokines in the infected respiratory tract correlated with a robust infiltration of leukocytes into the lung (Figure 1C).

We then assessed the expression of anti-viral genes in lung, spleen, BM and peripheral lymph nodes (pLNs) of infected animals. Transcription of ISG15, IRF7, Mx1, RIGI and MDA5 was observed in all the organs tested (Figure 1D and data not shown) following the same kinetics as the lung inflammatory response (Figure 1A). The enhanced anti-viral gene expression observed in the BM was not due to a relative increase in the pDC population which constitutively express IRF7 (Izaguirre et al., 2003) (Figure S1).

Lung-derived type I IFNs trigger the BM anti-viral response

Because the genes induced in the periphery are known targets of type I IFN signaling, we investigated whether these cytokines were responsible for the induction of anti-viral genes in the BM. We infected type I IFN receptor deficient (IFNRKO) mice with SeV or influenza virus and compared their gene expression the BM to that of WT controls. Infected IFNRKO mice exhibited impaired induction of anti-viral genes compared to controls (Figure 2A and 2B). The inflammatory cytokines IL12p70, IL6, IFNβ and IFNγ were produced to high levels in the lungs of SeV infected IFNRKO mice, demonstrating that the impairment observed in the BM of these mice was not due to reduced cytokine production in the lung (Figure 2C). In addition, in chimeric mice, WT donor hematopoietic cells upregulated the transcription of anti-viral genes in response to SeV infection while donor IFNRKO hematopoietic cells did not, regardless of the host responsiveness to type I IFN (Figure 2D). These results indicate that type I IFNs provide the primary signal for the induction of anti-viral genes in BM cells.

To more closely examine the source of the type I IFNs that activate the BM during infection, we measured the level of IFN α and β mRNAs in the lung, spleen, pLNs, blood and BM cells isolated from mice infected with SeV (Figure 2E and Figure S2A). Transcription of type I IFNs was only detected in the lung of infected mice. In addition, we were unable to detect expression of IFNs or any other cytokines (IL28α, IFNγ, CCL2, IL-6, G-CSF) in any discrete BM cell population (Figure S2B and data not shown), indicating that the source of IFNs activating the BM is solely the site of infection.

We then hypothesized that during infection type I IFNs were transported through the blood to peripheral organs at levels undetectable by commercially available ELISAs. To examine whether type I IFNs were present in the serum, we used a sensitive bioassay. In this assay, serum obtained at different times after infection with SeV was incubated ex-vivo with BM cells isolated from non-infected mice. The expression of anti-viral genes by the BM cells was then determined by qRT-PCR. BM cells isolated from uninfected mice and incubated with serum isolated from infected mice showed increased anti-viral mRNA transcripts (Figure 2F), similar to what we had observed in vivo. However, IFNRKO BM cells incubated with the same serum were unresponsive, indicating that type I IFNs present in the blood of infected mice were are least partially responsible for the observed gene expression. Altogether, these results show that type I IFNs originated in the infected lung are distributed systemically through the blood and instruct cells in the BM to confront the infection.

Induction of anti-viral programming of the BM does not result from direct virus infection

We next confirmed that in our system virus replication was restricted to the lung. Viral nuclear protein (NP) mRNA was detected at high levels in the lungs of mice infected with either SeV or influenza virus but was not detected in their blood or BM (Table 1). Furthermore, we did

not detect replicating SeV in the BM and blood of infected mice at any time analyzed, while infectious virus was generated in the lung to high titers (Table 1 and data not shown). Additionally, the expression of viral proteins and virus replication were significantly higher in the lungs of IFNRKO mice than in those of WT mice. Despite the higher level of virus in IFNRKO lung, viral mRNA was not detected in the BM of these mice confirming that SeV and influenza virus do not replicate in the blood or BM of infected animals. These results validate the use of these viruses to study the effects of a localized virus infection that does not reach the BM compartment.

BM cells from infected mice are protected from viral replication

We reasoned that the expression of anti-viral genes confers protection to leukocytes against virus infection while in the BM or when recruited to the infected lung. We therefore investigated if BM cells isolated from infected mice were resistant to virus infection. To do so, BM cells isolated at different times after infection with SeV (Figure 3A) or influenza virus strain X-31 (Figure 3B) were infected ex-vivo with either SeV, influenza PR8 virus, or Herpes Simplex virus (HSV) and viral replication was determined through the expression of viral mRNA. BM cells isolated from infected mice resisted infection by all three viruses. These results indicate that leukocytes in the BM of infected mice are programmed to limit virus replication.

B cells, pDCs and monocytes in the BM are instructed by type I IFN to confront a viral infection

To determine which leukocytes in the BM responded to type I IFNs produced in the infected lung, we performed flow cytometry analysis on cells obtained from the BM of Mx-Cre x Rosa26-stop^{flox}EGFP reporter mice. The activation of the type I IFN sensitive Mx promoter in these mice leads to the expression of Cre recombinase that removes a stop cassette upstream of the floxed reporter leading to EGFP expression (Kuhn et al., 1995; Mao et al., 2001). In these reporter mice, cells that are activated by type I IFN express EGFP permanently. BM pDCs, B cells and monocytes isolated from infected mice showed increased expression of EGFP compared to mock infected or Rosa26-EGFP littermates that do not express Mx-Cre (Figure 4A). Sorted cells from the BM of infected mice demonstrated an enhanced transcription of anti-viral genes compared to control mice demonstrating that these cells were instructed while residing in the BM (Figure 4B and Figure S2B).

Monocytes recruited from the BM have been proposed to contribute to immunity against respiratory viruses (Aldridge et al., 2009; Lin et al., 2008). In our system, monocytes from infected mice that were infected ex-vivo were less permissive to both SeV and influenza virus replication than monocytes from control animals (Figure 4C) similarly to total BM cells (Figure 3). To determine the role of these cells during the resolution of SeV infection, we infected CCR2 deficient mice (CCR2KO) that show impaired recruitment of monocytes in other infection models (Aldridge et al., 2009; Lin et al., 2008; Navarini et al., 2009; Serbina and Pamer, 2006; Tsou, 2007). As expected, CCR2KO mice had a reduced number of monocytes in the blood than WT mice. These animals also exhibited impaired recruitment of monocytes to the lung during the course of SeV infection while these cells accumulated in the BM (Figure S3A, S3B and S3C). In addition, CCR2KO mice showed delayed clearance of SeV as compared to WT controls (Figure 4D) suggesting that monocytes recruited from the BM and instructed during infection play a role in the clearance of SeV from the lung. The failure to promptly clear the virus from CCR2KO mice was independent of recruitment of T cells to the lung, as similar numbers of CD8+ virus specific T cells were present in the lung at various time points after infection (Figure S3D).

Type I IFN priming of cells newly recruited to the lung is needed for rapid viral clearance

We examined whether exposure of BM cells to type I IFN affected their function upon recruitment to the lung during infection. To do this, we determined the extent of viral replication and cytokine transcription in hematopoietic cells ($CD45^+$) purified from the infected lung of BM transplant recipients. IFNRKO $CD45^+$ cells showed higher level of both SeV NP and F mRNA than WT $CD45^+$ cells regardless of the host's IFN responsiveness (Figure 5A). However, despite exhibiting a lower level of viral mRNA, WT donors showed higher transcription levels of the pro-inflammatory genes IL12p40, CCL2 and CXCL10. SeV titers of whole lung homogenates corroborated the essential role of hematopoietic cells primed by type I IFN in controlling virus replication in vivo (Figure 5B).

Notably, in type I IFN receptor deficient mice whole lung homogenates showed enhanced cytokine production compared to WT mice (Figure 2C), differently from the response observed in pure hematopoietic cell preparations (Figure 5A). It is possible that the contribution of different lung cell types to the cytokine response is altered in the absence of type I IFN. To rule out the possibility that the differences between WT and IFNRKO cells recruited to the lung were due to the different virus burden and cytokine environment that these cells were exposed to in the lung, we generated mixed chimeric mice transplanted with a 1/1 ratio of WT and IFNRKO donor BM cells in a WT host. In these animals both WT and IFNRKO cells were exposed to the same environment. Analysis of gene expression in cells sorted from the lungs of infected mixed chimeras (Figure 5C) confirmed that responsiveness to type I IFN protects hematopoietic cells from infection in the lung and allows for their maximal contribution to the inflammatory process through the production of pro-inflammatory molecules.

BM leukocytes from infected mice are recruited to the lung and are protected from virus infection

To determine if BM leukocytes are recruited to the lung during infection, we followed the migration of adoptively transferred BM leukocytes in an infected animal. To do this, we isolated BM cells from $CD45.1^+$ WT or IFNRKO mice four days after infection with SeV (Figure S4). The cells were labeled with high and low levels of carboxyfluorescein diacetate succinimidyl ester (CFSE) respectively, and adoptively transferred to $CD45.2^+$ WT mice that had been infected for two days with SeV (Figure 6A). This timing was selected to ensure that the appropriate cytokine and chemokine environment had been established in the lung of host mice and that newly recruited cells had not yet saturated the lung. One day after the adoptive transfer $CD45.1^+$ cells from the lung and spleen of recipient mice were enriched and sorted based on their CFSE content. A slightly higher proportion of WT than IFNRKO donor cells were found in the infected host lung (Figure 6B). Most of the cells of either type found in the lung were of myeloid origin as indicated by their expression of CD11b.

Adoptively transferred BM cells that infiltrated the lungs but not the spleen expressed viral mRNA (Figure 6C), supporting the evidence that replication of SeV was restricted to the lung. Similarly to what we observed in chimeric animals (Figure 5), the level of virus replication was higher in IFNRKO cells present in the lung compared with WT cells. Additionally, transferred IFNRKO BM cells demonstrated impaired production of type I IFNs and other cytokines in the lung of host animals compared with WT transferred cells (Figure 6D). Cytokine induction was only observed in the cells infiltrating the lung, demonstrating that although the cells are primed in the BM they will only produce cytokines once they sense the virus in the lung. These data show that leukocytes primed in the BM can infiltrate the infected lung and become efficient cytokine producers while protected from virus infection.

Discussion

A number of different cell types are engaged in combating the pathogen in the lung during the days that immediately follow infection, before the activation of the adaptive immune response (Kohlmeier and Woodland, 2009). Since the BM is the major site of hematopoiesis in the adult mammal (Kondo et al., 2003) it is likely to contribute to replenishing and supplying most of the cells recruited to the lung during infection.

Infections with different viruses including human immunodeficiency virus (Alexaki and Wigdahl, 2008; Prost et al., 2008), vaccinia virus (Pratibha Singh et al., 2008), lymphocytic choriomeningitis virus (Binder et al., 1997), murine cytomegalovirus (Crane et al., 2009) and bacterial infections have a documented affect on the BM (Feng et al., 2008; Navarini et al., 2009; Serbina and Pamer, 2006; Tsou, 2007; Ueda et al., 2005). In addition, treatment of mice with Toll like receptors agonists was shown to drive differentiation of hematopoietic stem cells in the BM towards monocytes or DCs (Nagai et al., 2006; Welner et al., 2008). These models illustrate the ability of cells in the BM to respond to a systemic stimulus. However, the immune response to an infectious agent is tremendously complex. These studies do not discriminate between the direct detection of the pathogen that can breach the site of infection and the sensing of the pro-inflammatory environment derived from the primary infection site. SeV and most strains of influenza virus do not disseminate from the respiratory tract to the blood or other peripheral organs. These models permit the study of the BM responsiveness to the systemic inflammatory signals that originates in the infected lung without the complicating effect of direct viral infection of the cells in the BM.

Based on recent studies pointing out the responsiveness of dormant HSCs to type I IFNs (Essers et al., 2009; Sato et al., 2009), it is reasonable to hypothesize that due to the cytokine storm that follows infection with SeV and influenza virus, HSC activity in the BM is also influenced leading to the differentiation and release of cells specifically prepared to resist and fight infection. Interestingly, we were unable to detect transcription of IL6, IL12p40, CCL2 and type I IFNs (or other cytokines mRNA) in leukocytes from the BM of infected mice at any time post infection. Restricted cytokine production in the infected organ may avoid the risk of overwhelming the BM compartment with cytokines that will either over-drive cell proliferation or cause cell death leading to BM exhaustion.

The rate-limiting step for anti-viral induction in the BM appears to be the production of inflammatory cytokines at the site of infection (Moltedo et al., 2009). While the anti-viral activity of type I IFNs is extensively documented, it is usually considered to signal over short distances with a short half-life (Peleg-Shulman et al., 2004; Pepinsky et al., 2001). We have demonstrated that during a respiratory infection type I IFNs signal over long distances and that type I IFNs present in the serum are sufficient to instruct cells in the BM. Our data does not eliminate the possibility that other mechanisms complement the transport of type I IFN to the BM during infection. However, we were unable to detect transcription of type I IFNs in the BM, spleen, pLNs, thymus or blood at any time before or after infection. Although it has been suggested that type I IFNs are continually produced in the steady state (Lienenklaus et al., 2009; Wang et al., 1995) our results show that induction of anti-viral genes in non-infected tissues occurs only after type I IFNs are produced at the site of infection.

It has been reported that the BM B cells are reduced during influenza infection suggesting that cells residing in the BM respond to the lung inflammation (Sedger et al., 2002). Our data demonstrates that acute infection of the lung is sensed, mainly through type I IFN signaling, by different cells in the BM. This signal educates BM cells for their future encounter with viral cues making them resistant to virus infection and hyperresponsive to viral danger signals. These data demonstrate that the reported enhancement of cytokine production in response to virus

infection of DCs and monocytes previously exposed to type I IFN in vitro also occurs during the context of infection in vivo (Gerlini et al., 2008; Osterlund et al., 2005; Phipps-Yonas et al., 2008). The need for cells to be recruited from the BM to the lung in order to fight infection efficiently is highlighted by the delayed clearance of SeV virus from the lungs of CCR2KO mice in which monocytes do not efficiently infiltrate the site of infection. It stands to reason that recruitment of primed cells is even more advantageous than that of cells that did not acquire the anti-viral state. Future studies will need to address how various cell subtypes respond in the BM to the inflammatory environment and what are their relative contributions to virus control.

Viruses that employ distinct mechanism and strategies for entry, replication and antagonism failed to replicate in primed BM cells indicating that the anti-viral effect observed in the BM is broad and not specific to the original infectious agent. Immunosuppressed individuals, for example patients receiving BM transplants, are extremely prone to viral infections (Styczynski et al., 2009). These patients are currently treated with specific anti-viral drugs to avoid infection or reactivation of chronic viruses. Based on our studies it is reasonable to propose that priming of BM cells with low doses of type I IFNs might provide these patients with protection against unrestrained virus infection.

Experimental procedure

Mice and Viruses

C57BL/6 (CD45.2$^+$) and B6.SJL (CD45.1$^+$) mice were obtained from Taconic Farms. IFNRKO mice (C57BL/6 background) were a kind gift from Dr. Wilson CB (Department of Immunology, Washington University) and were backcrossed to a B6.SJL (CD45.1$^+$) background. CCR2KO from Jackson laboratories (Boring et al., 1997), Mx-Cre mice (Kuhn et al., 1995) were crossed to a reporter strain in which the Rosa26 promoter is upstream of a floxed stop cassette and the EGFP gene (Mao et al., 2001). Age and sex matched animals were used in all experiments. The animals were bred and housed in pathogen-free conditions and the experiments were performed according to institutionally approved protocols. SeV strain 52 and influenza virus strains PR8 and X31 were grown in 10-day embryonated chicken eggs (SPAFAS; Charles River Laboratories). Egg's allantoic fluid was snap frozen in ethanol-dry ice bath and stored at −80°C. HSV (Kos1.1 strain) was propagated and titrated on Vero 2.2 cells.

Mice infection and lung virus titration

Anesthetized mice were infected intranasally with 10 to 20 50% infectious doses (ID_{50}) of SeV-52 in phosphate-buffered saline (PBS) or by aerosol (Glass-Col Corp, Model A4212) with influenza strain PR8 or X31 solution consisting of $10^{7.9}$ virus particles/12 ml PBS for 30 min. For virus titration, the lungs were extracted, homogenized in PBS-gelatin (0.1%), and frozen in dry ice-ethanol for preservation. The presence of infectious SeV and influenza virus particles was evaluated by infecting LLCMK2 or MDCK cells (respectively) with 1:10 dilutions of the lung homogenates at 37°C. After 1 h of infection, 175 μl of medium containing 2 μg/ml trypsin was added and the cells were further incubated for 72 h at 37°C. A total of 50 μl of medium was then removed from the plate and tested by hemagglutination of chicken red blood cells (RBCs) for the presence of virus particles. Viruses at 1:4 dilutions in 0.5% chicken RBCs were incubated for 30 min at 4°C. The hemagglutination of RBCs indicated the presence of virus particles.

RNA isolation and qRT-PCR

Lungs, spleen, pLNs and thymus were harvested at different times after infection and homogenized in 3ml TRIzol reagent (Invitrogen) using GentleMACS Dissociator M tubes

(Miltenyi). BM from femurs and tibias was flushed with cold PBS and resuspended in TRIzol. Cells infected in vitro were resuspended in 500ul of TRIzol. RNA was isolated from TRIzol according to the manufacturer's protocol. Total mRNA was analyzed by qRT-PCR as previously described (Yount et al., 2008). See supplemental experimental procedure for list of primers.

Flow cytometry

Lungs were flushed with cold PBS containing 0.5mM EDTA and immediately grinded and digested with collagenase (Liberase Blendzymes, Roche, Indianapolis, IN) in RPMI supplemented with 0.5% FCS at 37°C for 45 min. EDTA was added to the samples for resuspension. BM was flushed with PBS supplemented with 2% FBS using a 25 gauge syringe. RBCs were lysed with lysis buffer (BD bioseciences). Single-cell suspensions were incubated with anti–mouse CD16-32 (BD Biosciences) for 10 min at 4°C. The following antibodies were used in different staining and magnetic cell separation protocols: B220 (RA3-6B2), CD3 (145-2C11), CD19 (1D3), NK1.1 (PK136), CD49b (DX5), Ter119, CD11b (M1/70), CD11c (HL3), CD45.1 (A20), CD45.2 (104), CD115 (AFS98), Ly6C (AL-21), Gr-1 (RB6-8C5) Ly6g (1A8), IA/IE (M5/114.15.2), SiglecH (eBio440c) from BD Biosciences or eBioscience. mPDCA-PE (JF05-1C2.4.1) was obtained from Miltenyi Biotec. Immediately before reading the samples, propidium iodide (PI) was added to exclude dead cells. PE conjugated SeV NP specific tetramer was purchased from Proimmune. Flow cytometry was performed in a Cytomic FC500 Coulter station (Beckman Coulter) and analyzed by the FloJo software. See supplemental experimental procedure for cell enrichment and sorting procedure.

Cytokine detection in serum and lung

Whole lung was grinded in 1.8 ml 0.1% gelatin/PBS. Cytokine concentration in the lungs and serum was analyzed by 22-plex multiplex ELISA (Milipore). IFNβ and IFNα were measured by ELISA (PBL biomedicals).

Ex vivo infections and Serum treatment

BM cells isolated from infected mice were incubated in RPMI containing gentamycin, β mercaptoethanol, and 2% normal mouse serum, and infected with SeV 52, influenza PR8 or HSV at MOI of 2. Cells were incubated for additional 6 h prior to collection for RNA extraction. Serum was isolated from infected mice at different times post-infection. Naive BM cells from WT or IFNRKO mice were incubated in media containing freshly isolated serum for 6 h at 37° C.

Irradiation and BM transplantation

Eight-week-old recipient mice were lethally gamma-irradiated with 1,300 rads delivered in two doses of 650 rads each, three hours apart. Mice were injected intravenously with 2–3×10^6 BM cells. Hematopoietic engraftment was analyzed by measurement of blood chimerism four weeks after transplantation by flow cytometry.

Adoptive transfer and isolation of cells

Donor WT (n=5) and IFNRKO (n=5) $CD45.1^+$ mice were infected for four days with SeV. BM WT and IFNRKO cells were isolated and labeled with 3.5μM or 0.5μM of CFSE respectively. Donor WT and IFNRKO BM cells were evenly mixed and 10^7 cells were injected intravenously to recipient WT CD45.2+ mice infected with SeV for two days (n=35). One day after the transfer $CD45.1^+CFSE^{hi/low}$ cells were separated from the lung and spleen of recipient mice by negative magnetic bead separation against CD45.2 followed by cell sorting for high and low CFSE content. mRNA was extracted and amplified by WT-Ovation™ RNA

Amplification System (NuGEN, San Carlos CA) according to the manufactures instructions prior to its analysis by qRT-PCR

Statistical analysis

We expressed the results as means±s.d. We determined the statistical significance of differences between two groups using one-sided Student's *t* tests. Values of at least $P < 0.05$ at $\alpha=0.05$ were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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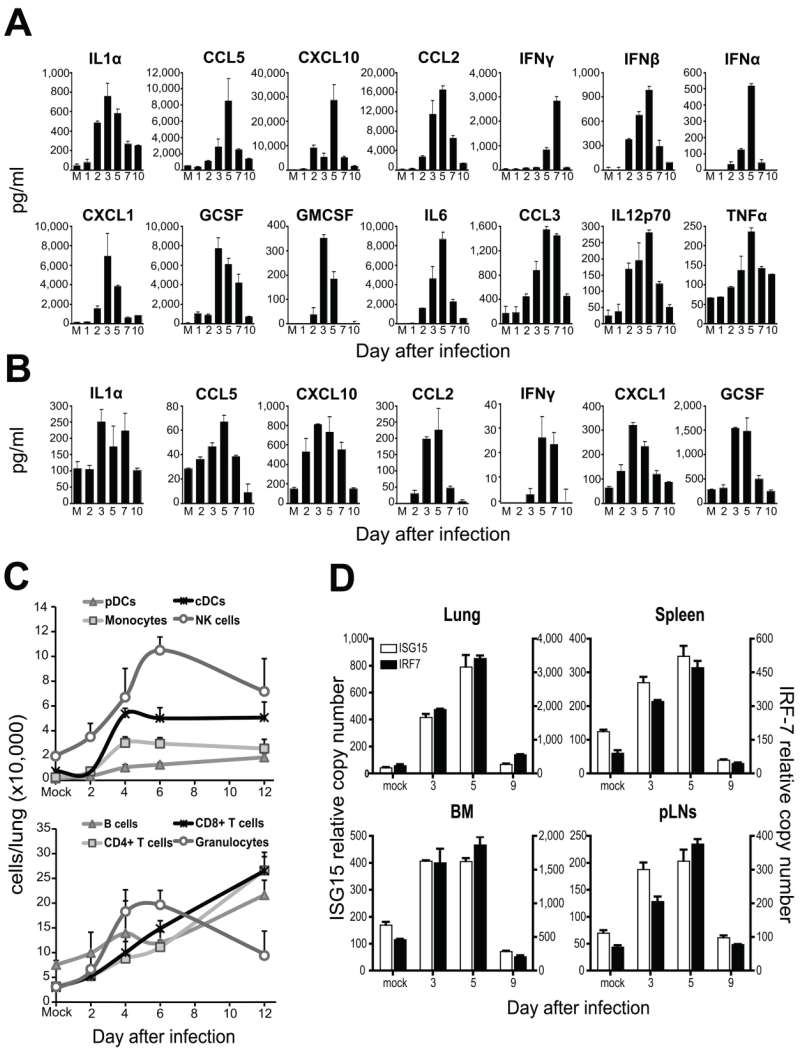

Figure 1.

Lung inflammation correlates with the systemic transcription of anti-viral genes. Cytokines from (A) fresh lung homogenates and (B) sera obtained from mice infected with SeV and sacrificed at different days post inoculation. Mock (M) infected animals were used as controls. (C) Lung cells count after tissue digestion. Cell numbers for specific cell population were extrapolated from flow cytometry analysis. The cells were pre-gated on live (PI^{-}) $CD45^{+}$ cells. B cells ($CD11b^{-}mPDCA^{-}B220^{+}$), pDCs ($CD11b^{-}mPDCA^{+}B220^{+}$), monocytes ($Ly6c^{+}Ly6g^{-}CD115^{+}$), Granulocytes ($Ly6c^{+}Ly6g^{+}$), NKs ($NK1.1^{+}CD11b^{+}$), cDCs ($CD11c^{+}MHCII^{+}CD11b^{+}$), $CD4^{+}$ T cells ($CD3^{+}CD4^{+}CD8^{-}$), CD8+ T cells ($CD3^{+}CD4^{-}CD8^{+}$). (D) ISG15 and IRF7 mRNA levels in the lung, pLNs, BM and spleen at different days after infection with SeV. mRNA was normalized to the house keeping genes α-tubulin, rps11 and β-actin of each sample analyzed. Error bars indicate the standard deviation of triplicate measurements in a representative experiment. See also Figure S1.

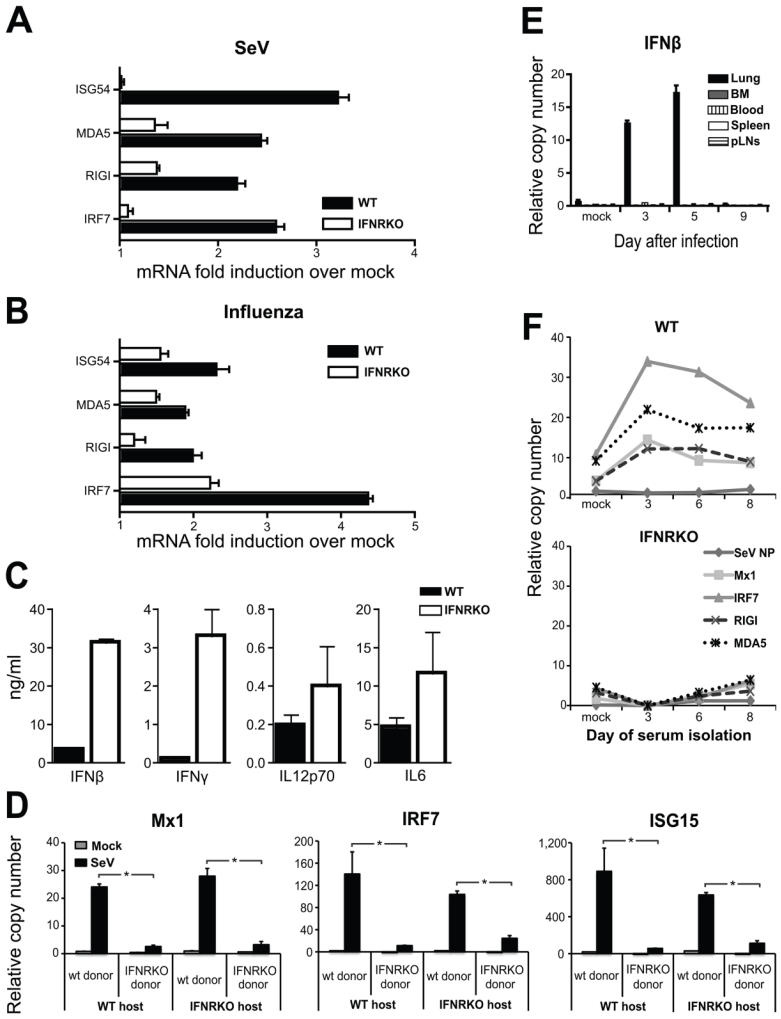

Figure 2.

Transcription of anti-viral genes in the BM is induced by type I IFNs transported in the blood. (A–B) Gene expression in BM of wild type mice or type I IFN receptor KO mice infected with (A) SeV two days after infection or (B) influenza PR8 virus three days after infection. (C) Cytokines in lung homogenates obtained from WT and IFNRKO mice 3 days post-infection. (D) Gene expression in the BM of complete chimeric mice infected for five days with SeV (n=4 mice in each group; $^{*}p< 0.05$). Error bars represent means±S.D. (E) IFNβ expression in the lung, BM, blood, peripheral lymph nodes (pLN) and spleen of wild type mice infected with SeV. See also figure S2. (F) Gene expression in naive BM cells isolated from WT (top panel) or IFNRKO (bottom panel) mice after a 6 h incubation with serum obtained from wild type mice at different times after infection with SeV. (n=3 mice in each group; $^{*}p< 0.05$).

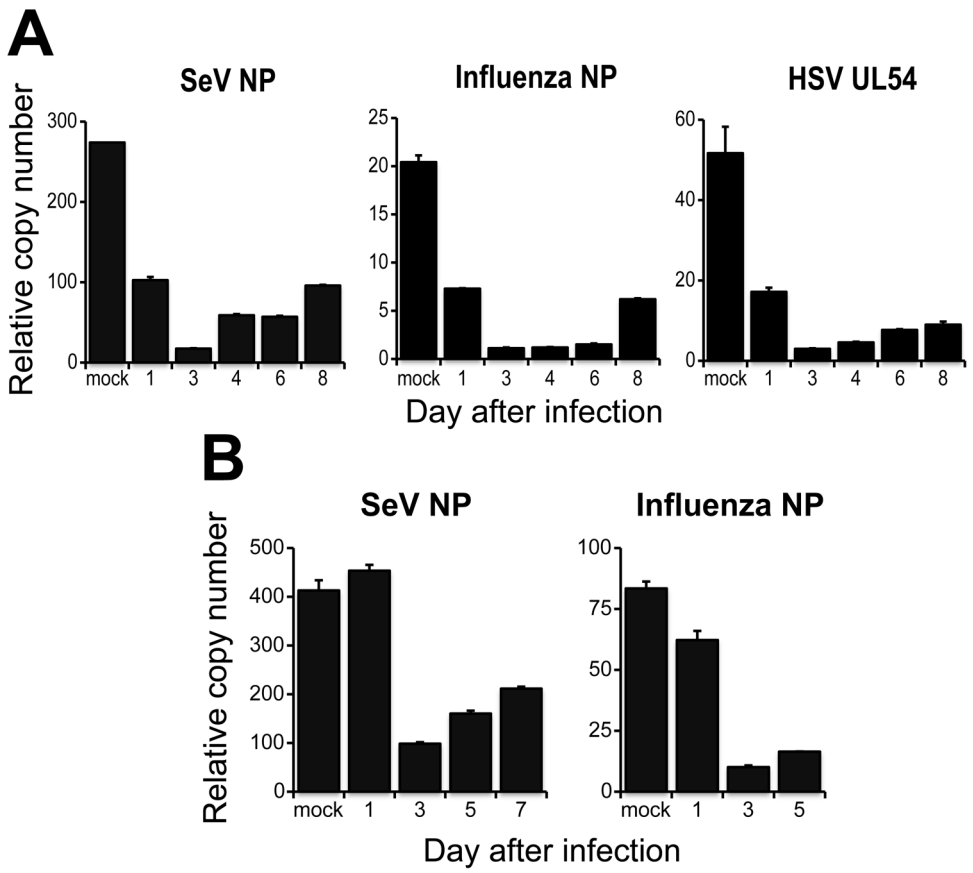

Figure 3.

BM cells of mice undergoing a respiratory infection are protected from viral replication. Expression of viral genes in BM cells isolated at different days after infection with (A) SeV or (B) influenza virus strain X31 after 6 h of infection ex-vivo with SeV, influenza strain PR8 or HSV at a MOI of 2. n=4 mice in each group. Error bars represent means±S.D

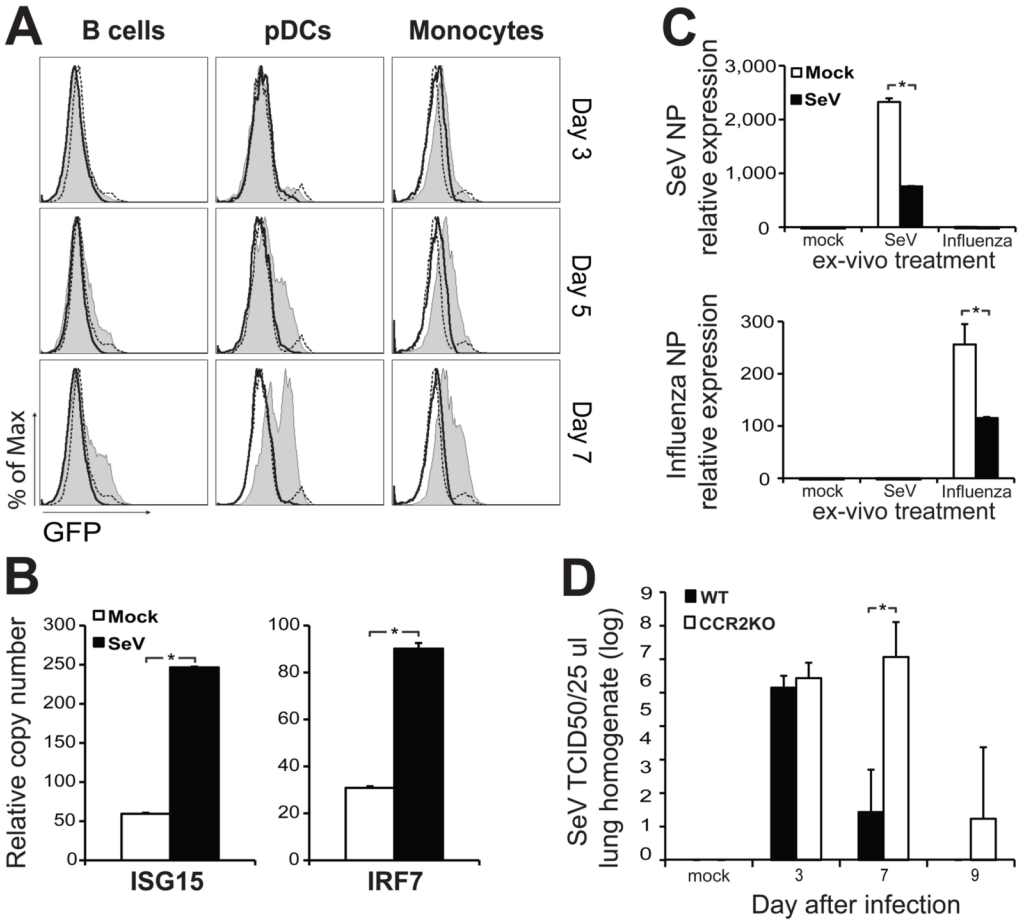

Figure 4.

Monocytes primed in the BM participate in the control of virus replication in the lung. (A) EGFP expression in BM B cells ($PI^{-}SS^{lo}B220^{+}CD11b^{-}mPDCA^{-}$), pDCs ($PI^{-}SS^{lo}B220^{+}CD11b^{-}mPDCA^{+}$) and monocytes ($PI^{-}SS^{lo}CD115^{+}CD11b^{+}Ly6c^{+}$) from Mx-Cre x Rosa26-$stop^{flox}$EGFP mice infected for 3, 5 or 7 days with SeV (tinted), mock infected (black dashed line) or Mx-Cre(neg)Rosa26-$stop^{flox}$EGFP litter mates (black line). (B) Gene expression in BM monocytes ($Ly6c^{+}Ly6g^{-}CD115^{+}$) sorted from mock or SeV infected mice at day 4 post-infection (n=6 mice in each group; $^{*}P < 0.05$). Error bars represent means±S.D. (C) Viral gene expression in BM monocytes isolated from mice four days post infection with SeV and after ex-vivo treatment with either SeV or influenza PR8. Mock infected animals were used as controls (n=6 mice in each group; $^{*}P < 0.05$). Error bars represent means±S.D.(d) SeV titers in lung homogenates of wild type or CCR2KO mice after infection with SeV. 50% Tissue culture infectious dose (TCID50). n=3–4 mice in each group; $^{*}P < 0.05$ Error bars represent means±S.D. See also figure S3

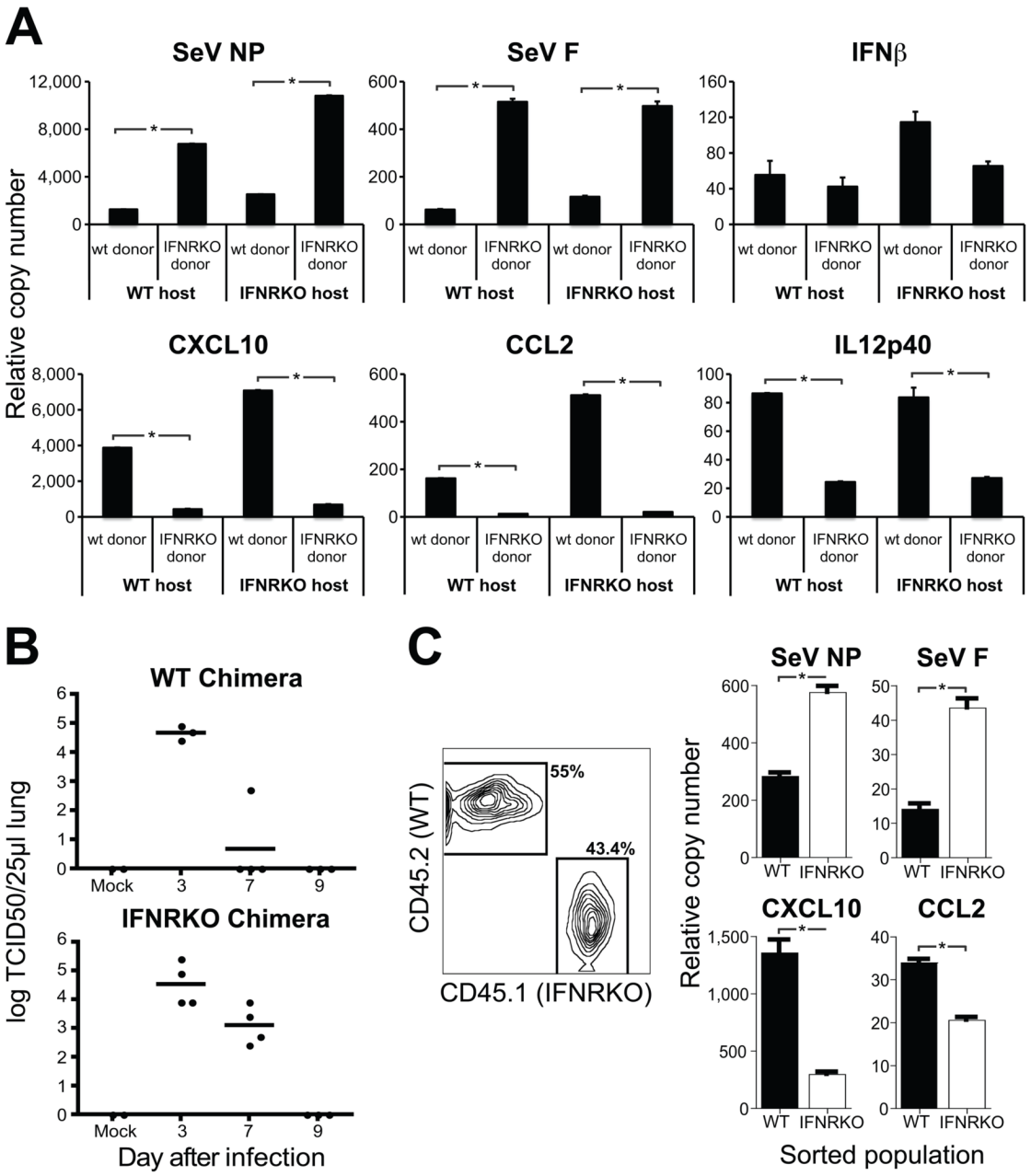

Figure 5.

Type I IFNs arm hematopoietic cells against viral replication in vivo.

(A) Gene expression on $CD45^+$ cells isolated by magnetic beads from the lung of complete chimeric WT or IFNRKO mice after five days of infection with SeV. (B) SeV titers in lung homogenates of chimeric mice at different days after infection. (C) Viral gene expression analysis of $CD45.2^+$ (WT) and $CD45.1^+$ (IFNRKO) cells isolated from the lung of mixed chimeric mice after four days of infection with SeV. (n=3 mice in each group; $^*P < 0.05$). Error bars represent means±S.D

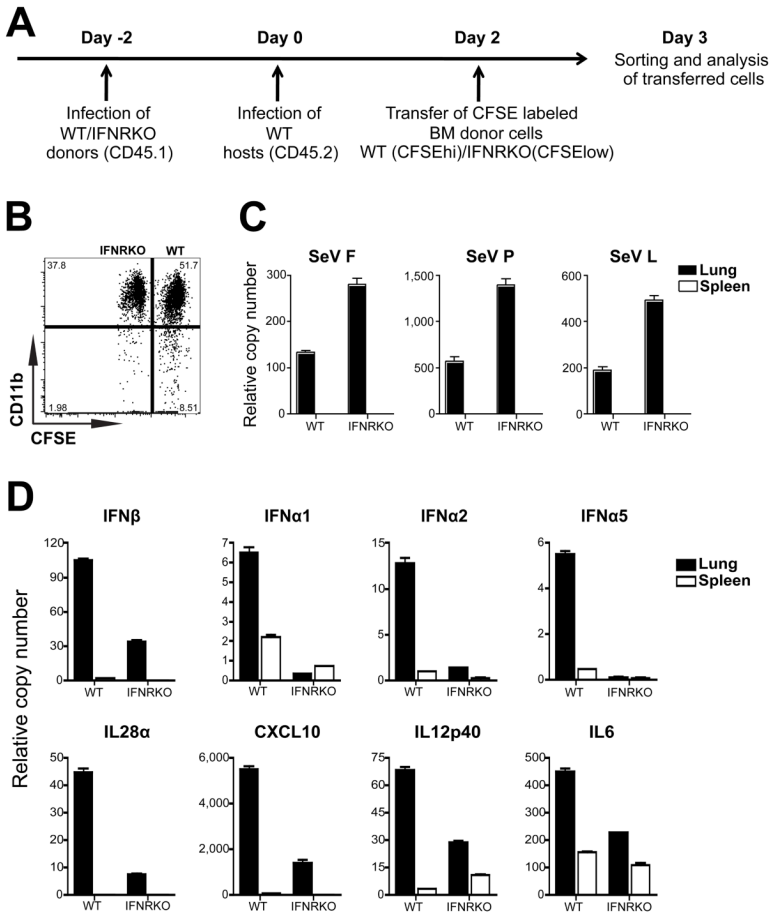

Figure 6.

Primed BM leukocytes are recruited to the lung and are protected from infection.

(A) Experimental design for adoptive transfer of primed BM leukocytes. CD45.1$^+$ WT or IFNRKO BM cells from mice infected with SeV for 4 days were labeled with 3.5μM or 0.5μM CFSE respectively, mixed at a 1/1 ratio and injected into WT CD45.2$^+$ mice infected for 2 days with SeV. (B) Expression of CD11b by CD45.1$^+$ CFSE high and low cells in the lungs of recipient mice. (C-D) qRT-PCR analysis of (C) viral mRNA and (D) cytokine mRNA in adoptively transferred BM WT and IFNRKO cells isolated from the lung and spleen.

Table 1

Virus titers upon infection with SeV and Influenza virus

		WT		IFNRKO	
		Viral NP[a]	**Virus titer**[b]	**Viral NP**	**Virus titer**
SeV	**Lung**	5557±36	5±0.36	16003±265*	5.7±0*
	BM	ND	ND	ND	NM
	Blood	ND	ND	ND	NM
Influenza	**Lung**	3035±59	5.7±0	3401±68	NM
	BM	ND	NM	ND	NM
	Blood	ND	NM	ND	NM

[a] Viral NP was measured on day 4 post infection by qRT-PCR. Expressed as relative copy numbers. n=3.

[b] Viral titers in the lung measured on day 4 post infection. BM and blood titers measured on day 5 after infection. Expressed as log TCID50/25ul Lung. n=4.

* $p<0.05$ compared to WT

ND-not detected

NM- not measured

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