DNGR-1 is dispensable for CD8\(^+\) T-cell priming during respiratory syncytial virus infection

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During respiratory syncytial virus (RSV) infection, CD8\(^+\) T cells both assist in viral clearance and contribute to immunopathology. CD8\(^+\) T cells recognize viral peptides presented by dendritic cells (DCs), which can directly present viral antigens when infected or, alternatively, "cross-present" antigens after endocytosis of dead or dying infected cells. Mouse CD8\(\alpha\)\(^+\) and CD103\(^+\) DCs excel at cross-presentation, in part because they express the receptor DNGR-1 that detects dead cells by binding to exposed F-actin and routes internalized cell debris into the cross-presentation pathway. As RSV causes death in infected epithelial cells, we tested whether cross-presentation via DNGR-1 is necessary for CD8\(^+\) T-cell responses to the virus. DNGR-1-deficient or wild-type mice were intranasally inoculated with RSV and the magnitude of RSV-specific CD8\(^+\) T-cell induction was measured. We found that during live RSV infection, cross-presentation via DNGR-1 did not have a major role in the generation of RSV-specific CD8\(^+\) T-cell responses. However, after intranasal immunization with dead cells infected with RSV, a dependence on DNGR-1 for RSV-specific CD8\(^+\) T-cell responses was observed, confirming the ascribed role of the receptor. Thus, direct presentation by DCs may be the major pathway initiating CD8\(^+\) T-cell responses to RSV, while DNGR-1-dependent cross-presentation has no detectable role.

Keywords: CD8\(^+\) T cell · Cross-presentation · DNGR-1 · Lung infection · Virus

Introduction

Respiratory syncytial virus (RSV) is an important respiratory virus of the single-stranded RNA virus family Paramyxoviridae. RSV disease burden is estimated at 64 million cases and up to 160,000 deaths every year worldwide (www.who.int). While RSV disease manifests as a simple common cold in the majority of cases, between 2 and 3% of children develop severe bronchiolitis. Although most of these children recover, they have a greater risk of developing recurrent wheeze and asthma-like symptoms in later childhood [1, 2]. RSV infection induces a robust virus-specific CD8\(^+\) T-cell response. These T cells play a key role in viral clearance by destroying infected cells [3] although, in mouse models, they also contribute to lung immunopathology following RSV infection [1, 4, 5].

Antigen processing and presentation of peptides on MHC class I (MHC-I) is crucial for CD8\(^+\) T-cell responses to RSV and other viruses. The classical pathway, whereby peptides from the cytosol are loaded onto MHC-I molecules in the ER and transported to the cell surface, is thought to be the major mechanism by which viral antigens are displayed on the surface of infected cells [6]. However, uninfected cells can also present viral antigens in some instances. This is termed “cross-presentation” and involves antigens released from virally infected cells, often in the form of debris of lysed cells, being taken up by bystander cells through...
endocytosis or phagocytosis and then diverted into the MHC-I presentation pathway [6]. The ability to cross-present exogenous antigens is particularly well developed in dendritic cells (DCs). DCs are professional antigen presenting cells (APCs) that have the ability to take up antigen in the periphery and then migrate to the lymph node where they can present antigen to naïve T cells. Cross-presentation has presumably evolved to ensure that DCs can present viral antigens to CD8+ T cells and prime cytotoxic T lymphocytes (CTLs) even in situations when they are not themselves infected or where viruses encode inhibitors of endogenous antigen processing.

Several distinct subsets of DCs are found across tissues in both mouse and man but it is unclear how their dynamics change during viral infection. In mouse models of RSV infection, lung CD103+ and CD11b+ DCs migrate to the lymph nodes [7, 8] while monocyte-derived DCs migrate into the infected lungs [9–11]. Lung CD103+ DCs are similar to lymphoid tissue-resident CD8α+ DCs in having a superior ability to cross-present antigens to CD8+ T cells [12–14]. The two DC subtypes are ontogenetically related and share the ability to avidly phagocytose dead or dying cells and cross-present antigens extracted from the phagocytosed material. This is partly dependent on the C-type lectin receptor, DNGR-1 (Dendritic cell NK Lectin Group Receptor 1) (also known as CLEC9A), which is uniquely expressed by CD103+ and CD8α+ DCs and their human equivalents [15–19]. DNGR-1 binds F-actin, which is exposed by dying cells at the point when they lose membrane integrity [20, 21]. Upon recognition of F-actin, DNGR-1 signals to prevent phagosomal maturation and this facilitates antigen presentation and subsequent cross-presentation, thereby favoring CD8+ T-cell responses against antigens present in the cellular debris, including ones of viral origin [22–24].

RSV infection induces cell death and syncytia formation [1, 25] but it is currently not known whether virus-induced cell death influences the initiation and magnitude of the ensuing immune response. Our work aimed to elucidate whether DNGR-1 and, by inference, cell death contributes to CD8+ priming in vivo during RSV infection. We found that DNGR-1-deficient mice mount reduced virus-specific CD8+ T-cell responses to intranasal inoculation with dead RSV-infected cells. However, upon live virus infection, RSV-specific CD8+ T-cell responses are equivalent between DNGR-1 knockout (KO) and wild-type (WT) mice. Thus, cross-presentation of epitopes from dead RSV-infected cells via DNGR-1 can influence the magnitude of CD8+ T-cell responses but is not crucial during RSV infection, suggesting that direct presentation of RSV epitopes by infected DCs may be the main pathway for CD8+ T-cell induction during RSV infection.

**Results**

DNGR-1 plays no role in viral load, cell infiltration or cytokine production during RSV infection

In order to evaluate whether DNGR-1 KO mice have any defects in innate anti-RSV immunity, WT and DNGR-1 KO mice were intranasally inoculated with $1 \times 10^6$ focus forming units (FFU) of RSV on day 0. Copies of RSV L gene RNA were detected on day 1, 4 (peak), and 8 post infection (p.i.) using qPCR (Fig. 1A). There was no noticeable difference in viral replication or clearance between WT and DNGR-1 KO mice. In order to assess lung inflammation, the cellular infiltration into the bronchoalveolar lavage (BAL) was investigated. The total number of cells in the BAL was not different between DNGR-1 KO and WT mice (Fig. 1B). Furthermore, in both DNGR-1 KO and WT mice, accumulation of neutrophils in the BAL was detected at day 1 p.i. and lymphocytes were detectable at day 4 and were further increased by day 8 p.i. (Fig. 1B). The frequency of macrophages was not different between DNGR-1 KO and WT mice and no eosinophils were detected in any BAL samples at any time point (data not shown). In addition, early inflammatory mediators (IFN-α, CXCL10, and IL-6) were analyzed in the BAL on day 1 and 4 and all were detected at similar levels in WT and DNGR-1 KO mice (Fig. 1C). Thus, DNGR1 expression does not appear to be essential for the early immune response or viral clearance during RSV infection.

**RSV pathology and T-cell infiltration to the lung is not dependent on DNGR-1**

In the mouse model, RSV disease severity as measured by weight loss has been linked to a potent response by RSV-specific T cells [1, 3, 26]. As DNGR-1 has been shown to mediate cross-priming of CTLs during virus infection [23, 24], the lack of DNGR-1 might lead to reduced T-cell priming and therefore decreased disease severity. RSV infected WT mice showed weight loss starting on day 5 p.i. with about 15% weight loss on day 6 and recovery on day 8 p.i. (Fig. 2A). Contrary to our hypothesis, DNGR-1 KO mice showed equal weight loss to the WT mice after RSV infection (Fig. 2A). In addition, total lung cell numbers after RSV infection were equivalent between DNGR-1 KO and WT mice (Fig. 2B) and there was no difference in frequency and absolute number of CD8+ T cells (Fig. 2C and E). The absolute numbers of CD4+ T cells in the lung did not increase after infection (Fig. 2C and D) as previously shown [27, 28]. Finally, no difference in RSV-specific antibodies was found after primary or secondary RSV infection in DNGR-1 KO mice compared with littermate controls (data not shown). Thus, the absence of DNGR-1 does not affect the influx of T cells into the lungs during RSV infection or the overall pathology.

**DNGR-1 deficiency does not affect the induction of RSV-specific CD8+ T-cell responses**

We further examined whether the RSV-specific CD8+ T-cell response was affected. First, we quantified the frequency of CD8+ T cells specific for an immunodominant epitope derived from the M2 protein [29, 30] by using Alexa Fluor-647 conjugated MHC class 1 tetramer-M2292-99 complexes (Fig. 3A). Tetramer+ CD8+ T cells were detected in the BAL and lungs at approximately 25% of CD8+ T cells on day 8 post RSV infection (the peak of T-cell
Figure 1. Viral load, cellular infiltration, and cytokine production into the BAL after RSV infection are not different in DNGR-1 KO mice compared with WT mice. Mice were intranasally infected with $10^6$ FFU of RSV A2. (A) Viral load was determined by measuring copies of RSV L gene RNA on day 1, 4, and 8 post infection (p.i.). The detection limit for the copy numbers of L gene according to the standard curve is indicated by the dotted line. Data are presented as the mean ± SEM (n = 4–5 mice per group) and are from a single experiment representative of at least two independent experiments. (B) Bronchoalveolar lavage (BAL) was obtained from naive or RSV-infected WT or DNGR-1 KO mice on day 1, 4, and 8 p.i. The total number of BAL cells was quantified and the frequency of neutrophils and lymphocytes were determined using differential cell counting of H&E stained cytospin slides. Data are shown as mean ± SEM of 8–22 mice per group pooled from three to four individual experiments. (C) Levels of IFN-α, CXCL10, and IL-6 in the BAL fluid were determined using ELISA. Data are presented as the mean ± SEM of 6–8 mice per group pooled from two experiments. Differences were not statistically significant by two-tailed, unpaired, nonparametric Mann–Whitney test.

Responses) but this was similar between DNGR-1 KO and WT mice (data not shown and Fig. 3A and B). We further analyzed the RSV specific CD8+ T-cell response on day 8 p.i. by quantifying IFN-γ+ CD8+ T cells in the lung following 4 h ex vivo restimulation with the immunodominant peptide M282-90 (Fig. 4A). Few IFN-γ+ CD8+ T cells were detected after restimulation with medium alone (Fig. 4) or with the irrelevant D'M187-195 RSV peptide (data not shown; see Fig. 5). However, around 40% of the CD8+ T cells produced IFN-γ after stimulation with M282-90 peptide but, importantly, there was no difference between the DNGR-1 KO and WT mice (Fig. 4B). In addition, IFN-γ levels in the BAL were examined at day 8 p.i. and similar IFN-γ production was detected in DNGR-1 KO and WT mice (Fig. 4C). Furthermore, total T-cell responses were quantified by restimulating lung single-cell suspensions with M282-90 peptide at various concentrations (5 ng/ml; 0.5 ng/ml; 0.05 ng/ml) or with RSV (MOI of 1) and measuring IFN-γ levels in culture supernatants, which again showed no difference between DNGR-1-deficient and WT mice (Fig. 5A).

The above analysis focused exclusively on the immunodominant epitope M282-90. We performed additional restimulations of lung cells from RSV infected mice with F85-93 and F93-106 peptides corresponding to two subdominant epitopes that, like M282-90, are presented by H-2Kd [31, 32]. Compared with restimulation in medium alone, F peptides induced IFN-γ production from lung cells above background although this response was much weaker than that seen with M2 restimulations. Importantly, there were no differences in the response to either F peptide between DNGR-1 KO and WT mice (Fig. 5B). Taken together, these data indicate that DNGR-1 expression is not critical for the induction of RSV specific CD8+ T cells in BALB/c mice.

Lack of DNGR-1 reduces RSV-specific CD8+ T cells after exposure to dead virus-infected cells

In order to assess whether DNGR-1 can have any impact on the induction of anti-RSV CD8+ T cells, mice were inoculated intranasally with UV-treated, RSV-infected human epithelial cell line 2 (HEp-2 cells). Using this model, we minimized the direct infection of DCs and increased the likelihood that any anti-RSV CD8+ T-cell responses resulted from cross-presentation of the inoculum. There was no weight loss detected in any of the groups after inoculation with infected dead cells (UV-RSV HEp-2; data not shown) and the number of total cells and CD8+ tetramer+ cells in the lung were similar in DNGR-1 KO and WT mice (Fig. 6A and B). However, fewer cells were found extravasating into the airways of DNGR-1 KO mice compared with WT controls as determined by BAL analysis (Fig. 6C). The number of RSV-specific CD8+ T cells in BAL was also reduced (Fig. 6D) and lower levels of IFN-γ in bronchoalveolar lavage fluid (BALF) were found in DNGR-1 KO mice compared with WT mice (Fig. 6E). We conclude that DNGR-1 deficiency can impact on anti-RSV immunity induced by intranasal immunization with dead infected cells.
Figure 2. Weight loss and T-cell infiltration into the lungs are not dependent on DNGR-1. Mice were intranasally infected with 10^6 FFU of RSV on day 0. Uninfected WT or DNGR-1 KO mice (Naïve; Na) were used as controls and received an equivalent volume of PBS. (A) Weight was recorded daily until day 8. The percentages of the weight compared with the original weight (%) on day 0 were calculated. Data are presented as the mean ± SEM (n = 4–5 mice per group) and are from a single experiment representative of four independent experiments. (B) Total lung cells were quantified in WT and DNGR-1 KO mice. Data are shown as mean ± SEM of 10–15 mice per group, pooled from three individual experiments. (C) Representative plots of nondebris (SSC-A vs FSC-A), singlet (FSC-W vs SSC-A), live (Dead cells vs. SSC-A) cells analyzed for expression of CD3 and CD19. CD3^+ cells were further gated on CD4 and CD8 expression. Frequencies and absolute numbers of (D) CD4^+ T cells and (E) CD8^+ T cells were determined by flow cytometry on day 0 and 8 after RSV infection. Data are presented as mean ± SEM of 4–5 mice per group from one experiment representative of three independent experiments. There were no significant differences between the groups using unpaired, two-tailed, unpaired Mann–Whitney test.

Discussion

CD8^+ T cells are an important part of the immune response against RSV infection. They are important for viral clearance but at the same time can contribute to immunopathology, at least in mouse models. It is not clear to what extent cross-presentation of viral antigens from dead cells contributes to the CD8^+ T-cell response during respiratory infections. As RSV is cytopathic and can cause syncytia formation in epithelial cells, we wanted to investigate whether RSV-triggered cell death is important for the initiation of CD8^+ T-cell responses. One way to address this issue is to use mice lacking DNGR-1, which is known to bind to the actin cytoskeleton from damaged cells and ferry associated antigens into the cross-presentation pathway [33]. DNGR-1^+ CD103^+ DCs are present in the lung and are thought to be one of the main migratory lung DC populations [7, 10, 11, 34, 35]. In previous studies, DNGR-1 KO mice were shown to be defective in mounting specific CD8^+ T-cell responses to cytopathic lung infection with herpes simplex virus 1 (HSV-1) or skin infection with vaccinia virus [23, 24]. Notably, we show a dependence on DNGR-1 for the RSV-specific CD8^+ T cells in the airways when mice are inoculated with UV-killed RSV-infected cells. This demonstrates that DNGR-1 can play a role in the anti-RSV response if the lung is exposed to infected dead cell material but, interestingly, we only detect a difference in number of RSV-specific CD8^+ T cells in the airways (BAL) and not in the lung tissue. This could be due to a decreased inflammation of the lungs of DNGR-1 KO mice with less infiltration of cells into the airways or that RSV-specific CD8^+ T cells in WT mice are more activated and have the ability to migrate into the airways. Independently of these considerations, we found that DNGR-1 is dispensable for the anti-RSV CD8^+ T-cell response in the lungs during live virus infection. This is in contrast to what has been found during HSV-1 lung infection [23]. It is possible that the quantity of virally infected dead cell material and/or the host cell for viral replication differ during RSV and HSV infection and therefore the two models differentially depend on cross-presentation via DNGR-1 for mounting CD8^+ T-cell responses. Alternatively, or in addition, the rapid decrease of CD103^+ DC from the lung early after RSV infection [7] may lead to the contribution of this DC subset, and, consequently, the contribution of DNGR-1, being...
Figure 3. Quantification of RSV-specific (M2 Tetramer+) CD8+ T cells from RSV-infected DNGR-1 KO and WT mice. DNGR-1 KO and WT mice were infected with 10^6 FFU RSV. Mice were sacrificed on day 8 and lung cells were stained with MHC class I tetramer complexes specific for the RSV epitope K^dM2_82-90 and analyzed by flow cytometry. (A) Representative plots for the various groups examined on day 0 (Naïve) and day 8 post RSV infection. CD3+CD19- cells expressing M2 Tetramer and CD8 are shown. (B) Quantification of the percentages of RSV K^dM2_82-90 specific CD8+ T cells of total CD8+ T cells. Each symbol represents an individual mouse and data are shown as mean ± SEM of 6–11 mice per group, pooled from two to three individual experiments. Differences were not statistically significant using unpaired, two-tailed, unpaired Mann–Whitney test.

Overall, our data show that cross-presentation of RSV infected dead cell material via DNGR-1 is not a major pathway for initiating the CD8+ T-cell response during RSV infection. We cannot exclude the possibility that cross-presentation via DNGR-1 might contribute to the priming of a small population of CD8+ T cells specific for epitopes other than the three studied here. It is also still possible that other, DNGR-1-independent routes of cross-presentation are important. Nevertheless, the results from this study suggest that DNGR-1 is not a major player in the initiation of RSV immunity and help our understanding of the basic biology behind immunity to this virus.

Materials and methods

Mice, virus stocks, and infection

Clec9a^gfp/gfp (DNGR1-deficient) mice [22] were kindly provided by C. Reis e Sousa (Cancer Research UK London Research Institute, London, UK). These were backcrossed six generations onto a BALB/c background, then bred and housed in specific-pathogen free conditions according to the UK Home Office guidelines. Clec9a^+/+ (wild type) and Clec9a^+/gfp (heterozygous) littermates or age and sex-matched WT BALB/c mice purchased from Harlan Laboratories (UK) or Charles River Laboratories (UK) were used as controls. No differences were ever noticed between these control groups and are all therefore referred to as WT mice.
Immunity to infection

1) for 3 days. Supernatants were quantified for levels of IFN-γ by ELISA. Each symbol represents an individual mouse and data are shown as mean ± SEM of 9–21 mice per group, pooled from two to five individual experiments. (B) Lung cells were re-stimulated with the subdominant M2*82-90* and FII/III+ receptors and surface protein μ, and RSV. Lung cells from DNGR-1 and WT BALB/c mice were lightly anesthetized with isofluorane and challenged intranasally (i.n.) with a dose of 1 × 10^6 FFU of RSV on day 0.

To prepare UV-killed RSV-infected HEP-2 cells, 80% confluent HEP2 cells were infected with RSV at an MOI of 0.5 in serum-free media (Dulbecco’s Modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine). After 2 h incubation at 37°C, serum-containing media (DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine) was added. Infected cells were harvested after 24 h, washed and resuspended in sterile PBS and UV-killed using 750 mL/cm² treatment twice in a CX-2000 UV cross-linker (UPV). Cells were further rested for 2 h at room temperature (RT) and mice were then inoculated i.n. with 2 × 10^6 UV-killed RSV-infected HEP-2 cells.

Cell isolation and processing

Mice were culled using a fatal dose of pentobarbital injected intraperitoneally (i.p.) according to UK home office guidelines.

BAL was collected by flushing the lungs three times with 1 mL of PBS supplemented with 0.5 mM EDTA (Life Technologies). Lung lobes were collected and digested with Collagenase D (1 mg/mL, Roche) and DNase I (30 μg/mL; Sigma-Aldrich) using a gentleMACs cell dissociator (Miltenyi Biotec) according to the manufacturer’s protocol and incubated at 37°C for 30 min. Red blood cells were lysed by treating lung cells with ACK lysis buffer and lung cells were passed through 100 μM cell strainers to create single-cell suspensions. Total cell counts were determined using hemocytometer slides and dead cells were excluded by Trypan blue staining (Sigma-Aldrich). To determine the cellular composition in the BAL, cells were transferred onto a microscope slide (Thermo Scientific, UK) using a Shandon Cytospin 3 Centrifuge and slides were stained with hematoxylin and eosin (H&E; Reagen, Gamidor, UK). Cells were categorized as macrophages, lymphocytes, neutrophils, and eosinophils based on their morphology and size under a light microscope.

Flow cytometry

For flow cytometry analysis, dead cells were discriminated using LIVE/DEAD Fixable Aqua Dead Cell Stain (Molecular Probes, Life Technologies) according to the manufacturer’s instruction. Cells were then incubated in FACS Staining Buffer (PBS containing 1% BSA and 5 μM EDTA) with antibody recognizing Fcγ II/III receptor (CD16/CD32; BD Biosciences). Surface receptor staining was performed using the following antibodies, all purchased from BD Bioscience unless otherwise stated: PE-Cy7 conjugated anti-CD3 (clone 145–2C11); allophycocyanin-H7 or allophycocyanin-Cy7 conjugated anti-CD4 (clone GK1.5); AlexaFluor 700 conjugated anti-CD8a (clone S3–6.7); and FITC conjugated anti-CD19 (clone 1D3, eBioscience) in FACS Staining Buffer.

Biotinylated RSV M2*82-90* peptide monomers (H-2K或其他) were obtained from the NIH Tetramer Core Facility (Emory University Atlanta, GA, USA) and tetramerization was performed in-house using allophycocyanin-conjugated Streptavidin (Molecular Probes) using a protocol provided by the NIH Tetramer Core Facility. M2 tetramer staining was performed on lung and BAL cells following Fc block steps and prior to surface receptor staining. Cells were incubated with APC-conjugated M2 tetramer in FACS staining buffer for 30 min in the dark at RT. Cells were fixed for 30 min in BD Cytofix/Cytoperm fixation buffer at 4°C, washed and then analyzed on the flow cytometer.

For intracellular detection of IFN-γ, lung cells were stimulated in 96-well plates with 2 μg/mL of the immunodominant RSV M2*82-90* peptide (H-2K或其他) and NAITNACKII; Synthetic Biomolecules, San Diego, CA, USA) or the irrelevant M*187–195* peptide (H-2D或其他) (Synthetic Biomolecules, San Diego, CA, USA) identified to be immunodominant in C57BL/6 (H-2D或其他) mice [5] in complete DMEM (dDMEM; supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin). After 1 h incubation, monensin (Golgi Stop, BD) was added. Cells were incubated a further 3 h, washed and incubated with live-dead fixable stain, Fcγ II/III receptor and surface
Figure 6. DNGR-1 is important for induction of RSV-specific CD8+ T cells responses after intranasal dead cell inoculation. DNGR-1 KO and WT mice were inoculated with $2 \times 10^6$ UV-irradiated, RSV-infected HEp-2 cells (UV-RSV HEp-2). Mice were sacrificed on day 8 and airway (BAL) and lung cells were enumerated and stained with MHC class I tetramer complexes specific for the RSV epitope K$^d$M232-90 and analyzed by flow cytometry. (A) The total number of lung cells was quantified in naïve or UV-RSV HEp-2 inoculated mice. (B) Quantification of the total number of RSV K$^d$M232-90 specific CD8+ T cells in the lung of naïve or UV-RSV HEp-2 inoculated mice. (C) The total number of BAL cells was quantified in naïve or UV-RSV HEp-2 inoculated mice. (D) Quantification of the total number of RSV K$^d$M232-90 specific CD8+ T cells in the BAL of naïve or UV-RSV HEp-2 inoculated mice. (E) Levels of IFN-γ in the BAL fluid were determined using ELISA. Each symbol represents an individual mouse and data are shown as mean ± SEM of 6–10 mice pooled from two individual experiments except in (B) were data are presented as the mean ± SEM ($n = 4–5$ mice per group) and are from a single experiment representative of two independent experiments. ***$p \leq 0.001$, *$p \leq 0.05$, unpaired, two-tailed, unpaired Mann–Whitney test.

receptor antibodies as described above. Intracellular cytokine staining was performed using the Cytofix/Cytoperm kit (BD) according to manufacturer’s instructions. Briefly, cells were fixed in BD Cytofix/Cytoperm buffer for 30 min at 4°C, washed with FACS staining buffer and then incubated with Fcγ II/III receptor (CD16/CD32) antibody in BD Perm Wash Buffer for 15 min. Cells were then incubated with PE-conjugated anti-IFN-γ antibody (clone XMG1.2, BD biosciences) in Perm Wash buffer for 1 h at 4°C, washed and transferred to FACS staining buffer for analysis. Cells were acquired on the LSR Fortessa flow cytometer (BD) and data were analyzed using Flowjo Software (Version 9.6.4). Cells were gated for nondebris, live cells, and singlets and then analyzed for indicated markers.

Gene Expression

Total RNA was extracted from homogenized lung tissue using TRIzol RNA Isolation reagent in combination with a chloroform separation, followed by washes with isopropanol and 75% ethanol as per the protocol provided (TRIzol Reagent, Ambion, Life Technologies, UK). RNA purity and concentration was determined using the Nanodrop 1000 (Thermo Scientific) and 1–2 μg of total RNA was reverse-transcribed to cDNA using the high capacity RNA-to-DNA kit according to the manufacturer’s instructions (Invitrogen, Life Technologies). Real-time quantitative PCR was performed to determine mRNA levels of RSV L gene using primers (Invitrogen) and probes (Eurofins) [36]. Results were normalized to Gapdh (Applied Biosystems) and the exact copy numbers of L gene was calculated using an internal plasmid standard to give an absolute quantification. Analysis was performed using the Quantitect Probe PCR Master Mix (Qiagen) and the 7500 Fast Real-time PCR System (Applied Biosystems).

Chemokine and cytokine protein detection

To quantify RSV-specific IFN-γ levels from infected lung cells, $4 \times 10^5$ cells/well in 96-well round-bottomed plates were stimulated with either RSV (MOI of 1) or RSV M232-90 (H-2K$^d$/SYIGSINNI), F85-93 (H-2K$^d$/KYKNAVTEL), F92-106 (H-2K$^d$/ELQLLMQSTPTNNR) or control M187-195 (H-2D$^b$/NAITNAKII) peptide in indicated concentrations (Synthetic Biomolecules, San Diego, CA, USA) in cDMEM for 72 h at 37°C. Supernatants were assayed using an IFN-γ ELISA kit (R&D Systems) according to the manufacturer’s instructions.

Levels of IL-6, CXCL10 (IP-10) and IFN-γ protein were measured in the BAL fluid using ELISA kits and following the
manufacturer's instructions (all from R&D Systems). IFN-α levels in the BAL were measured by ELISA as previously described [37]. Data was acquired on a SpectraMax Plus plate reader (Molecular Devices) and analyzed using SoftMax software (version 5.2).

Statistical analysis

Results are presented as mean ± SEM. Statistical significance was determined using a two-tailed, unpaired, nonparametric Mann–Whitney t-test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Values of p < 0.05 were considered significant (Prism software; Graph-Pad Software Inc.).

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References


Abbreviations: BAL: bronchoalveolar lavage · DNGR-1: DC NK Lectin Group Receptor 1 · FFU: focus forming units · HEp-2: human epithelial cell line 2 · HSV-1: herpes simplex virus 1 · p.i.: post infection

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