CD47 restricts antiviral function of alveolar macrophages during influenza virus infection

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Highlights
CD47 restricts influenza A virus (IAV) clearance by alveolar macrophages (aMφ)
Hemoglobin (HB) expression is restricted by CD47 in aMφ after IAV infection
HB shows antiviral properties against IAV and SARS-CoV2

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CD47 restricts antiviral function of alveolar macrophages during influenza virus infection

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SUMMARY

CD47 is an ubiquitously expressed surface molecule with significant impact on immune responses. However, its role for antiviral immunity is not fully understood. Here, we revealed that the expression of CD47 on immune cells seemed to disturb the antiviral immune response as CD47-deficient mice (CD47<sup>−/−</sup>) showed an augmented clearance of influenza A virus (IAV). Specifically, we have shown that enhanced viral clearance is mediated by alveolar macrophages (aMΦ). Although aMΦ displayed upregulation of CD47 expression during IAV infection in wildtype mice, depletion of aMΦ in CD47<sup>−/−</sup> mice during IAV infection reversed the augmented viral clearance. We have also demonstrated that CD47 restricts hemoglobin (HB) expression in aMΦ after IAV and severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infection, with HB showing antiviral properties by enhancing the IFN-β response. Our study showed a negative role for CD47 during antiviral immune responses in the lung by confining HB expression in aMΦ.

INTRODUCTION

Influenza A viruses (IAV) are a common cause of lower respiratory infections. During seasonal epidemics up to 5 million people suffer from severe IAV infections with up to 650,000 deaths annually.1 Although vaccines as well as antiviral drugs against IAV are available to date, their efficacy is highly limited because of the antigenic variability of the virus and the occurrence of drug-resistant strains.2,3 Nevertheless, relevant immune, viral, and host factors defining either the clearance or the severity of the disease are still unclear. Same applies to the precise mechanisms defining why some patients only experience mild symptoms while others struggle with life-threatening viral pneumonia.4 Thus, novel treatment strategies consider the manipulation of IAV-related immune responses to ameliorate the clearance of the virus.

Different lung resident as well as recruited immune cells are involved in the clearance of influenza viruses. For example, alveolar macrophages (aMΦ) reside in the alveolar lumen and recognize invading pathogens such as IAV.5,6 Yet, pro-inflammatory immune responses were demonstrated to contribute to the severity of IAV infection-associated pathology as well.7,8 Thus, pulmonary immunity needs to be highly regulated. Type I interferons (IFN-I) partially take over this task. IFN-I are released after recognition of pathogen associated molecular patterns (PAMPs) via innate receptors like toll-like receptor (TLR) 7, retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated protein 5 (MDAS).9 During influenza virus infection, interferon-β (IFN-β) modulates a type I immunity.10

CD47 is an ubiquitously expressed cell surface protein with immunoregulatory function. It is best noted for its function as a “don’t eat me signal” exploited by tumor cells to avoid proper antitumor immunity via the interaction with signal regulatory protein α (SIRPα).11 an inhibitory receptor expressed on phagocytes. Another known ligand of CD47 is thrombospondin 1 (TSP-1), an extracellular homotrimeric matrix protein, which links CD47 to cellular processes such as adhesion and apoptosis.12,13 Although previous studies implicated CD47 in antimicrobial immune responses,14 its role for antimicrobial immunity remains controversial and our current understanding of the effect of CD47 on antiviral immunity is even more fragmentary. Along this line, the oxygen carrying protein hemoglobin (HB) exerts multiple functions, including the regulation of innate immunity and resistance to the invasion of pathogens.15,16 Furthermore, it was recently...
Figure 1. CD47<sup>−/−</sup> mice have an enhanced clearance of influenza virus
Wildtype (WT) and CD47<sup>−/−</sup> mice were i.n. infected with a single dose of 75 PFU of influenza virus A/PR8/34.

(A–C) (A) F4/80<sup>+</sup> macrophages (MΦ) in the lung were analyzed 3 days post infection (dpi) via flow cytometry regarding the mean fluorescence intensity (MFI) of CD47. Viral loads in the lung of mice infected with 75 PFU IAV was determined at day 3 (B) and 7 (C) after infection via plaque assay. (D) Immune induced histopathology in the lung was determined at 7 dpi. (E) Body weight change after infection. (F) Pro-inflammatory cytokines including TNF-α, IL-1β and IFN-α were determined in the BALF at indicated timepoints via Luminex and ELISA. (G) Alveolar type II epithelial cells were isolated from the lung and ex vivo infected with indicated doses. Virus titers were assessed by qRT-PCR 2 dpi (n = 4). Results are pooled from two or more independent experiments.

Data shown are mean ± SEM. Statistics were done by Student’s t test in (A). Two-way ANOVA with Sidak’s multiple-comparisons post-test was used in all other figures. one-way ANOVA with Tukey’s multiple-comparisons post-test was performed in (F). * = p < 0.05, ** = p < 0.01.

We next focused on MΦ immunity to delineate the role of CD47 during IAV infection. By histological analysis of the lung, we noticed increased number of MΦ in the alveolar lumen of CD47<sup>−/−</sup> compared to WT mice 3 dpi (Figures 2A and 2B). aMΦ are among the first immune cells to encounter influenza virus infected cells because of their localization and are critical for modulating the disease severity. Therefore, we next analyzed the proportion of the aMΦ population by flow cytometry. Of interest, the frequencies of aMΦ in WT mice decreased during infection.
significantly compared to CD47−/− mice (Figures S3, 2C, and 2D). At the same time, numbers of aMϕ in CD47−/− were increased 3 dpi compared to WT mice (Figure 2E). Next, we also analyzed the expression of CD47 and SIRPα, the myeloid cell-expressed ligand of CD47 on aMϕ of infected WT mice. aMϕ displayed an increased expression of CD47 (Figure 2F). Likewise, expression level of SIRPα was constant on IAV infection. These results indicate that CD47 could influence aMϕ during IAV infection.

**CD47 deficiency enhances viral clearance by alveolar macrophages**

To assess whether elevated frequencies of aMϕ are linked to reduced viral loads in CD47−/− mice, aMϕ were specifically depleted by intranasal (i.n.) application of clodronate-containing liposomes resulting in a decrease of ~75% of aMϕ from the lung (Figure 3A). Importantly, depletion of aMϕ before infection reversed the beneficial effect on viral clearance in CD47−/− mice, whereas the viral load in WT mice was still unaffected (Figure 3B). Moreover, specific depletion of interstitial macrophages (iMϕ) by intraperitoneal (i.p.) application of clodronate containing liposomes (efficiency ~50%) did neither affect the viral load of WT nor CD47−/− mice. Hence, aMϕ seemed to be responsible for the improved viral clearance in CD47−/− mice. Accordingly, adoptive transfer of aMϕ isolated from CD47−/− but not WT donor mice into aMϕ-depleted WT recipient mice before infection reduced the viral load of IAV (Figure 3C). Thus, genetic ablation of CD47 resulted in an enhanced viral clearance of IAV by aMϕ.
CD47 restricts the antiviral activity of alveolar macrophages independent of SIRPα

Although aMφs observed to enhance viral clearance in CD47−/− mice, aMφs from IAV infected CD47−/− mice showed significantly less expression of costimulatory molecules compared to WT mice (Figure S4A). Also, similar values for the proportion of iNOS or arginase expressing aMφs between CD47−/− and WT mice were detected (Figure S4B).

To address the impact of CD47 deficiency on IAV phagocytosis during infection, particles labeled with a pH-sensitive dye were intranasally applied into naive and infected (3 dpi) WT and CD47−/− mice (Figure 4A). The uptake of fluorescent particles by aMφs was determined by flow cytometry. Of interest, the phagocytic activity of aMφs was reduced on IAV infection, however the genetic deletion of CD47 expression did not influence the engulfment of foreign particles (Figure 4B). Thus, the observed protective function of CD47−/− aMφs on adoptive transfer into WT recipient mice is rather not because of enhanced phagocytosis.

To further examine the role of CD47-SIRPα pathways, SIRPα−/− mice were infected with IAV. Importantly, the prevented interaction of CD47-SIRPα did not affect the clearance of IAV from infected tissue as similar virus titers were detected in WT and SIRPα−/− mice (Figure 4C). Thus, the antiviral effect of aMφs cannot be explained by an increase in phagocytosis and the disrupted CD47-SIRPα signaling does not seem to be responsible for the enhanced viral clearance in CD47−/− mice.

CD47 controls in the expression of hemoglobin in alveolar macrophages

Our previous experiments demonstrate an inhibitory role of CD47 on antiviral function, especially on aMφs during IAV infection. To define the mechanism of how CD47 restrains aMφs during IAV infection in more detail, we performed transcriptome analyses from aMφs that were isolated from either naive or IAV infected WT and CD47−/− mice (3 dpi). Already at steady state, CD47−/− aMφs displayed an altered expression profile showing an increased expression of genes involved in cell adhesion (e.g., SELP = selectin platelet) and inflammation (e.g., C4a = complement component 4a). In contrast, genes involved in adaptive immune responses...
(e.g., H2-Ab1 = histocompatibility 2, class II antigen A, beta 1) and apoptosis (e.g., ACVR1C = activin A receptor, type IC) were downregulated in absence of CD47 (Figure 5A). Surprisingly, an increased expression of hemoglobin-ß (HBB) was detected in naive CD47−/−/−aMVs compared to WT aMVs. This augmented expression of HB was even more prominent during IAV infection as both hemoglobin-α (HBA) and HBB chains were found to be increased 3 dpi (Figure 5A). Importantly, HBA and HBB were also detectable in aMVs by flow cytometry (Figures 5B and 5C). Although aMVs generally expressed HBA and HBB, the expression was slightly increased in CD47−/−/−aMVs compared to WT aMVs (Figures 5B and 5C). Other immune cell populations such as iMVs, DCs, PMN, CD4+T cells or B cells also seemed to express HBA and HBB, albeit at a significantly lower level compared to aMVs (Figures S5A and S5B). In accordance to this, we also detected a significantly higher concentration of HB in the BALF of CD47−/−/− mice compared to WT mice after IAV infection (Figure 5D). This HB could be derived from aMVs, because we were able to detect HB in the supernatants of ex vivo cultured aMVs compared to WT aMVs. To investigate whether HB expression is regulated by CD47 signaling, we additionally stimulated these aMVs with the CD47 ligand TSP-1. In fact, we found that TSP-1 leads to significantly decreased secretion of HB by WT aMVs in a dose-dependent manner (Figure 5E). Importantly, this was not the case when CD47−/−/−aMVs were stimulated with TSP-1. Hence, aMVs in CD47−/− mice could produce more HB and secrete it into their environment, where it could act on surrounding cells. To test this hypothesis, we isolated AEC II as well as pulmonary immune cells from naive WT mice and incubated them ex vivo with fluorescently labeled HB. Of interest, HB seems to be taken up by AEC II within 30 min (Figures 5F and 5G). aMVs also appeared to be able to take up HB efficiently (Figure 5H). Of interest, this was not the case for iMVs and DCs. Thus, although the precise mechanism is still elusive, our data suggests that CD47 is involved in the negative regulation of HB expression in aMVs.

**Hemoglobin restrains influenza virus infection**

Although HB is mainly known for its role in oxygen transport, it was recently associated with antiviral responses against several RNA viruses. It is not yet known whether HB also has an antiviral function against IAV. We thus hypothesized, that HB might play a role in the defense against IAV infection and the corresponding inflammation. Therefore, we next examined whether BALF of infected CD47−/− mice could restrain IAV infection. Of interest, in vitro treatment of Madin-Darby canine kidney (MDCK) cells before

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**Figure 4.** CD47 restricts the antiviral activity of alveolar macrophages independent of SIRPα

At day 3 after influenza virus infection mice were i.n. treated with pH sensitive fluorescent particles and the phagocytosis of particles by aMΦs was determined via flow cytometry after 1 h.

(A) The uptake of fluorescent particles is shown in representative histograms.

(B) The mean fluorescence intensity (MFI) for FITC of aMΦs was determined (n = 10).

(C) The viral load in the lung of WT and SIRPα−/− mice was assessed by plaque assay 7 dpi. Representative results from one of two independent experiments are shown.

Data shown are mean ± SEM. Mann-Whitney test was performed. **** = p < 0.0001.
**Figure 5.** CD47 is involved in the regulation of hemoglobin expression in alveolar macrophages

Mice were i.n. infected with IAV. aMΦs were isolated from WT and CD47−/− mice 3 dpi. (A) Transcriptome analysis of isolated WT and CD47−/− aMΦs were assessed by an Affymetrix MicroArray. Hemoglobin alpha (HBA) (B) and beta (HBB) (C) chains were detected by flow cytometry analysis in aMΦs isolated from WT and CD47−/− mice. Representative histograms for HBA and HBB are shown. Mean fluorescence intensity (MFI) for HBA and HBB from aMΦs are shown (n = 4). (D) Whole hemoglobin (HB) concentration was assessed in BALF at indicated time points by ELISA (n = 7–17). (E) aMΦs were isolated from infected WT and CD47−/− mice 3 dpi and stimulated ex vivo with thrombospondin-1 (TSP-1). After 24 h, HB concentration in the supernatants was measured by ELISA (n = 4). (F) Type II alveolar epithelial cells (AEC II) were isolated from the lung of naive WT mice and incubated with 1 μg/mL Alexa Fluor 647 (AF647)-labeled HB for 30 min. Representative flow cytometry plots are shown. (G) Isolated cells from lungs of WT mice were incubated with 1 μg/mL AF647-labelled HB for 30 min. Percentages of HB+ aMΦs, iMΦs, and DCs are shown. Results depicted were obtained from one (A) or two independent experiments (C, D, E, G, and H). Data shown are mean ± SEM. One-way ANOVA with Tukey’s multiple-comparisons post-test (D and E) or Student’s t test (F) were performed. * = p < 0.05, ** = p < 0.01, **** = p < 0.0001.

IAV infection with BALF obtained from infected CD47−/− mice, but not WT mice suppressed the infectivity (Figure 6A). Because the antiviral activity of BALF from infected CD47−/− mice could be because of the increased HB concentrations, we next investigated the possible role of HB in the defense against IAV. To this end, we infected MDCK cells in presence or absence of HB prior or post infection. Pretreatment with HB was already sufficient to significantly decrease viral replication whereas the addition of HB post infection had a similar effect confirming an antiviral role for HB (Figure 6B). To analyze whether this effect could be related to an enhanced IFN-I response induced by HB, we analyzed gene expression of IFN-β and the interferon inducible gene (ISG) Xx1 by flow cytometry analysis in aMΦs isolated from WT and CD47−/− mice. Representative histograms for HBA and HBB are shown. Mean fluorescence intensity (MFI) for HBA and HBB from aMΦs are shown (n = 4). (D) Whole hemoglobin (HB) concentration was assessed in BALF at indicated time points by ELISA (n = 7–17). (E) aMΦs were isolated from infected WT and CD47−/− mice 3 dpi and stimulated ex vivo with thrombospondin-1 (TSP-1). After 24 h, HB concentration in the supernatants was measured by ELISA (n = 4). (F) Type II alveolar epithelial cells (AEC II) were isolated from the lung of naive WT mice and incubated with 1 μg/mL Alexa Fluor 647 (AF647)-labeled HB for 30 min. Representative flow cytometry plots are shown. (G) Isolated cells from lungs of WT mice were incubated with 1 μg/mL AF647-labelled HB for 30 min. Percentages of HB+ aMΦs, iMΦs, and DCs are shown. Results depicted were obtained from one (A) or two independent experiments (C, D, E, G, and H). Data shown are mean ± SEM. One-way ANOVA with Tukey’s multiple-comparisons post-test (D and E) or Student’s t test (F) were performed. * = p < 0.05, ** = p < 0.01, **** = p < 0.0001.

The antiviral abilities of HB might not be IAV specific, but also applicable to other (RNA) viruses such as SARS-CoV-2. Therefore, we tested whether HB can exert an antiviral effect on SARS-CoV-2 infection and added HB to A549-AT cells before or after infecting target cells with SARS-CoV-2. Importantly, we found dose-dependent significant reductions in infection under both conditions (Figures 6G and 6H). This again confirms the potent antiviral effect of HB even across different virus types. Thus, enhanced expression of antiviral HB in aMΦs of CD47−/− mice might augment the clearance of IAV during infection by enhanced IFN-β induction, which means that disruption of CD47 signaling could elevate its expression.

**DISCUSSION**

A deeper understanding of the immunological mechanisms during an influenza virus infection remains an urgent need, as the efficiency of existing vaccines or antiviral drugs is limited. Here, the manipulation of antiviral immune responses is of special interest, as they contribute to disease severity. Among the known immunoregulatory proteins, CD47 is currently widely studied, as it appeared to be a promising target in cancer therapy, whereas its impact on antiviral immunity is a matter of debate. Within the present study, we defined the role of CD47 for innate and adaptive immune responses during an acute IAV infection. Upon infection with IAV, significantly lower viral loads were observed in CD47−/− mice compared to WT mice on day 3 after infection (Figure 6D). To investigate whether IFN-β can be specifically induced by administration of HB, we treated WT mice with HB during IAV infection. We observed, that HB treatment increases the secretion of IFN-β in the lungs of infected mice compared to untreated infected animals (Figure 6E). In addition, intranasal administration of HB reduced the viral loads in the lung compared to only infected mice (Figure 6F).

We found increased cell surface levels of CD47 during acute IAV infection on different immune cells. Inflammatory stimuli such as pro-inflammatory cytokines (e.g., TNF-α, IFN) or activation of pattern recognition receptors (e.g., TLR7) have been described to induce the expression of CD47 during cancer as well as infection. Although the role of CD47 as an innate immune checkpoint is unambiguous during cancer, its effect on the course of infectious diseases is varying depending on the infectious agent and the site of infection. On the one hand, CD47 was shown to be involved in protective immunity against systemic bacterial (Escherichia coli) or fungal (Candida albicans) infection as well as the clearance of chronic LCMV
Clodronate containing liposomes is a suitable method to deplete Mφ as day 5 after infection. Importantly, viral loads were already decreased 5 dpi in the lung of CD47+/− mice. Nasal treatment worsened condition of the mice, so that a twofold increase in viral load occurred early. Significantly reduced viral loads in CD47+/− mice were observed already 6 dpi. Ultimately, this could also be because of increased retention of aMφ in lung homogenates. Of interest, we determined a shift in the proportion of aMφ within the lung at day 3 after influenza infection. Plasmodium yoelii and acute LCMV infection. Within the present study, we detected significantly reduced viral loads in CD47−/− compared to WT mice at day 7 after IAV infection implying a repressive role of CD47. This is interesting because CD47 was previously described as a negative regulator of type 1 immune responses. Commonly, bone marrow chimeric mice would be used to unravel the distinct role of CD47 expressed on tissue and immune cells. Yet, as CD47 functions as a marker of self-phagocytosis and subsequent presentation, CD8+ T cell frequencies were not enhanced in the context, the initially high number of viral particles and their replication could indeed be important for the effects observed by us.

In contrast to previous studies showing a major effect of CD47 on CTL responses by the inhibition of antigen presentation and subsequent presentation, CD8+ T cell frequencies were not enhanced in the lung of CD47−/− mice during IAV infection. Instead, a reduced expression of cytotoxic effector molecules (e.g., GzmB) was observed in absence of CD47. Thus, CD47 might have a dual role for CTL immunity during infectious diseases depending on the location of and time point of infection. In line with this, CD47 was described to both contribute to and limit T cell activation. The underlying mechanisms appear to be individual and have yet to be elucidated. In accordance with a previous study, which investigated the role of CD47 during vaccination we did not find augmented levels of IgA, IgM and IgG antibodies in the BALF of CD47−/− mice. Two independent experiments are shown unless otherwise noted.

Figure 6. Hemoglobin has antiviral function and reduces influenza virus replication
(A) MDCK cells were incubated with the BALF of WT and CD47−/− mice isolated at the indicated time points. After 30 min cells were infected with 1000 PFU of influenza virus and 3 dpi the antiviral activity of BALF samples was determined based on plaque count (n = 8).
(B) MDCK cells were incubated with 1 μg/mL hemoglobin (HB) for 30 min. Afterward, cells were infected and 3 dpi the antiviral activity of HB was determined based on plaque count. For post infection treatment, HB was added 2 h after infection.
(C) MDCK cells were incubated prior or after infection with 1 μg/mL HB as described. 48 h after infection cells were harvested and analyzed for IFN-β gene expression by qRT-PCR (n = 6). Non infected (n.i.) cells served as controls.
(D) Mice were i.n. infected with IAV. 3 dpi IFN-β concentrations were assessed in lung homogenates (n = 4). One representative experiment of two is shown.
(E) IAV-infected mice were intranasally treated with 50 μg HB protein on day 0 and 3 post infection. IFN-β expression by ELISA was analyzed 6 dpi from lung homogenates (n = 3). One representative experiment of two is shown.
(F) Virus titers were assessed by qRT-PCR from lung homogenates of HB treated and untreated mice 6 dpi. One representative experiment of two is shown.
(G) A549-AT cells were incubated with indicated HB concentrations for 30 min. Afterward, cells were infected and 24 h later the antiviral activity of HB was determined by in cell ELISA.
(H) For post infection treatment, HB was added after SARS CoV-2 infection of A549-AT cells. Two independent experiments are shown unless otherwise noted.

Data shown are mean ± SEM. One-way ANOVA with Tukey’s multiple-comparisons post-test was performed in (A–C, G, and H). Two-way ANOVA with Sidak’s multiple-comparisons post-test was performed in (D). Student’s t test was performed in (E and F). * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001.

In contrast to previous studies showing a major effect of CD47 on CTL responses by the inhibition of antigen phagocytosis and subsequent presentation, CD8+ T cell frequencies were not enhanced in the lung of CD47−/− mice during IAV infection. Instead, a reduced expression of cytotoxic effector molecules (e.g., GzmB) was observed in absence of CD47. Thus, CD47 might have a dual role for CTL immunity during infectious diseases depending on the location of and time point of infection. In line with this, CD47 was described to both contribute to and limit T cell activation. The underlying mechanisms appear to be individual and have yet to be elucidated. In accordance with a previous study, which investigated the role of CD47 during vaccination we did not find augmented levels of IgA, IgM and IgG antibodies in the BALF of CD47−/− mice compared to WT mice after IAV infection.

Of interest, we determined a shift in the proportion of aMφ within the lung at 3 dpi after influenza virus and 3 dpi aMφ increased compared to WT mice 3 dpi. Ultimately, this could also be because of increased retention of aMφ in the event of CD47−/−. aMφ are a highly specialized subset of Mφ, which derive during embryogenesis and form a self-renewing population. Because of their specific localization in the alveolar lumen they are the first immune cells to encounter invading influenza viruses and significantly shape subsequent immunity. The application of clodronate containing liposomes is a suitable method to deplete Mφ in vivo. However, repeated intranasal treatment worsened condition of the mice, so that we had to measure the viral load here as early as day 5 after infection. Importantly, viral loads were already decreased 5 dpi in the lung of CD47−/− mice treated with PBS containing liposomes. However, depletion of aMφ by intranasal application of clodronate containing liposomes impaired this viral clearance in IAV infected CD47−/− mice. The fact that aMφ depletion in WT mice had no such effect on viral loads suggests a crucial role of CD47 for the antiviral
function of aMΦ. This is also in accordance with our finding that the transfer of CD47\(^{-/-}\) aMΦ into WT recipient mice was able to significantly augment the clearance of IAV suggesting a negative role for CD47 in the regulation of antiviral function of aMΦ.

In this regard, aMΦ have been shown to contribute to the clearance of IAV by the phagocytosis of aggregated viral particles as well as the engulfment of dying or dead infected epithelial cells.\(^{34,35}\) Although the binding of CD47 to SIRP\(\alpha\) was previously described to restrain the phagocytic activity of MΦ,\(^{36}\) no enhanced phagocytosis by CD47\(^{-/-}\) aMΦ were observed within this study. Furthermore, the viral clearance after IAV infection was not improved in SIRP\(\alpha\) deficient mice compared to WT mice. Thus, other proteins such as surfactant proteins A and D might still interact with SIRP\(\alpha\) in the alveolar lumen in absence of CD47.\(^{37,38}\) At the same time, it suggests that the negative regulation of the antiviral function of aMΦ could rely on another ligand of CD47, such as TSP-1. TSP-1 is a multi-domain matrix glycoprotein that has been shown to be involved in inflammation.\(^{39}\) Different functions of TSP-1 for macrophages have been described so far controlling pro-inflammatory immunity.\(^{40}\) Well in line, in our experimental setting TSP-1 decreased aMΦ mediated HB secretion from WT but not from CD47\(^{-/-}\) mice. Thus, our data strongly suggests TSP-1 as a negative regulator of the HB expression and antiviral function of macrophages via CD47. To what extent TSP-1 is involved in the antiviral role of CD47 during IAV infection requires further investigation. However, transcriptome analysis revealed elevated transcript levels of HBA and HBB in CD47\(^{-/-}\) aMΦ compared to WT aMΦ before and after IAV infection. This was surprising because previous studies did not indicate an induction of HB expression, i.e., by AEC II after IAV infection.\(^{41}\)

HB is essential for oxygen transport by erythrocytes, and the gene expression of non-erythroid HB in macrophages was initially described in 1999.\(^{42}\) Our current study confirms now that HB is expressed and exists at the protein level in aMΦ. The fact that we could not see any significant differences in the HB protein level between WT and CD47\(^{-/-}\) MΦ here could possibly be because of ingested HB. In fact, the uptake of fluorescently labeled HB by aMΦ and AEC II that we noticed in our study indicates that HB can both be secreted and taken up by these cells. Moreover, additional functions of HB in antimicrobial and antiviral defense have been demonstrated. For example, C-terminal fragments of HBB have been shown to inhibit entry of herpes simplex virus 2 (HSV-2) and to limit the growth of different bacteria.\(^{43,44}\) In addition, HBB has been demonstrated to restrict infectivity of different RNA viruses by regulating RIG-I mediated type I IFN response.\(^{17}\) Influenza viruses are also recognized via RIG-I.\(^{9}\) Importantly, to our best knowledge we show here for the first time that HB inhibited influenza virus replication in vitro and in vivo, which was accompanied by enhanced IFN-\(\beta\) expression. Beside acceleration of viral clearance by modulating type 1 immunity, IFN-\(\beta\) mediates inhibition of viral replication by induction of ISGs such as Mx1 that interestingly cannot be compensated for by IFN-\(\alpha\).\(^{10,45}\) Of note, although we used mouse-derived HB, sequence analyses revealed that there are high similarities between mouse, dog and human HB. This suggests that HB mediates an improved antiviral response via similar mechanisms as described by Yang et al.\(^{17}\) Hence, our data indicate, that the CD47\(^{-/-}\) aMΦ do not directly enhance the clearance of virus, e.g., via phagocytosis of infected cells, but alter the cytokine response (i.e., IFN-\(\beta\)) possibly in target cells such as AEC II via HB secretion. This indirect effect of CD47\(^{-/-}\) aMΦ could result in a delayed effect on the antiviral response and explain the discrepancy between the late decreased viral load in the CD47\(^{-/-}\) mice at day 7 post-infection and the early altered aMΦ response at day 3. However, we were able to detect significant reduction in viral load in lungs of CD47\(^{-/-}\) compared to WT mice as early as day 5 after infection (Figure 3B), indicating that the enhanced antiviral response in CD47\(^{-/-}\) mice must develop directly after the 3 dpi time point examined. Thus, our data suggests that aMΦ initiate the enhanced virus clearance indirectly. A MΦ-specific knockdown of HB could clarify the connection between CD47-deficient macrophages and the increased antiviral effect via HB. Unfortunately, such transgenic mice that would allow to specifically delete HB in aMΦ are not available. Thus, future studies still need to further specify the role of MΦ-derived HB. However, the direct treatment of IAV-infected WT mice with HB led to the effects we postulate: reduced viral load with simultaneously increased IFN-\(\beta\) concentration in the lungs.

Importantly, we could also demonstrate for the first time an antiviral effect of HB on SARS-CoV-2 infection, indicating that the observed effects are IAV non-specific processes induced by HB and thus could be of therapeutic interest to treat acute viral respiratory infections. The fact that we identified increased levels of HB after IAV infection in the BALF of CD47\(^{-/-}\) mice compared to samples from WT mice could indicate a secretion of HB by aMΦ and subsequent uptake by surrounding cells, such as AEC II and DCs. Our uptake
studies demonstrate the general possibility of uptake of secreted HB. The uptake of HB by innate immune cells may then lead to increased recognition of influenza virus or SARS-CoV-2 RNA, which ultimately results in increased IFN-β expression. In line with this, we were able to detect higher IFN-β concentrations in the lung of infected CD47−/− compared to WT mice as well as after the treatment of infected WT mice with HB. However, subsequent studies have yet to identify the exact source of this IFN-β.

Taken together, our study is the first to demonstrate CD47 as a negative regulator of antiviral aMΦ immunity during acute IAV infection. Improved viral clearance of IAV in absence of CD47 seemed to be related to the expression of HB. Yet, further studies are of need to define the detailed mechanisms of the induction and function of antiviral HB. Importantly, due to the non-specific nature of CD47 signaling as well as the evolutionary conservation of HB, CD47 targeting therapies or application of HB may allow the treatment of various respiratory infections such as IAV, SARS-CoV-2 or even the respiratory syncytial virus (RSV).

Limitations of the study
HB is a tetramer protein. Although we were able to demonstrate an antiviral effect of the entire HB protein, it remains unclear whether this effect can also be achieved by the α- or β-subunit. The commercial availability of murine HB-α and HB-β is currently limited. Transgenic mice that would allow to specifically delete HB in aMΦ are not available to investigate the direct relationship between MΦ-derived HB and antiviral effects.

STAR★METHODS
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SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
T.K., A.M.W., and J.B. conceived of the study. T.K. and C.W. designed the experiments and wrote the paper. C.W., T.K., P.S., F.W., K.S., J.D.B., R.K., D.B. V.J., and R.G. substantially contributed to the acquisition and analysis of the data. C.W. and T.K. wrote the original manuscript and all authors edited and approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | Cat# |
|---------------------|--------|-----|
| **Antibodies**      |        |     |
| Anti-mouse CD47 antibody, PeCy7 | Biolegend | 127524, RRID:AB_2629545 |
| Anti-mouse CD19 antibody PE PeCy7 | BD Biosciences | 557399, RRID:AB_396682 |
| Anti-mouse CD31 antibody, PE | Biolegend | 102407, RRID:AB_312902 |
| Anti-mouse CD11b antibody PE PerCP-Cy5.5 APC | BD Biosciences, ThermoFisher Scientific | 557397, RRID:AB_396680, 550993, RRID:AB_394002, 17-0112-82, RRID:AB_469343 |
| Anti-mouse CD31 antibody, PE | Biolegend | 102407, RRID:AB_312902 |
| Anti-mouse CD16/CD32 monoclonal antibody, PE | eBioscience | 120161-82, RRID:AB_465568 |
| Anti-mouse CD45 antibody, PE APC V450 | BD Biosciences, ThermoFisher Scientific | 553081, RRID:AB_394611 |
| Anti-mouse CD45 antibody, PE APC V450 | BD Biosciences | 550261, RRID:AB_398460, 560521, RRID:AB_1727423 |
| Anti-mouse CD4 antibody PerCP PE | BD Biosciences | 561090, RRID:AB_10562560 |
| Anti-mouse CD4 antibody PerCP PE | BD Biosciences | 553653, RRID:AB_394973 |
| Anti-mouse CD93 antibody, APC | Biolegend | 136509, RRID:AB_2275879 |
| Anti-mouse CD11c antibody APC V450 | BD Biosciences | 550261, RRID:AB_398460 |
| Anti-mouse CD8 antibody, Pacific Blue APC BV421 | BD Biosciences | 558106, RRID:AB_397029 |
| Anti-mouse CD8 antibody, Pacific Blue APC BV421 | BD Biosciences | 127614, RRID:AB_227348 |
| Anti-mouse I-A/I-E antibody, BV510 FITC | Biolegend | 561105, RRID:AB_10562567 |
| Granzyme B monoclonal antibody, APC | ThermoFisher Scientific | MHGB05, RRID:AB_10373420 |
| Anti-mouse CD107a antibody, PE | BD Biosciences | 558661, RRID:AB_1645247 |
| iNOS monoclonal antibody, PeCy7 | ThermoFisher Scientific | 255920-82, RRID:AB_2573499 |
| Human/mouse Arginase 1 antibody, APC | Bio-Techne | IC5868A, RRID:AB_2810265 |
| Anti-mouse CD80 antibody, V450 | BD Biosciences | 560523, RRID:AB_1727515 |
| Anti-mouse CD86 antibody, PeCy7 | BD Biosciences | 560582, RRID:AB_1727518 |
| Anti-mouse I-A/I-E antibody, BV510 | Biolegend | 107636, RRID:AB_2734168 |
| Anti-rabbit IgG DyLight 649 | Biolegend | 406406, RRID:AB_397029 |
| Anti-hemoglobin alpha (HBA) antibody | Novus Biologicals | NBP2-67716, RRID:AB_2809609 |
| Anti-hemoglobin beta (HBB) antibody | Invitrogen | PA-576877, RRID:AB_2720604 |
| Anti-SARS-CoV-2-NP | Bioss | ABIN6952435, RRID:AB_2890255 |
| Peroxidase-AffiniPure Goat | Dianova | 115-035-003, RRID:AB_10015289 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | Cat# |
|---------------------|--------|------|
| Anti-mouse MAC-3    | Biolegend | 108502, RRID:AB_313383 |
| HRP-Goat Anti-Rat IgG (H+L) | ThermoFisher Scientific | A18865, RRID:AB_2535642 |

**Bacterial and virus strains**

| Name               | Source                                      | Cat# |
|--------------------|---------------------------------------------|------|
| Influenza A/PR/8/34| Prof. Dr. P. Stäheli (Department of Virology, University Freiburg) | N/A |
| SARS-CoV-2 B.1.1.10| Patient material (Heilingloh et al.) | N/A |

**Chemicals, peptides, and recombinant proteins**

| Name                                         | Source                    | Cat#         |
|----------------------------------------------|---------------------------|--------------|
| 2N H2SO4                                     | Roth                      | X873         |
| Avicel                                       | Sigma Aldrich             | 11365        |
| Bovine Serum Albumin (BSA)                   | Biornol                   | 01400.100    |
| Brefeldin A                                  | Sigma Aldrich             | B 7651       |
| Clodronate Liposomes & Control Liposomes (PBS)| Liposoma B.V.             | N/A          |
| Collagenase D                                | Roche Life Science        | 11088866001  |
| Corning® Dispase                             | Corning                   | 354235       |
| Crystal violet                               | Roth                      | T 123.1      |
| Diaminobenzidine (DAB)                       | Dako Agilent              | K3467        |
| Dispase                                      | Corning                   | 354235       |
| DNase I                                      | Roche Life Science        | 10104159001  |
| DNase I from bovine pancreas                 | Merck KGaA                | D4263        |
| Ethylenediaminetetraacetic acid (EDTA)       | Roth                      | 8043.2       |
| Fixable viability dye, eFluor 780            | ThermoFisher Scientific   | 65-0865-18   |
| Gentamycin                                   | Sigma Aldrich             | G 1272       |
| Goat Serum                                   | Invitrogen                | 31872        |
| GoTaq® DNA Polymerase                        | Promega                   | M3001        |
| Igepal                                       | Sigma Aldrich             | I8896        |
| Ionomycin                                    | Sigma Aldrich             | I 0634       |
| L-Glutamine                                  | Sigma Aldrich             | G 7513       |
| LE Agarose                                   | Biozym                    | 840004       |
| M-MLV Reverse Transcriptase                  | Promega                   | M 3683       |
| Matrigel (Basement Membrane Matrix) Matrigel | Corning                   | 354230       |
| Murine Hemoglobin protein                    | Fitzgerald Industries     | FGI-30-1135  |
| Paraformaldehyde (PFA)                       | Roth                      | 0335.2       |
| pHrodo Green E. coli BioParticles            | ThermoFisher Scientific   | P35366       |
| Recombinant Mouse Thrombospondin-1 (TSP-1)   | R&D Systems               | 7859-TH-050  |
| RNase-Free DNase Set                         | Qiagen                    | 79254        |
| ROTI Histofix                                | Roth                      | P087.3       |
| TMB Substrate Solution                       | Biolegend                 | 421101       |
| Triton X-100                                 | Roth                      | 3051.4       |
| Trypsin/EDTA                                 | Sigma Aldrich             | T4174        |
| Trypsin from bovine pancreas                 | Sigma Aldrich             | T8802        |
| Tween 20                                     | Roth                      | 9127         |

**Critical commercial assays**

| Name                                            | Source                    | Cat#         |
|-------------------------------------------------|---------------------------|--------------|
| AlexaFluor 647 Protein Labeling Kit             | ThermoFisher Scientific   | A20173       |
| FoxP3 Staining Buffer Set                       | eBioscience               | 00-5523-00   |

(Continued on next page)
### REAGENT or RESOURCE

| REAGENT or RESOURCE | SOURCE | Cat# |
|---------------------|--------|------|
| Luminex Assay       | Bio-Techne | N/A  |
| LumiKine Xpress mIFN-α/β 2.0 ELISA Kit | Invivogene | Luxex-mifnav2 / Luxex-mifnrbv2 |
| Maxima SYBR Green qPCR Kit | ThermoFisher Scientific | K0222 |
| MouseFree Hemoglobin ELISA Kit | Novus Biologicals | NBP2-59999 |
| Nucleo Spin RNA XS | Macherey-Nagel | 740902-250 |
| RNeasy Mini Kit | Qiagen | 74106 |

### Deposited data

| MicroArray data | This paper | GEO: GSE201825 |

### Experimental models: Cell lines

| Cell line | Depositing source | Cat# |
|-----------|-------------------|------|
| A549-AT  | Dr. Marek Widera (Institute of Virology, University Frankfurt) | N/A  |
| Madin-Darby canine kidney (MDCK) | Prof. Dr. P. Staheli (Department of Virology, University Freiburg) | N/A  |

### Experimental models: Organisms/strains

| Organism/Strain | Depositing source | Cat# |
|----------------|-------------------|------|
| CD47<sup>−/−</sup> mice | Jackson Laboratory | 003173 |
| C57BL/6 wildtype mice | Envigo | 057 |
| SIRPα<sup>−/−</sup> mice | Riken Bioresource Center | RBRC01544 |

### Oligonucleotides

| Primer | Source | N/A |
|--------|--------|-----|
| Forward primer for matrix gene M1 5′- CTTCTAACCAGGTCGAACG-3′ | Eurofins | N/A |
| Reverse primer for matrix gene M1 5′- CTTCTAACCAGGTCGAACG-3′ | Eurofins | N/A |
| Forward primer for Rps9 5′- CTGAATGATCTGAGTGAAGC-3′ | Eurofins | N/A |
| Reverse primer for Rps9 5′- CTGAATGATCTGAGTGAAGC-3′ | Eurofins | N/A |
| Forward primer for canis Ifnb 5′- CAGTCCAGAAGGAGGACA-3′ | Eurofins | N/A |
| Reverse primer for canis Ifnb 5′- CAGTCCAGAAGGAGGACA-3′ | Eurofins | N/A |
| Forward primer for canis Mx1 5′- GAATCCTGGGAGTTTCTATG-3′ | Eurofins | N/A |
| Reverse primer for canis Mx1 5′- GAATCCTGGGAGTTTCTATG-3′ | Eurofins | N/A |
| Forward primer for canis Rps9 5′- TGCCGAGGTTTCTATG-3′ | Eurofins | N/A |
| Reverse primer for canis Rps9 5′- TGCCGAGGTTTCTATG-3′ | Eurofins | N/A |

### Software and algorithms

| Software | Source | N/A |
|----------|--------|-----|
| BD FACSDivaTM software v 8.0.1 | BD Biosciences | N/A |
| Fiji | Schindelin et al., 2012<sup>46</sup> | N/A |
| Prism 7 | GraphPad Software | N/A |

### Other

| Resources | Source | Cat# |
|-----------|--------|------|
| Dulbecco’s Modified Eagle Medium | Invitrogen | 41966029 |
| Opti-MEM | Invitrogen | 31985047 |
| Fetal Bovine Serum | Sigma Aldrich | F7524-500ML |
| Roswell Memorial Park Institute Medium | Invitrogen | 72400-021 |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Torben Knuschke (torben.knuschke@uk-essen.de).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Microarray data were deposited in NCBI’s Gene Expression Omnibus and are publicly available as of the date of publication. Accession number is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Cell culture
Madin-Darby canine kidney (MDCK) cell line was a kind donation from Professor Peter Staeheli, Department of Virology, University of Freiburg. MDCK cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) medium complete containing 10% endotoxin-free fetal bovine serum (FBS) and 50 μg/mL penicillin/streptomycin. Cell lines were maintained in a humidified 5% CO2 atmosphere at 37°C.

Animals
Wildtype C57BL/6 mice were purchased from Envigo Laboratories (Envigo CRS GmbH). CD47−/− mice were purchased from Jackson laboratories (JAX stock #003173; Jackson Laboratory). SIRPα−/− mice used in the present study were kindly provided by K. Lang (Institute for Immunology, University Hospital Essen). Male and female mice were used in the experiments. All mice used in the experiments were 7–9 weeks old at the time point of infection and housed under specific-pathogen-free conditions in the Laboratory Animal Facility of the University Hospital Essen. Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The experimental protocols were approved by the North Rhine-Westphalia State Agency for Nature, Environment and Consumer Protection (LANUV). All efforts were made to minimize suffering.

Viruses
Infection studies were performed by using the Influenza A/PR/8/34 (H1N1) strain (a kind donation by Professor Peter Staeheli, Department of Virology, University of Freiburg) or the SARS-CoV-2 strain B.1.1.10.

METHOD DETAILS

Influenza A virus infection
Wildtype mice, CD47−/− mice and SIRPα−/− were anesthetized by ketamine (100 mg/kg body weight)/xylazine (5 mg/kg body weight) and intranasally infected with sublethal (8 PFU) or lethal dose (75 PFU) influenza virus A/PR/8/34 (25 μL). Upon infection mice were monitored daily and weight loss was recorded.

Histopathological analysis
Lungs were rinsed in ice-cold PBS and fixed in 4% paraformaldehyde (PFA), followed by embedding in paraffin and sectioning at 4 μm thickness. After staining with hematoxylin and eosin (H&E) the severity of the histopathology was scored in a blinded manner according to the inflammation markers, including inflammatory cell infiltration, cell necrosis, granulocyte infiltration and histiocytosis. Each marker was scored from 0 (= no signs of inflammation) to 4 (= severe signs of inflammation).
Ex vivo AEC II infection assay

The specific isolation of alveolar epithelial cells was performed as described previously. Briefly, lungs were perfused with phosphate buffer saline (PBS) and filled with Dispase Solution (Corning) followed by 1% Agarose Solution (Biozym). After incubation for 2 min at 4°C, lungs were dissected and digested for 45 min at RT in Dispase Solution. Next, the lobes were rigorously minced and DNase I (Merck KGaA) as well as TruStain feX™ (Biolegend) were added. The samples were incubated for 10 min at RT on a rocker and the obtained cell suspension was sequentially filtered through a 100, 70, and 30 μm strainer. Erythrocyte lysis was performed using Ammonium-Chloride-Potassium (ACK) buffer and cells were stained with following antibodies: CD19-PE, CD31-PE, CD11b-PE, CD16/32-PE, CD45 PE, F4/80-APC, CD93-APC, and CD11c-APC. The AEC II cells were sorted with the BD FACSAria II and isolated AEC II cells were plated on a 96-well plate, which was coated with 3 mg/mL Matrigel (Corning). Before further use the AEC II were allowed to rest for 12 h at 37°C and 5% CO2 in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% FBS, 50 U/mL penicillin, and 25 mM HEPES (Invitrogen). Next, AEC II were infected with different doses of IAV and incubated for 48 h at 37°C. Thereafter, the cells were lysed, and the RNA was isolated in accordance with the manufacturer’s instructions of NucleoSpin® RNA XS kit (Macherey-Nagel). The obtained RNA was transcribed into cDNA and the viral titer of IAV in infected AEC II was determined by qRT-PCR using the Maxima SYBR Green qPCR Kit (Thermo Scientific). A conserved sequence in the matrix gene (M1) of influenza A virus was detected using the specific primers (5′- CTTCTAACCGAGGTCGAAACG-3′; 5′-AGGGCATTTTGGACAAAGTCGTCTA-3′) described elsewhere.

Cell recovery

To obtain single cell suspensions of pulmonary immune cells, mice were euthanized at indicated time points, the lung tissue was perfused with PBS and dissected. Digestion of the tissue was performed at 37°C in Iscove’s Modified Dulbecco’s Medium (IMDM) (Invitrogen) containing 0.5 mg/mL DNase I (Roche Life Science), 0.16 mg/mL Collagenase D (Roche Life Science) and 5% heat-inactivated FBS. After 45 min the tissue was placed on a 70 μm nylon cell strainer and erythrocytes were lysed using ACK buffer. Finally, the cells were resolved in PBS containing 2% heat-inactivated FBS and 2 mM Ethylenediaminetetraacetic acid (EDTA) for further use.

For generation of BALF, the trachea was surgically exposed and intubated with a syringe catheter. The lungs underwent lavage with 1 mL pre-warmed PBS containing 2 mM EDTA and 0.5% FBS for 5 times. In total, 5 mL BALF was obtained from each mouse and cells in BALF were pelleted by centrifugation (1,200 rpm for 10 min at 4°C). Cells were then BAL cells were erythrocyte-depleted using ACK buffer and resuspended in PBS containing 2% heat-inactivated FBS and 2 mM EDTA or medium.

Flow cytometric analysis

According to Misharin et al. aMΦ were classified by the expression of CD11ch and CD11bl (Figure S3) for investigation of the proportion of aMΦ. The gating was adjusted by previously gating on CD103+ cells to exclude DCs. For staining of CD107a lymphocytes were restimulated for 4 h with PMA and ionomycin in the presence of brefeldin A and CD107a antibody. Subsequent intracellular staining for granzyme B was performed according to the Foxp3 Staining Buffer Set (eBioscience). For intracellular HBA/HBB staining cells were fixed with 2% PFA (Roth) for 30 min at 4°C. Afterward, cells were washed and treated for 10 min with 0.1% Igepal (Sigma Aldrich) at room temperature. Subsequently, intracellular staining for HB alpha and beta was performed for 30 min at 4°C followed by staining with anti-rabbit IgG (Biolegend) for 30 min at 4°C. For all stainings a Fixable Viability Dye (Thermo Scientific) was used to exclude dead cells from analysis. Data was acquired by using a LSR II or Canto™ instrument together with DIVA software (BD Biosciences Pharmingen).

Immunohistology

Immunohistochemical staining of MΦ responses were performed as described previously. Briefly, the lungs were perfused and fixed, embedded in paraffin and sectioned with a thickness of 5 μm. Subsequently, the paraffin was removed from the sections and the samples were steam boiled in citrate buffer (pH 6). Endogenous peroxidase activity was blocked with 3% H2O2 and MΦ were labeled based on the expression of MAC-3. Visualization of labeled cells was accomplished via horseradish peroxidase (HRP) coupled secondary antibodies and a subsequent dianamobenzidine (DAB) staining. Moreover, the sections were counterstained with hematoxylin.
Influenza virus plaque assay and suppression assay

Lungs were obtained from IAV infected mice at indicated time points and subsequently homogenized in PBS containing 0.3% bovine serum albumin (BSA). Viral loads were determined by plaque assay as described previously. Homogenized lungs were centrifuged for 8 min at 8,000 RPM. Lung supernatants were serially diluted in Opti-MEM (Invitrogen) containing 0.3% BSA and added to MDCK cells on a 12 well plate for 1 h at room temperature. Afterward, supernatants were discarded and DMEM complete containing 3% Avicel, trypsin (0.4 μg/mL) and gentamycin (100 μg/mL) was added for 72 h at 37°C. To count the plaques MDCK cells were fixed for 30 min with 4% PFA. Afterward MDCK cell layer was stained with crystal violet (Roth).

In vivo macrophage depletion

Specific depletion of macrophages was achieved by administration of dichloromethylene bisphosphonate (clodronate) containing liposomes (Liposoma B.V. - Amsterdam, Netherlands). A single intranasal instillation of 75 μL of clodronate containing liposomes was performed to deplete aMΦ from the alveolar lumen. Depletion of iMΦ was achieved by twice intraperitoneal administration of 200 μL of clodronate containing liposomes at an interval of 48 h.

Adoptive transfer of murine alveolar macrophages

Adoptive transfer of aMΦ was accomplished by an initial depletion of macrophages in the alveolar lumen of recipient mice as described before. After 48 h aMΦ isolated from wildtype and CD47−/− donor mice were transferred intranasally into recipient mice. Isolation of murine aMΦ was achieved by repeated BAL and a subsequent sorting of the cells based on their autofluorescence via the BD FACS Aria II to obtain an untouched aMΦ population. The purity of the population was checked by staining for F4/80+ and CD11Chi cells as described previously.

In vivo phagocytosis assay

The in vivo phagocytic activity of macrophages in the lung was determined by intranasal administration of 0.05 mg pHrodo Green E. coli BioParticles (Thermo Scientific) in 75 μL of PBS. Anesthesia was performed as mentioned above and after 1 h cells of the BAL were isolated for flow cytometric analyses. The pH-sensitive pHrodo Green dye enables a specific determination of phagocytosed BioParticles as it emits green light in the acidic environment of phagosomes.

In vivo treatment of mice with hemoglobin

Mice were infected with 75 PFU as described and intranasally treated on day 0 and 3 post infection with 50 μg murine hemoglobin protein (Fitzgerald Industries) diluted in PBS.

Transcriptome analysis

Global gene expression of WT and KO aMΦ was assessed by Affymetrix MicroArray. Quality and integrity of total RNA was controlled on Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). RNA was extracted using Qiagen RNeasy according to manufacturer’s manual. 2–10 ng of total RNA were used for biotin labeling according GeneChip Pico Kit (Affymetrix). 5.5 μg of biotinylated cDNA were fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix Clarion™ S (400 Format) for 17 h at 45°C. Hybridization was done for 16 hat conditions recommended by the manufacturer. Clarion™ S chips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GCS 3000. Image analysis was done by Affymetrix® GeneChip® Command Console® Software (AGCC) and Affymetrix® Expression Console™ Software.

Raw data obtained after image analysis were further analyzed by R/BioConductor packages “oligo” and “Biobase”. Raw signal intensities of each probeset (gene feature) were summarized by median polish method. Summarized probeset data was log2 transformed followed by RMA normalization procedure. Finally, the obtained dataset was annotated by NetAffx (Affymetrix). Normalized datasets were filtered for informative genes (showing at least expression values > log2(10) in more than two samples). Datasets were tested across all groups (ANOVA) or pairwise using linear models to assess differential expression in context of the multifactorial designed experiment. For statistical analysis and assessing differential
expression the R/BioConductor package “limma” was used that utilizes an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. Functional analysis was performed by R package “clusterProfiler”.

Ex vivo TSP-1 stimulation of aMΦ
WT and CD47−/− mice were infected with 75 PFU as described. 3 dpi aMΦ were isolated by repeated BAL as described. 2x10⁶ BAL cells were then incubated in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics for 90min at 37°C, 5% CO2 in cell culture plates. Non-adherent cells were then removed by extensively rinsing the surface with PBS. To verify effective purification, adherent cells were detached by treatment with 0.05% Trypsin/EDTA (Sigma Aldrich), stained with antibodies against F4/80 and CD11c and analyzed by flow cytometry. Cells were incubated with 1 μg/mL and 5 μg/mL murine TSP-1 (R&D) in 300 μL Medium on a 24 well plate for 24 h. Afterward, supernatants were collected and analyzed for HB concentration by ELISA.

Uptake of hemoglobin by cells
Murine hemoglobin protein was fluorescently labeled using the Alexa Fluor 647 (AF647) Protein Labeling Kit (Invitrogen) according to manufacturer’s instructions. To examine the uptake by AEC II the cells were isolated as described. 1 x 10⁶ AEC II were then incubated with 1 µg/mL of AF647-labelled hemoglobin for 30min at 37°C. Afterward, cells were washed. CD45− CD326+ AF647+ cells were identified via flow cytometry as having taken up hemoglobin. For the investigation of the uptake of HB by pulmonary immune cells single cell suspensions were prepared as described. Then 2.5×10⁶ pulmonary immune cells were incubated with 1 µg/mL of AF647-labelled HB for 30min at 37°C. Indicated AF647+ cells were identified as having taken up HB.

Suppression assay to determine the antiviral activity of hemoglobin
The suppressive activity of BALF samples and hemoglobin was determined based on plaque assay as described. Apart from this, MDCK cells were pretreated for 30min at room temperature with the BALF from WT and CD47−/− mice of indicated time points. BALF was diluted 1:3 in Opti-MEM Medium (Invitrogen). Subsequently BALF was removed and MDCK cells were infected with 1000 PFU of A/PR8/34 influenza virus for 1 h. 72 h later plaques were counted. The suppressive activity of the BALF samples was calculated relative to a medium-treated control.

To determine the antiviral activity of HB, MDCK cells were pretreated for 30min at room temperature with indicated concentrations of murine whole HB protein (Fitzgerald Industries). Afterward, HB was removed and MDCK cells were infected as described. 72 h later plaques were counted. For post infection treatment of MDCK cells 1 μg/mL HB was added to the cell culture 2 h after infection.

Generation of infectious SARS-CoV-2 stocks
The SARS-CoV-2 strain used in this study was isolated from a nasopharyngeal swab of a patient suffering from COVID-19 disease using a Virotech vial (Sigma, Germany). For propagation, the Virotech medium was then incubated on 2x10⁶ VeroE6 cells that were seeded in a T75 flask and maintained in DMEM supplemented with 10% fetal bovine serum, L-glutamine, penicillin (100 IU/mL) and streptomycin (100 μg/mL). After 3 days of incubation, the supernatant was harvested and cell debris was removed by centrifugation. Aliquots of the supernatant were prepared and stored at −80°C. Viral titers were determined by endpoint dilution assay and the 50% tissue culture infective dose (TCID50) was calculated as previously reported. The virus stock was sequenced and assigned to B.1.1.10 according to Pangolin database accession number EPI_ISL_602518.

Stimulation of A549-AT cells with HB
A549 cells, which constitutively express ACE2 and TMPRSS2 (A549-AT) were kindly provided by Marek Wi dera (University Hospital Essen). A549-AT cells were seeded in 24 well plates. Next day, medium was removed and cells were either stimulated 30 min with HB prior to infection with 350 PFU/mL SARS-CoV-2 for 1 h (HB pre) or left untreated. Fort post treatment with HB, virus-containing medium was removed 1 h post infection and cells were stimulated with HB or left untreated. After 24 h of culture in 37°C Cat 5% CO2 atmosphere cells were analyzed by in-cell ELISA.
In-cell ELISA

The in-cell (ic) ELISA was performed based on the previously published protocol. Briefly, infected and HB-treated VeroE6 cells were fixed with 4% ROTI Histofix (Roth) for 2 h at room temperature to fix the cells and inactivate the virus 24 h post infection.

Afterward, the plate was washed thrice with PBS. The PBS was aspirated and 200 μL of freshly prepared permeabilization buffer (PBS, 1% Triton X-100 (Roth)) were added to the cells and the plate was incubated for 30 min at room temperature under constant shaking. Subsequently, the permeabilization buffer was aspirated and 200 μL of blocking buffer (PBS, 3% FBS) were added for 1 h. Then, the blocking buffer was aspirated and 50 μL of primary antibody solution (anti-SARS-CoV-2-NP (RRID: AB_2890255) 1:5,000 diluted in PBS + 1% FBS) was added to each well. The plate was incubated overnight at 4°C. The next day, the primary antibody solution was aspirated and the plate was washed thrice with wash buffer (PBS, 0.05% Tween 20 (Roth)). Thereafter, 50 μL of the secondary antibody solution (Peroxidase-AffiPure Goat Anti-Mouse IgG (H + L) (RRID: AB_10015289) 1:2,000 in PBS, 1% FBS) was added to the wells and the plate was incubated for 2 h at room temperature. After the incubation period, the wells were washed 4 times with 250 μL wash buffer. Afterward 100 μL of TMB substrate solution (Biolegend) were added and the plate was incubated about 20 min at room temperature in the dark. The reaction was stopped by addition of 100 μL 2 NH2SO4 (Roth). The absorbance was measured at 450 nm with a reference wavelength of 620 nm using Spark 10M multimode microplate reader (Tecan).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantitative data are expressed as the mean ± SEM. Statistical analyses were performed using Prism 7.0 software (GraphPad). One-way or two-way ANOVA with multiple-comparisons post-test, Student’s t test or Mann-Whitney test were performed as indicated in the figure legend. A p value of <0.05 was considered statistically significant. The details of the statistical analysis used in each experiment are found in the figure legends.

DAB quantification of slides stained for MAC-3 and counterstained with hematoxylin was performed by semi-quantitative analyses. Ten random, non-overlapping fields (magnification, x200) of lung tissue from each specimen were photographed and pictures were automated single cell counted for DAB using the “Fiji” version of ImageJ from http://fiji.sc. The following adjustments for automatic counting were used: threshold 140, particle size 200–8000, Circularity 0.14–1.00. From all fields, the mean counts were averaged to yield the final score for each specimen.