The Natural Cytotoxicity Receptor 1 Contribution to Early Clearance of Streptococcus pneumoniae and to Natural Killer-Macrophage Cross Talk

Shirin Elhaik-Goldman1,2,*, Daniel Kafka1,2,*, Rami Yossef1, Uzi Hadad1, Moshe El kabets1, Alexandra Vallon-Eberhard3, Luai Hulihel1, Steffen Jung3, Hormas Ghadially4, Alex Brainman1, Ron N. Apte1, Ofer Mandelboim5, Ron Dagan3, Yaffa Mizrahi-Nebenzahl1,2, Angel Porgador1*

1 The Shraga Segal Department of Microbiology and Immunology and the National Institute for Biotechnology in the Negev, Ben Gurion University of the Negev, Beer Sheva, Israel, 2Pediatric Infectious Disease Unit, Soroka University Medical Center, Ben Gurion University of the Negev, Beer Sheva, Israel, 3Department of Immunology, Weizmann Institute of Science, Rehovot, Israel, 4The Lautenberg Center for General and Tumor Immunology, The Hebrew University Hadassah Medical School, Jerusalem, Israel

Abstract

Natural killer (NK) cells serve as a crucial first line of defense against tumors, viral and bacterial infections. We studied the involvement of a principal activating natural killer cell receptor, natural cytotoxicity receptor 1 (NCR1), in the innate immune response to S. pneumoniae infection. Our results demonstrate that the presence of the NCR1 receptor is imperative for the early clearance of S. pneumoniae. We tied the ends in vivo by showing that deficiency in NCR1 resulted in reduced lung NK cell activation and lung IFN-γ production at the early stages of S. pneumoniae infection. NCR1 did not mediate direct recognition of S. pneumoniae. Therefore, we studied the involvement of lung macrophages and dendritic cells (DC) as the mediators of NK-expressed NCR1 involvement in response to S. pneumoniae. In vitro, wild type BM-derived macrophages and DC expressed ligands to NCR1 and co-incubation of S. pneumoniae-infected macrophages/DC with NCR1-deficient NK cells resulted in significantly lesser IFN-γ levels compared to NCR1-expressing NK cells. In vivo, ablation of lung macrophages and DC was detrimental to the early clearance of S. pneumoniae. NCR1-expressing mice had more potent alveolar macrophages as compared to NCR1-deficient mice. This result correlated with the higher fraction of NCR1-ligand high lung macrophages, in NCR1-expressing mice, that had better phagocytic activity compared to NCR1-ligand dull macrophages. Overall, our results point to the essential contribution of NK-expressed NCR1 in early response to S. pneumoniae infection and to NCR1-mediated interaction of NK and S. pneumoniae infected-macrophages and -DC.

Introduction

The gram positive bacterium Streptococcus pneumoniae belongs to the commensal flora of the human respiratory tract. However S. pneumoniae also causes clinical infections including pneumoniae, meningitis and sepsis [1]. Pneumococcus is the fifth leading cause of death worldwide [2]. Considering the spread of antibiotic resistance to the bacterium, it is crucial to understand the host response to pneumococcal infection in order to improve therapy.

The host immune response to pneumococcal lung disease has been characterized as an intense inflammatory reaction, initially involving resident alveolar and interstitial macrophages, followed by lung infiltrating neutrophils [2]. Yet, the contribution of immune components, other than phagocytic cells, has also been demonstrated to be important [3–6]. It has emerged that chemokines and pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), interleukin (IL)-6 and IL-1β have a crucial role in defense against S. pneumoniae. These mediators recruit and activate inflammatory cells to the site of infection. Several studies, including our own, showed that depletion or genetic ablation of these cytokines resulted in impaired host defense [7–9]. Interferon gamma (IFNγ) is another critical immunomodulator in early host defense against a variety of infections. IFNγ is a key activator of macrophage killing activity and also recruits circulating neutrophils and lymphocytes to the sites of infection. The role of IFNγ in natural immunity to S. pneumoniae infection is not clear as reports are contradictory [10–12]. Natural killer (NK) cells are bone marrow derived lymphocytes that constitute a key frontline defense against a wide range of pathogens such as viruses, bacteria, intracellular parasites [13–15], as well as tumors [16]. NK cells are believed to release the prominent fraction of the IFNγ evident during Gram-positive infection [17] and they are recruited to the lung during pneumococcal pneumonia within 6 hours of infection [18]. Although NK cells can kill target cells spontaneously without prior
stimulation, a delicate balance between inhibitory and activating receptors tightly regulate their activation. Among these, natural cytotoxicity receptor-1 (NCR1, also named NKp46) is the only receptor reported so far to be expressed specifically on NK cells in all mammals tested, including humans [19] and mice [20]. NKp46 is a transmembrane type I glycoprotein containing two immunoglobulin domains and positively charged arginine residue in the transmembrane domain, which associates with the CD3ζ or the FceRIγ signaling adaptor molecules [19,21]. Several in vitro studies have demonstrated that NKp46 is important in the recognition and destruction of various tumors [16,22] and virus infected cells [23,24]. In addition, we have recently demonstrated a critical function for murine NCR1 (murine NKp46) in the in vivo eradication of influenza virus [25]. There are contradicting reports about the role of NK cells and IFNγ in pneumococcal infections, thus their exact effect is not yet defined. Rubins et al. showed that IFNγ+/- mice demonstrated increased mortality during S. pneumoniae lung infection, suggesting a protective role for IFNγ in host response to pneumococcal disease [10]. In contrast, Rijneveld et al. demonstrated that IFNγ does not serve a protective role during pneumococcal pneumonia. IFNγR−/− mice and IFNγ−/− mice had relatively increased resistance to S. pneumoniae infection, exhibiting significantly fewer pneumococci in their lungs in comparison to wild type (WT) mice [11]. NK cells were shown to be detrimental in pneumococcal pneumonia and sepsis in immunocompromised mice; depletion of NK cells in SCID mice resulted in significantly lower bacteremia and inflammatory cytokine production [26]. In this present study we employed C57BL/6 mice in which a gene encoding GFP was inserted into the Ncr1 locus, thereby rendering the Ncr1 gene nonfunctional. Using these mice we assessed the involvement of the NCR1 receptor in the activation of NK cells following S. pneumoniae infection, and the role of NCR1 in the reciprocal interaction between NK cells, DC and macrophages following S. pneumoniae infection. The involvement of NCR1 was shown to contribute to NK cell activation together with S. pneumoniae clearance at early stages following inoculation.

Materials and Methods

Mice

C57BL/6 mice strain Ncr1+/+gfp, Ncr1−/−gfp and Ncr1+/+ wild type littermates were used. In these mice, as described previously [25], the gene encoding the NCR1 receptor (Ncr1) was replaced with a green fluorescent protein (GFP) reporter cassette. This study involved also the use of the C57BL/6 mouse strain CD11c: Diphtheria toxin (DTx) receptor (DTR) transgenic mice (B6.FVB-Tg [Hgax-DTR/GFP/57Lan/]); The Jackson Laboratory) that carry a human DTR transgene under the murine CD11c promoter [27]. All experiments were done in the animal facilities of Ben Gurion University according to guidelines of the ethical committee.

Bacteria

The S. pneumoniae strain WU2 (capsular serotype 3) was used in this study [28]. This strain was grown to mid-late log phase as determined by OD in Todd-Hewitt broth supplemented by yeast extract. Aliquots of bacteria were harvested by centrifugation, resuspended in sterile PBS containing 10% glycerol and stored at −70°C. Colony-forming units (CFU) counts were verified in each experiment on blood agar plates at 37°C under microaerobic conditions.

Infection of mice with S. pneumoniae

For mice infection, aliquots of bacteria were thawed rapidly and samples of serial 10-fold dilutions were plated onto blood agar plates for determining bacterial concentration. Each mouse was anaesthetized with Terrel isoflurane (MINRAD, New York, USA) and inoculated intranasally with 5 × 10³, 1 × 10⁴ or 4 × 10⁴ bacteria (in 25 µl PBS). Survival was monitored daily until 8 days after inoculation.

Determination of bacterial load in the nasopharynx and lungs

Mice were sacrificed by Terrel isoflurane inhalation at 3, 6 and 24 h after inoculation. Nasopharynx and lungs were removed and homogenized in 1 ml of sterile PBS using the Polytron PT-10 homogenizer (Kinematik, Lucerne, Switzerland). Samples (25 µl) in serial dilutions were then plated onto blood agar plates and grown overnight (18 h) at 37°C in anaerobic jars for CFU determination.

Real time PCR

Total RNA was extracted from C57BL/6 mice lungs by TRI-Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Purified RNA was converted to cDNA utilizing Promega’s First Strand Synthesis (Madison, WI, USA). 20–100 ng RNA was subsequently used as a template for each Real-Time-PCR reaction. All PCR reactions for cytokine-specific mRNA were preformed on ABI PRISM 7500 Real-Time PCR System (ABI Applied Biosystems, USA) using proprietary cytokine-specific primers from ABI Applied Biosystems, USA. Relative cytokine mRNA levels were determined by normalization of signal with that for GAPDH mRNA. In initial studies, fivefold dilutions of cDNA generated a linear signal curve over at least a 50-fold range of cDNA concentrations. mRNA induction in pulsed lung tissue was reported as-fold increases over uninfected lung mice. We thank Mr. Shahar Dotan (Ben Gurion University of the Negev, Beer-Sheva, Israel) for supplement of the primers.

Depletion of CD11c+ cells following intratracheal (i.t.) instillation of DTx

PBS (80 µl) containing DTx (100 ng/gr, List Biological Laboratories) was applied to the mouse tracheae. Mice were lightly anesthetized using isoflurane and placed vertically and their tongues were pulled out. Using a long nasal-tip, liquid was placed at the top of tracheae and was then actively aspirated by the mouse. Gasping of treated mice verified liquid application to the alveolar space.

Cell isolations and generation of Bone Marrow Derived DCs/Macrophages

For bronchoalveolar lavage (BAL), the trachea was exposed to allow insertion of a catheter, through which the lung was filled and washed 10 times with 5 ml of PBS without Ca²⁺/Mg²⁺. Bone marrow (BM)-derived DC (BMDC) and bone marrow (BM)-derived macrophages (BMMQ) were generated from Bone marrow which obtained from C57BL/6 mice by flushing femoral cells with a 23-gauge needle with RPMI. Low-density mononuclear bone marrow cells were isolated using red blood cell lysing buffer (SIGMA) and centrifuged. Cells (5 × 10⁶) were cultured in 10 cm tissue dishes with the fresh complete Iscove’s Modified Dulbecco’s Medium (cIMDM) with recombinant GM-CSF (100 ng/ml) or 20% Lymphocyte conditioned medium (LCM) respectively. The medium was changed every 2–3 days and replaced with fresh medium supplemented with GM-CSF (100 ng/ml) or 20% LCM respectively. For experiments, the slightly adherent cells (DC) and/or the adherent cells (macrophages) were harvested on days 6 to 8 of culture.
For purification of NK cells, spleens were harvested and purified from RBC using the ACK lysing buffer (Quality Biological, Inc., Gaithersburg, MD). NK cells were then negatively selected by EasySep NK Selection Kit (StemCell Technologies, Inc., Vancouver, Canada). The percentage of NK cells in the isolated population was evaluated using PE-conjugated anti-CD3 mAb and APC-conjugated anti-CD56 or anti-NK1.1 mAb (eBioscience) by flow cytometry. Recombinant IL-2 (100 IU/ml) was added in order to obtain a polyclonal NK cell population.

Antibodies and Flow cytometry

Flow cytometry analysis was employed for analysis of cell surface marker expression. Splenocytes, total lung cells, BM cells (1×10^6 per well) or bronchoalveolar lavage fluid (BALF) cells (0.5×10^6 per well) were plated in 96-well U-bottom plates. Cells were washed and non specific binding was blocked with anti-CD16/CD32 in 0.5% FCS/0.5% mouse serum/PBS for 15 min on ice. Cells were then stained for 25 min with the following specific mAbs: Biotin-conjugated-anti-mF4/80, FITC-anti-mCD14, PE-anti-mNK1.1, PE-anti-mCD69, PE-anti-mCD115, PE-anti-mCD3, PE-anti-mCD11b, PerCP-Cy5.5-anti-mCD11c, Pacific Blue-anti-mCD11b, Alexa flour 647-anti-mCD107a, APC-anti-NK1.1, APC-anti-mCD3, APC-anti human Fc-IgG, APC-conjugated streptavidin, all purchased from eBioscience. For staining with fusion-lg's, cells or bacteria were incubated with 4 or 2 μg of the mNCR1-Ig, mNKG2D-Ig, LIR-Ig or CD99-lg fusion proteins for 2 h at 4°C, washed, and stained with APC-conjugated-F(ab')2 goat-anti-human-human-IgG-Fc (109-136-098, minimal cross-reaction to bovine, horse and mouse serum proteins, Jackson Immuno Research, West Grove, PA). Staining and washing buffer consisted of 0.5% (w/v) BSA and 0.05% sodium azide in PBS. Propidium iodide or AmCyan dye was added prior to reading for exclusion of dead cells. Stained cells were analyzed using either FACS Calibur or FACScanto II (Becton Dickinson, Mountain View). The data were then analyzed either with BD CellQuest™ 3.3 software or FlowJo software version 6.3.4 (Tree Star). Fluorescence data was acquired using logarithmic amplification and reported fluorescence intensity units represent conversion of channel values according to the logarithmic scale (range 10^4 to 10^7). Results are shown as the geometric mean fluorescence intensity (MFI) of the stained populations.

NK/BMDC and NK/BMMQ co-cultures

NK cells and BMDC or BMMQ co-cultures were preformed in RPMI 1640 +10% FCS in 24 well plates. Live S. pneumoniae were added to BMDC/BMMQ cultures (10^6 cells/well) for 1.5 h at noted multiplicity of infection (MOI) ratio. NK cells were added to BMDC (1:10) or BMMQ (1:4) cultures 5 hours after infection followed by bacterial washing with Tetramycin (30 μg/ml), Gentamycin (50 μg/ml), Penicillin (100 U/ml) and Streptomycin (100 μg/ml). After additional incubation for 24 h cells were collected and analyzed.

ELISA for IFNγ in supernatant

The concentration of IFNγ secreted into the media of BMDC/ BMMQ cultures was measured using optimized standard sandwich ELISA. Media samples were centrifuge at 1200 rpm for 10 min and supernatant was collected and stored in -70°C. Recombinant mouse IFNγ (mIFNγ) which was used as standard, as well as the capture mAb, biotinylated mAb which was used for detection, and Streptavidin-HRP were purchased from BioRad. The samples were tested in triplicates. The concentration of the cytokine was determined relative to a standard curve of recombinant IFNγ (ranging from 10,000 pg/ml to 125 pg/ml at 1:2 serial dilutions).

Depletion of NK cells following intraperitoneal administration of anti-asialo-GM1

NK cells were depleted using polyclonal anti-asialo-GM1 rabbit antibody (Wako Bioproducts, Neuss, Germany). Mice were injected with 50 μl of anti-asialo-GM1 in 200 μl of PBS or with equivalent volume of PBS 24 h prior to intranasal infection with S. pneumoniae. 27 hours post-infection the lung and spleen were collected and subjected to a CFU assay and to FACS staining in order to check the efficacy of treatment.

CFDA staining of bacteria

Bacteria were grown to mid-late log phase as determined by OD in Todd-Hewitt broth supplemented by yeast extract. Aliquotes of bacteria were harvested by centrifugation (13000 rpm), resuspended in sterile PBS and washed twice. Bacteria (1 ml suspension, 1×10^9 cell concentration) were then stained with carboxyfluorescein diacetate (CFDA) using 200 μl of stock solution (0.5 mM), incubated with shaking for 30 min at 37°C, and washed twice following incubation.

Immunocytochemistry

BALF cells were grown on chamber µ-slide (Ibidi, Germany), incubated alone or with 1 MOI CFDA-labeled S. pneumoniae for 1 h. Cells were then rinsed with PBS, fixed for 10 minutes in 2% paraformaldehyde and blocked with PB/A blocking solution (PBSX1, 5% BSA, 0.5% NaN3) for 30 minutes. Fixed cell were stained with mNCR1-lg and rat anti mouse F4/80-biotin conjugated. Secondary staining was made using APC-conjugatetd-F(ab')2 goat-anti-human-IgG-Fc and PE-Cy7-Streptavidin. Nuclear staining was made using Hoechst 33342 1:10000 (Fuka, Switzerland). All incubations were made in room temperature. All antibodies were diluted in PB/A. After each step the wells were rinsed for three times. Images acquisition and analysis were made using either Olympus IX70 fluorescent microscope or Olympus Fluoview 1000 Laser Scanning Microscopy system equipped with a 40x/1.3 oil immersion objective.

Statistical analysis

Results obtained from groups of 3 to 12 mice were expressed as the mean ±SD. Mann-Whitney U-test analysis was used. Differences in the number of bacterial loads in the lungs were analyzed by using two-tailed Student’s t test. Differences in cells number between two groups were analyzed by using two-tailed Student’s t test or by one-way ANOVA. Values for p<0.05 were considered to be statistically significant.

Results

Survival of Ncr1+/-gfp, Ncr1+/+gfp and Ncr1+/+ mice following intranasal challenge with S. pneumoniae

We first aimed to evaluate the influence of deficiency of the activating natural killer cell receptor gene, Ncr1, on the survival of mice after infection with S. pneumoniae. C57BL/6 Ncr1+/-gfp, Ncr1+/+gfp and Ncr1+/+ WT littermate mice were infected with high lethal dose (4×10^5) of S. pneumoniae serotype 3 strain WU2 and survival was monitored for 8 days (Fig. 1). The survival rate of Ncr1+/-gfp was significantly lower than Ncr1+/+ mice (p<0.05). The Ncr1+/-gfp shows the same pattern of survival as Ncr1+/+ due to small number of mice. Challenging the mice with l×10^9 or
With 5 x 10^7 of *S. pneumoniae* resulted in similar survival rate for all 3 mice groups (data not shown). These data suggest that the NCR1 receptor plays an important role in the survival of mice only following challenge with high dose of *S. pneumoniae*.

**Bacterial load in lungs of Ncr1<sup>+/−/gfp</sup>, Ncr1<sup>+/+gfp</sup> and Ncr1<sup>+/−</sup> mice following intranasal challenge with *S. pneumoniae***

We next assessed the importance of the NCR1 receptor in the clearance of *S. pneumoniae* from the lungs at the early stages of infection. We compared the bacterial load in this organ of Ncr1<sup>+/−/gfp</sup>, Ncr1<sup>+/+gfp</sup> and Ncr1<sup>+/−</sup> mice, at 3, 6 and 24 h after intranasal inoculation with 5 x 10^7 *S. pneumoniae* strain WU2 (Fig. 2). In accordance with the survival results, 24 h after infection, the bacterial load in the lungs of Ncr1<sup>+/−/gfp</sup> mice was comparable to the levels observed in the lungs of Ncr1<sup>+/−</sup> and Ncr1<sup>+/+</sup> mice (Fig. 2C). In sharp contrast, during the first 6 hours of infection, the bacterial load in the lungs of Ncr1<sup>+/−/gfp</sup> mice was significantly higher than in Ncr1<sup>+/+</sup> and Ncr1<sup>+/−</sup> mice (Fig. 2A, 2B, p<0.01). The number of bacteria in the lungs of Ncr1<sup>+/−</sup> and Ncr1<sup>+/+</sup> mice was similar during all the above-described time points (Fig. 2A–C).

This is in accordance with the observation that NK cells from Ncr1<sup>+/−/gfp</sup> mice are fully competent [25]. Overall, bacterial load numbers in the lungs of Ncr1<sup>+/−</sup> and Ncr1<sup>+/+</sup> mice increased 5.7 fold in average between 3 to 24 h post challenge, while for Ncr1<sup>+/−/gfp</sup> mice the average fold increase was only 1.7. These data suggest that the NCR1 receptor plays an important role in mediating the clearance of *S. pneumoniae* in the lungs early after infection.

**Contribution of NCR1 to NK cells activation by *S. pneumoniae***

To further investigate the NCR1-dependent activation of NK cells following *S. pneumoniae* infection, we studied activation of NK cells and IFNγ production in the lungs 3 h following intranasal challenge with the bacteria. Enhancement of CD107a expression on NK cell membranes represents the fusion of secretory granules with the plasma membrane and not de-novo protein synthesis. Therefore, CD107a enhancement is a marker for NK cell activation at early stages reflecting cytokine secretion and release of the content of lytic granules [29]. In vivo, 3 h following *S. pneumoniae* challenge, membrane-associated expression of CD107a by lung NK cells appeared in both Ncr1<sup>+/−/gfp</sup> and Ncr1<sup>+/+gfp</sup> mice compared to uninfected mice (p<0.05 for both, Fig. 3A). Yet, this increase was significantly higher in lung NK cells from NCR1-expressing mice (Ncr1<sup>+/−/gfp</sup>) compared to NCR1-deficient mice (Ncr1<sup>+/+gfp</sup>) (52% vs. 28%, p<0.01, Fig. 3A). NK cell number in lungs of infected mice didn’t differ between Ncr1<sup>+/−/gfp</sup> and Ncr1<sup>+/+gfp</sup> mice (data not shown).

To assay local IFNγ production in vivo, total RNA was extracted from lungs of naive and 3 h-infected Ncr1<sup>+/−/gfp</sup> and Ncr1<sup>+/+gfp</sup> mice. IFNγ mRNA was quantified by real-time PCR. In accordance with the CD107a levels (Fig. 3B), IFNγ transcript in 3 h-infected lungs increased considerably compared to non-infected lungs for both Ncr1<sup>+/−/gfp</sup> and Ncr1<sup>+/+gfp</sup> mice (Fig. 3B). Yet, the fold increment of IFNγ was significantly higher in NCR1-expressing mice compared to NCR1-deficient mice (Fig. 3B, p<0.01). Challenge with *S. pneumoniae* induces a local production of
proinflammatory cytokines [2,26,30]. Therefore we investigated whether the observed differences in 3 h following challenge bacterial load could correlate with differential induction of other proinflammatory cytokines. TNFα and IL-6 mRNA levels were quantified. Similarly to the IFNγ, TNFα and IL-6 transcripts in the lung were greatly induced 3 h following challenge (Fig. 3C, D). However, the opposite trend to IFNγ results was observed when comparing between NCR1-expressing and -deficient mice; fold increase was significantly higher in Ncr1^gfp/gfp mice (Fig. 3C, D, p < 0.001). This data suggest that the NCR1 receptor plays an important role in activation of NK cells during early stages of S. pneumoniae infection by activating IFNγ production and enhancing CD107a expression.

Bacterial load in the lungs of NK-depleted mice following intranasal challenge with S. pneumoniae

To validate the role of NK cells in mediating early clearance of S. pneumoniae in the lungs, we depleted NK cells using anti-asialo-GM1 (one injection of 50 μl per mouse). More than 95% of NK cells were removed following depletion as tested 24 h later (Fig. 4B, shown for both Ncr1^gfp/gfp and Ncr1^+/+ groups). Twenty-four hours following NK cell depletion or mock treatment, we challenged Ncr1^gfp/gfp and Ncr1^+/+ mice with 5 x 10^7 CFU S. pneumoniae strain WU2 and compared the bacterial load in the lungs 3 h later (Fig. 4A). In both mice groups, depletion of NK cells 24 h prior to infection resulted in a significant increase of bacterial load 3 h following inoculation compared to the mock-treated mice (3.8 and 2.57 fold increase, p < 0.01 and p < 0.05 for Ncr1^+/+ and Ncr1^gfp/gfp, respectively). These results point to the involvement of NK cells in early clearance of S. pneumoniae challenge. As expected (Fig. 4A), 3 h after inoculation, bacterial load in mock-treated Ncr1^gfp/gfp was significantly higher compared to mock-treated Ncr1^+/+ mice (4.4 fold increase, p < 0.01). Increased bacterial load was also observed between NK-depleted Ncr1^gfp/gfp and NK-depleted Ncr1^+/+ mice, yet to a lower extent (2.9 fold, p < 0.05). This indicates that in addition to NK cells contribution to early clearance upon

Figure 3. NK cells activation and cytokines mRNA levels in lungs following intranasal challenge with S. pneumoniae. Lungs were harvested from Ncr1^+/gfp (Het group) and Ncr1^gfp/gfp (KO group) C57BL/6 mice 3h after intranasal inoculation with 5 x 10^7 CFU of S. pneumoniae strain WU2, and from non-infected mice. (A) NK cells were gated as CD45+NK1.1+ and analyzed for the expression of membrane-associated CD107a by flow cytometry. The bar graphs show the fraction of CD107a positive NK cells (average of 3 mice per group, ±SD). ** p < 0.01 compared to Het group in 3h-infected mice (ANOVA test). Total RNA was extracted from naive lungs and from 3h-infected lungs of Ncr1^+/gfp (Het) and Ncr1^gfp/gfp (KO). Infection was performed with 5 x 10^7 CFU of S. pneumoniae. Level of cytokines mRNA of IFNγ (n = 4), TNFα (n = 4) and IL-6 (n = 3) were analyzed by RT-PCR and calibrated to mRNA level of GAPDH. Results are from one representative experiment of two. *** p < 0.001, ** p < 0.01, compared with the Het group +SD (ANOVA test).

doi:10.1371/journal.pone.0023472.g003
NCR1 Role in Streptococcus pneumoniae Clearance

Bacterial load in the macrophage/DC-depleted lungs following intranasal challenge with S. pneumoniae

To assay in vivo the contribution of lung phagocytes to S. pneumoniae clearance at early stages following infection, the extent of bacterial load in Ncr1<sup>−/−</sup>/gfp, Ncr1<sup>+/+</sup>/gfp and mice ablated from lung CD11c<sup>+</sup> mononuclear phagocytes was compared. We took advantage of CD11c:DTx transgenic mice that allow specific depletion of CD11c<sup>high</sup> cells [27]. The intratracheal DTx installation into CD11c:DTx transgenic mice results in the specific local ablation of CD11c<sup>+</sup> lung mononuclear phagocytes, including macrophages and DC [27] (Fig. 5B). Mice were inoculated with 5 × 10<sup>7</sup> S. pneumoniae strain WU2 and lungs were harvested 3 h after challenge (Fig. 5A). The bacterial load in the lungs of DTx-treated CD11c<sup>+</sup>: DTR mice was significantly higher than the load in lungs of mock-treated CD11c: DTR mice (p < 0.01), and was similar to that of mock-treated Ncr1<sup>−/−</sup>/gfp mice. DTx-treated and mock-treated Ncr1<sup>−/−</sup>/gfp mice had low bacterial load similar to that of mock-treated CD11c<sup>+</sup>:DTR mice. Fold increase between mock-treated and DTx-treated CD11c<sup>+</sup>: DTR mice was similar to that between mock-treated Ncr1<sup>−/−</sup>/gfp and Ncr1<sup>+/+</sup>/gfp (6 fold increase, p < 0.01). Thus, lung CD11c<sup>+</sup> mononuclear phagocytes play a major role in the clearance of S. pneumoniae inoculated intranasally.

NCR1 involvement in NK cells activation following infection of bone marrow-derived macrophages and bone marrow-derived DC with S. pneumoniae

We studied the expression of ligands to NCR1 by BMMQ and BMDC. We stained BMMQ and BMDC from 6-day cultures with mNCR1-Ig and mNKG2D-Ig fusion proteins. Human protein LIR1-Ig served as negative control for the staining. F4/80<sup>−</sup>CD115<sup>+</sup> BMMQ and CD11c<sup>+</sup> BMDC were stained positively with the mNCR1-Ig (Fig. 6A–D) and mNKG2D-Ig (Fig. 6C–D). The positive staining was specific, as no staining was observed with the control fusion protein LIR1-Ig (Fig. 6A–B). These results imply that macrophages and DC are able to express ligands to NCR1 and could be involved in the innate immune response of NCR1<sup>+/+</sup> mice to S. pneumoniae challenge. We then investigated whether S. pneumoniae-infected BMMQ or BMDC could activate NK cells via the NCR1 receptor; we studied IFNγ levels in the co-cultures of BMMQ/BMDC from WT mice with NK cells from NCR1-deficient isolated NK cells. In brief, WT BMMQ/BMDC were stained positively for the control fusion protein LIR1-Ig (Fig. 6A–B). We then investigated whether S. pneumoniae-infected BMMQ or BMDC could activate NK cells via the NCR1 receptor; we studied IFNγ levels in the co-cultures of Ncr1<sup>−/−</sup>/gfp mice treated intraperitoneally either with 50 μl of anti asialo GM1 (WT-anti NK and KO-anti NK) or with 50 μl of PBS (mock treatment, WT-mock and KO-mock). 24 h after anti asialo GM1 or mock treatment, all mice were challenged with 5 × 10<sup>7</sup> CFU of S. pneumoniae strain WU2 and lungs were harvested 3 h after challenge. (A) CFU of S. pneumoniae in lungs, 3 h after bacterial challenge (n = 3 to 5 per group). Results are presented as box plot of CFU. ** p < 0.01 compared with WT-mock; * p < 0.05, compared with WT-anti NK, ± SD (ANOVA test). (B) Flow cytometry analysis of spleen cells from depleted and non-depleted mice for CD3<sup>−</sup> NK cells expressing and NCR1-deficient isolated NK cells. In brief, WT BMMQ/BMDC from WT mice with NK cells from NCR1- and/or NKG2D-deficient isolated NK cells, e.g. phagocytes, might be better conditioned to cope with S. pneumoniae infection at early stages.

Figure 4. Bacterial load in lungs following in vivo depletion of NK cells. Ncr1<sup>−/−</sup> and Ncr1<sup>+/+</sup>/gfp C57BL/6 mice treated intraperitoneally either with 50 μl of anti asialo GM1 (WT-anti NK and KO-anti NK) or with 50 μl of PBS (mock treatment, WT-mock and KO-mock). 24 h after anti asialo GM1 or mock treatment, all mice were challenged with 5 × 10<sup>7</sup> CFU of S. pneumoniae strain WU2 and lungs were harvested 3 h after challenge. (A) CFU of S. pneumoniae in lungs, 3 h after bacterial challenge (n = 3 to 5 per group). Results are presented as box plot of CFU. ** p < 0.01 compared with WT-mock; * p < 0.05, compared with WT-anti NK, ± SD (ANOVA test). (B) Flow cytometry analysis of spleen cells from depleted and non-depleted mice for CD3<sup>−</sup> NK cells expressing and NCR1-deficient isolated NK cells. In brief, WT BMMQ/BMDC from WT mice with NK cells from NCR1- and/or NKG2D-deficient isolated NK cells, e.g. phagocytes, might be better conditioned to cope with S. pneumoniae infection at early stages.

challenge, other cells in Ncr1<sup>+/+</sup> mice, e.g. phagocytes, might be better conditioned to cope with S. pneumoniae infection at early stages.
levels in co-cultures of Ncr1+/+ derived NK cells with WT BMMQ/BMDC (p<0.01, Fig. 6E-F ‘BMMQ+SP’, ‘BMDC+SP’). To summarize, these results indicate a significance of NCR1 for NK cell activation following the cross-talk with S. pneumoniae-infected BMMQ and BMDC.

NCR1 ligand expression on macrophages and DC

To further investigate NK cell-expressed NCR1 involvement in S. pneumoniae, we first tested whether NCR1 ligands are expressed by the bacteria. A recent study suggested that NKp44 on human NK cells may be involved in the direct recognition of bacterial pathogens [31]. To investigate whether NCR1 could interact directly with the bacterium, we stained S. pneumoniae with soluble mNCR1-Ig and mNKG2D-Ig fusion proteins. Both proteins did not stain S. pneumoniae indicating that neither NKG2D- nor NCR1-ligands are expressed on the bacteria (Fig. 7A). These results imply that the involvement of NCR1 in S. pneumoniae infection could be indirectly mediated via the interaction of NK cells with NCR1 ligand-expressing macrophages or DC. The importance of the lung macrophage response during infection with S. pneumoniae implied from Fig. 5, and knowing that macrophages comprise 98% of lung lavage (BAL) leukocytes cells, led us to investigate the phenotypic characterization of macrophages in lung BAL and whether the presence of the NCR1 receptor in mice plays a role in this phenotype. Cells from lung BAL, BM and spleen were harvested from Ncr1+/+, Ncr1+/Δp and Ncr1Δp/Δp naive C57BL/6 mice and stained with mNCR1-Ig and mNKG2D-Ig for detection of their ligands. Human fusion proteins LIR1-Ig and CD99-Ig served as negative staining controls. Staining with mNCR1-Ig of lung BAL showed two different sub-populations: F4/80+CD115+NCR1-ligandhigh and F4/80+CD115+NCR1-liganddull (Fig. 7B). Similarly, staining of BM F4/80+CD115+ macrophages also revealed the NCR1-ligandhigh and NCR1-liganddull sub-populations (Fig. 7C). The NCR1-ligandhigh macrophage sub-population was significantly higher in BAL and BM of Ncr1+/+ compared to Ncr1Δp/Δp (2 fold, P<0.001, Fig. 7E); likewise, it was higher in lung and BM of Ncr1+/+ compared to Ncr1Δp/Δp (1.4 fold, P<0.05, Fig. 7F). The results in panels 7E-F represent normalized values as the actual fraction of NCR1-ligand high macrophage sub-population varied between experiments and mice and ranged between 5% to 25% for Ncr1+/+ mice. Yet, it was significantly lower in lung BAL and BM of Ncr1Δp/Δp mice (Fig. 7E-F). Notably, the NCR1-ligandhigh BAL macrophages had higher expression of CD115 and residual staining with LIR1-Ig that might point to high expression of Fc receptors though the blocking of those receptors during staining (see Methods). The staining of macrophages from spleen did not reveal the NCR1-ligandhigh sub-population in any of the three mice types (Fig. 7D). We observed this direct correlation between NCR1 presence and NCR1-ligandhigh macrophage sub-population in additional genetic backgrounds of

Figure 5. Bacterial load in lungs following in vivo ablation of lung CD11c+ cells. CD11c-DTR mice (C57BL/6 genetic background) were treated intratracheally with DTx (100 ng/gr, DTR-tox group), CD11c-DTR littermates treated intratracheally with PBS (‘DTR-mock’) served as control. Ncr1+/gfp C57BL/6 mice treated intratracheally with DTx (‘Het-tox’) or with PBS (‘Het-mock’) and Ncr1Δp/gfp C57BL/6 mice treated with PBS (‘KO-mock’) served as additional control groups. One day after DTx or mock treatment, all mice were challenged with 5×10^7 CFU of S. pneumoniae strain WU2 and lungs were harvested 3 h after challenge. (A) CFU of S. pneumoniae in lungs, 3 h after bacterial challenge (n = 3 to 5 per group). Results are presented as box plot of CFU. *** p<0.001 compared with DTR-mock; ** p<0.01, compared with Het-mock, ± SD (ANOVA test). (B) Flow cytometry analysis of lung cells for CD11c and CD11b expression, 3 h after bacterial challenge (representative mouse). Numbers indicate percentage of gated cells from total white blood cells. Results are from one representative experiment of two.
mice (129/Sv and BALB/c, data not shown). This phenomenon was not observed for the staining with mNKG2D-Ig. Macrophages from lung, BM and spleen did not manifest heterogeneous phenotype for NKG2D-ligands expression which did not differ between Ncr1<sup>+</sup>/<sup>+</sup>, Ncr1<sup>+</sup>/gfp and Ncr1<sup>gfp/gfp</sup> (Fig. 7B, C and D). In addition, we studied CD11c<sup>+</sup> DC in both BM and spleen. The NCR1-ligandhigh DC sub-population in BM and spleen (data not shown) was low to negligible and did not differ significantly between the mice groups (1.4% vs. 3.3%, Fig. 7G). These results show, for the first time, a unique macrophage sub-population, located in BM and lung, which is characterized by high expression of ligands for NCR1.

**NCR1 contribution to activation of alveolar macrophages**

We further investigated the NCR1 involvement in the activation of naive alveolar macrophages. Macrophage activation state can be determined by (i) up regulation of activation molecules and by (ii) phagocytic capacity. BALF cells were taken from Ncr1<sup>+/</sup><sup>+</sup> or Ncr1<sup>gfp/gfp</sup> C57BL/6 mice lungs and the alveolar macrophages were gated by staining with antibodies to CD45 and F4/80 and analyzed for the expression of CD11b activation markers. CD45<sup>+</sup>F4/80<sup>+</sup> cells consisted the majority of BALF cells for both Ncr1<sup>+/</sup><sup>+</sup> and Ncr1<sup>gfp/gfp</sup> mice types (97% and 96% respectively, Fig. 8A). Higher fraction of CD11b<sup>+</sup> alveolar macrophages was observed in Ncr1<sup>+/</sup><sup>+</sup> compared to Ncr1<sup>gfp/gfp</sup> mice (6% vs. 2.5%, Fig. 8B). Macrophages number in the BALF was similar between the Ncr1<sup>+/</sup><sup>+</sup>/gfp and Ncr1<sup>gfp/gfp</sup> mice group as determined by cell counting (403,333 vs. 487,667 per lung; data not shown). Phagocytic capacity was evaluated by the incubation of CFDA-stained bacteria with freshly isolated BALF cells and direct visualization with either conventional fluorescent or confocal microscope. We observed that significantly higher numbers of BALF cells from Ncr1<sup>+/</sup><sup>+</sup> mice were associated with CFDA-stained bacteria as compared to BALF cells from Ncr1<sup>gfp/gfp</sup> mice (2.2 fold, p<0.05, Fig. 8C). Confocal-based analysis of WT BALF cells showed that F4/80<sup>+</sup> cells were either NCR1-ligand high or NCR1-ligand none (Fig. 8D, representative image). The ratio between the two populations (0.3 vs. 1, p<0.01, Fig. 8E) was similar to the results obtained by flow cytometry (Fig. 7E). The NCR1-ligand dull observed by flow cytometry parallel to the NCR1-ligand none observed by confocal microscopy. Importantly, there was a significantly higher proportion of phagocytosed CFDA-stained bacteria in F4/80<sup>+</sup>/NCR1-ligandhigh as compared to F4/80<sup>+</sup>/NCR1-ligandnone (2.6 fold, p<0.05, Fig. 8E). These results imply that high NCR1 ligand expression is correlated with enhanced phagocytic activity of macrophages and further support our assumption that NCR1 expression by NK cells is associated with priming of alveolar macrophages.

![Figure 6. NCR1 involvement in NK cells activation following infection of BMMQ/ BMDC with S. pneumoniae.](image-url)
Discussion

NK cells constitute a key frontline defense against a range of viruses and bacteria. Different studies have demonstrated the importance of NK cells in controlling bacterial infections in mice, in particular with Shigella flexneri [32]. Human NKp46 is a key NK activating receptor that was reported to be involved in the response against Mycobacterium tuberculosis [14]. In the current study, we investigated the involvement of the mouse NCR1 (murine NKp46) in the innate immune response to Streptococcus pneumoniae infection. We showed the following: (i) NCR1 receptor plays a role in S. pneumoniae induced mortality in mice only when high challenge doses are applied (Fig. 1); yet NCR1 plays an imperative role in reducing S. pneumoniae bacterial load in the lungs at early stages after infection. (Fig. 2); (ii) In vivo, NCR1 expression activates IFNγ production in the lung and enhances the expression on lung NK of the membrane-associated CD107a early after infection with S. pneumoniae (Fig. 3); (iii) In vivo, NCR1 expression activates the function of the membrane-associated CD107a early after infection with S. pneumoniae (Fig. 3); (iv) In vitro, NCR1 is important for NK cell activation during the interaction with S. pneumoniae-infected BMMQ and BMDC (Fig. 6); (v) NCR1 presence in mice is directly correlated with the magnitude of NCR1-ligand high macrophage sub-population, located in lungs and BM (Fig. 7); and (vi) NCR1 presence in
mice is correlated with higher activation state and phagocytic capacity of alveolar macrophages (Fig. 8). Taken together, these findings suggest that NCR1 plays an important role in mediating the killing of \textit{S. pneumoniae} in the lungs during the initial stages of infection.

We studied survival rate following \textit{S. pneumoniae} infection of \textit{Ncr1}^{+/+} and \textit{Ncr1}^{gfp/gfp} naive C57BL/6 mice. Deficiency of the key NK activating receptor, NCR1, resulted in lower survival rate of mice infected with high lethal dose. Lower lethal doses did not induce differences in survival rate; yet, during the first 6 hours of infection, a significantly reduced bacterial load was observed in the lungs of \textit{Ncr1}^{+/+} and \textit{Ncr1}^{gfp/gfp} mice compared to \textit{Ncr1}^{gfp/gfp} mice.

In accordance with the survival results following lower lethal doses challenge, 24 h after inoculation, bacterial load in the lungs of \textit{Ncr1}^{+/+} mice was comparable to the levels observed in the lungs of \textit{Ncr1}^{gfp/gfp} mice. These results suggest that the NCR1 receptor plays an important role in mediating the clearance of \textit{S. pneumoniae} in the lungs only during the initial stages of infection. In the late stages of infection, the intense inflammatory response that evolves as a result of the large number of bacteria in the lungs may conceal the NCR1 receptor effect. Alternatively, since inflammation contribute to \textit{S. pneumoniae} proliferation in lungs [1,33] and NK cell activity and IFN$\gamma$ could enhance inflammation [11], the lesser contribution of NCR1-deficient NK cells to inflammation...
could oppose the picture in the later stages of the infection. This is in accordance with the report on the detrimental effect of NK cells in *S. pneumoniae* infection [26]. The plausible divergent contribution of NCR1-mediated IFNγ production at early stages (clearance of *S. pneumoniae*) and late stages (enhancement of inflammation) could also explain the contradictory reports on the role of IFNγ in response to *S. pneumoniae* [10-12].

No differences in the bacterial load and survival between the *Ncr1*+/+ and the *Ncr1*−/− mice could be observed. This was in accordance with a previous study, which demonstrated that NK cells from *Ncr1*+/+ mice are fully functional while NK cells from *Ncr1*−/− mice manifest reduced activity following influenza infection and tumor development [25]. The reduced bacterial load in the *Ncr1*+/+ and *Ncr1*−/− mice suggested that the NK cell were activated via NCR1 following infection. To evaluate the extent of NK cell activation we investigated the expression of CD107a and IFNγ production following bacterial challenge. CD107a expression on NK cell membrane represents the fusion of secretory granules with the plasma membrane and characterize early stages of NK cell activation [29]. Three hours following inoculation of mice enhancement of CD107a expression on NCR1-expressing NK cells was higher than that found on NCR1-deficient NK cells (Fig. 3A). In accordance with the CD107a levels, 3h following infection the IFNγ transcript level in the lungs was significantly higher in *Ncr1*+/+ mice compared to *Ncr1*−/− infected mice (Fig. 3B). In contrast, 3h following infection, TNFα and IL-6 transcripts in the lungs of *Ncr1*−/− mice were significantly higher compared to the transcripts of these cytokines in *Ncr1*+/+ mice (Fig. 3C, D). This could be explained by the significantly higher bacterial load in *Ncr1*−/− mice 3h after infection (Fig. 2A). Indeed, we did not observe this result for TNFα and IL-6 in the in vitro assays (data not shown) since *S. pneumoniae* was removed after 1.5 hrs and antibiotics were added.

A recent study suggested that NKP44 may be involved in the direct recognition of bacteria [31]. To investigate the possibility of direct recognition of the bacterium by NCR1 receptor, we stained *S. pneumoniae* with soluble mNCR1-Ig and mNKG2D-Ig fusion proteins (Fig. 7A). Our results suggest the absence of NCR1 ligands on *S. pneumoniae* and thus preclude NCR1-mediated direct recognition of the bacteria by NK cells. However, these results suggest that the enhanced *S. pneumoniae* clearance may be mediated by innate immune accessory cells. The first immune cells to encounter the bacteria are the lung macrophages and DC [2]. To investigate macrophages and DC involvement in the *S. pneumoniae* clearance at early stages following infection we compared the bacterial load level of *S. pneumoniae* in *Ncr1*−/−, *Ncr1*+/+ mice and in DTR:CD11c transgenic mice in which the lung mononuclear phagocytes including alveolar macrophages were ablated (Fig. 5). Our findings suggest that (i) the presence of macrophages and dendritic cells in the lungs is imperative for early clearance *S. pneumoniae*, (ii) the lack of lung mononuclear phagocytes and the absence of NK-expressed *Ncr1* result in the same bacterial load level. Our findings are supported by Sun et al. who demonstrated that the resident alveolar macrophages bind *S. pneumoniae* within 4 hours and their depletion with liposomal clodronate led to enhanced bacterial outgrowth in both lung tissues and alveoli [34]. We then searched for the existence of NCR1 receptor ligands on BMMQ and BMDC. Using the mNCR-Ig fusion protein we have found that BMMQ and BMDC express ligands for NCR1 receptor as well as ligands for mNKG2D receptor (Fig. 6A-D). Next we investigated whether the cross talk between NK cells and *S. pneumoniae* infected macrophages and DC is mediated through NCR1 by assessing the extent of IFNγ production. Supernatants of *S. pneumoniae* infected BMMQ and BMDC co-cultured with NK cells showed enhanced IFNγ levels in an NK-NCR1-dependent manner (Fig. 6E-F). We further demonstrated that upon challenge with *S. pneumoniae*, BMDC up-regulate their co stimulatory molecules (data not shown). This up-regulation was previously demonstrated to be useful for their interaction with NK cells which then improves the immune response against bacterial infections [35,36]. Together these results imply the importance of the cross talk between DC, macrophages and NK cells following infection with *S. pneumoniae* in *vivo*. According to Ferlazzo et al., the biological relevance of NK cell activation mediated by DCs during bacterial infections resides mainly in the secretion of IFNγ [36]. The higher secretion of IFNγ found in our study in *vivo* and in vitro in the *Ncr1*+/+ mice may also result also from the improved NK-DC/macrophage cross talk which in turn augments bacterial clearance by the DC/macrophages. Our results suggest that NK-expressed NCR1 is mediating a direct cross talk between NK cells and macrophages/D C which is contributing to *S. pneumoniae* clearance by macrophages and DC. Reciprocal activating interaction between NK cells and DC was already reported for several bacterial pathogens [35-37]. Yet, our results extend it to NK-macrophages interaction for *S. pneumoniae* infection and point to the involvement of NK-expressed NCR1 in this cross talk. To further assess the role of NK cells and NCR1 in resistance to *S. pneumoniae* infection, NK cells were depleted from *Ncr1*−/− and *Ncr1*+/+ mice using anti-asialo GM1 and then challenged with *S. pneumoniae* (Fig. 4). In both strains, depletion of NK cells prior to infection resulted in a significant increased bacterial load 3h following inoculation in comparison to the mock-treated mice. Surprisingly, the bacterial load level in the lungs of NK-depleted *Ncr1*−/− mice was significantly higher than in the NK-depleted *Ncr1*+/+ mice. These results suggest that in addition to NK cells contribution to the early clearance of *S. pneumoniae* in the first 3 h, *Ncr1*+/+ mice are better conditioned to clear *S. pneumoniae*. Indeed, alveolar macrophages from NCR1-positive mice were more potent than alveolar macrophages from NCR1-deficient mice prior to any *S. pneumoniae* infection (Fig. 8). This conditioning may occur in the lung prior to infection and could indicate NCR1-mediated pathogen-independent priming of lung macrophages by NK cells. Alternatively, it could be the result of NCR1-mediated cross talk between NK cells and lung macrophages in response to commensal pathogens. Recently published studies [38,39] described that activation of NK cells prior to infection resulted in macrophage priming, which improved their microbial clearance abilities. We therefore investigated the expression of NCR1 ligands on macrophages and DCs. The results show, for the first time, a unique macrophage sub-population, located in the BM and in the lungs, which is characterized by a high expression of the NCR1 ligand (Fig. 7, B-F). This population was significantly higher in *Ncr1*+/+ compared to *Ncr1*−/− mice. Recently it was reported [35-37] that NK cell-mediated NCR1-dependent priming of lung macrophages by NK cells results from the improved NK-DC/macrophage cross talk which in turn augments bacterial clearance by the DC/macrophages. Our results suggest that NK-expressed NCR1 is mediating a direct cross talk between NK cells and macrophages/DC which is contributing to *S. pneumoniae* clearance by macrophages and DC. Reciprocal activating interaction between NK cells and DC was already reported for several bacterial pathogens [35-37]. Yet, our results extend it to NK-macrophages interaction for *S. pneumoniae* infection and point to the involvement of NK-expressed NCR1 in this cross talk. To further assess the role of NK cells and NCR1 in resistance to *S. pneumoniae* infection, NK cells were depleted from *Ncr1*−/− and *Ncr1*+/+ mice using anti-asialo GM1 and then challenged with *S. pneumoniae* (Fig. 4). In both strains, depletion of NK cells prior to infection resulted in a significant increased bacterial load 3h following inoculation in comparison to the mock-treated mice. Surprisingly, the bacterial load level in the lungs of NK-depleted *Ncr1*−/− mice was significantly higher than in the NK-depleted *Ncr1*+/+ mice. These results suggest that in addition to NK cells contribution to the early clearance of *S. pneumoniae* in the first 3 h, *Ncr1*+/+ mice are better conditioned to clear *S. pneumoniae*. Indeed, alveolar macrophages from NCR1-positive mice were more potent than alveolar macrophages from NCR1-deficient mice prior to any *S. pneumoniae* infection (Fig. 8). This conditioning may occur in the lung prior to infection and could indicate NCR1-mediated pathogen-independent priming of lung macrophages by NK cells. Alternatively, it could be the result of NCR1-mediated cross talk between NK cells and lung macrophages in response to commensal pathogens. Recently published studies [38,39] described that activation of NK cells prior to infection resulted in macrophage priming, which improved their microbial clearance abilities. We therefore investigated the expression of NCR1 ligands on macrophages and DCs. The results show, for the first time, a unique macrophage sub-population, located in the BM and in the lungs, which is characterized by a high expression of the NCR1 ligand (Fig. 7, B-F). This population was significantly higher in macrophages from *Ncr1*+/+ and *Ncr1*+/+ compared to *Ncr1*−/− mice. We did not observe this correlation for DC (Fig. 7G). This NCR1-ligand expression sub-population was responsible for the better phagocytic activity of the NCR1-expressing mice as it manifested efficient phagocytosis compared to the NCR1-ligand expressing macrophages (Fig. 8). Therefore, this NCR1-ligand expression sub-population could be involved in the conditioning/priming and/or activation of the macrophages by NCR1-expressing NK cells in a cell-to-cell contact interaction prior to infection and during the early phase of infection. To summarize, we showed the involvement of NK cells and NK-expressed NCR1 in the early clearance of *S. pneumoniae* and the contribution of NCR1 to the cross talk of NK and macrophages/DC. The plausible phenomenon of NK-NCR1-mediated pre-conditioning of lung phagocytes and the NCR1-dependent condition of lung phagocytes and the NCR1-dependent
sub-population of NCR1-ligated macrophages should be further explored.

Acknowledgments

Shirin Elhaik-Goldman is an ISEF scholar.

References

1. Dagan R, Greub G, Jacobs N (2004) Pneumococcal infections. Friggin R CJ, Demmler GJ, Kaplan S, editor. Philadelphia: Saunders. pp 1204–1238.
2. Kadioglu A, Andrew PW (2004) The innate immune response to pneumococcal lung infections: the untold story. Trends Immunol 25(5): 143–149.
3. McCoil TL, Weiser JN (2006) Limited role of antibody in clearance of Streptococcus pneumoniae in a murine model of colonization. Infect Immun 72(10): 5807–5813.
4. Zhang Z, Clarke TB, Weiser JN (2009) Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. J Clin Invest 119(7): 1809–1909.
5. Bogaert D, Weinberger D, Thompson C, Lipshitz M, Malley R (2009) Impaired innate and adaptive immunity to Streptococcus pneumoniae and its effect on colonization in an infant mouse model. Infect Immun 77(4): 1615–1622.
6. Bogaert D, De Groot R, Hermans PW (2004) Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis 4(3): 144–154.
7. van der Poll T, Keogh CV, Buurman WA, Lowry SF (1997) Passive immunization against tumor necrosis factor-alpha impairs host defense during pneumococcal pneumonia in mice. Am J Respir Crit Care Med 155(2): 603–605.
8. van der Poll T, Keogh CV, Guirao X, Buurman WA, Kopf M, et al. (1997) Interferon-γ gene-deficient mice show impaired defense against pneumococcal pneumonia. J Infect Dis 176(2): 439–444.
9. Kafka D, Ling E, Feldman G, Benharroch D, Voronov E, et al. (2008) Contribution of IL-1 to resistance to Streptococcus pneumoniae infection. Int Immunol 20(9): 1139–1146.
10. Rubin JB, Pomeroy C (1997) Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. Infect Immun 65(7): 2975–2979.
11. Rijnveld AW, Lauter FN, Schultz MJ, Florquin S, Te Velde AA, et al. (2002) The role of interferon-gamma in murine pneumococcal pneumonia. J Infect Dis 185(3): 91–97.
12. Schultz MJ, Rijnveld AW, Speelman P, van Deventer SJ, van der Poll T (2001) Endogenous interferon-gamma impairs bacterial clearance from lungs during Pseudomonas aeruginosa pneumonia. Eur Cytokine Netw 12(1): 39–44.
13. Yokoyama WM, Kim S, French AR (2004) The dynamic life of natural killer cells. Annu Rev Immunol 22: 405–429.
14. Garg A, Barnes PF, Porgador A, Roy S, Wu S, et al. (2006) Vimentin expressed by B-cell leukemia (BCL1) cells as a target for NK cell-mediated immunotherapy. Bone Marrow Transplant 38(11 suppl 1): 1–9.
15. Warfield KL, Perkins JG, Swenson DL, Deal EM, Bosio CM, et al. (2004) Role of natural killer cells in innate protection against lethal ebola virus infection. J Exp Med 195(1): 1–13.
16. Weiss L, Reich S, Mandelboim O, Slavin S (2004) Murine B-cell leukemia (BCL1) cells as a target for NK cell-mediated immunotherapy. Bone Marrow Transplant 38(11 suppl 1): 1–9.
17. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 203(9): 2680–2689.
18. Gazit R, Gruda R, Elboim M, Arnon TI, Katz G, et al. (2006) Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. Nat Immunol 7(5): 517–523.
19. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 409(6825): 1055–1060.
20. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol 31(9): 2680–2689.
21. Mandelboim O, Lieberman N, Lev M, Paul L, Arnon TI, et al. (2001) Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. Nature 409(6825): 1055–1060.
22. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. Eur J Immunol 31(9): 2680–2689.
23. Gazit R, Gruda R, Elboim M, Arnon TI, Katz G, et al. (2006) Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. Nat Immunol 7(5): 517–523.
24. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
25. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
26. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
27. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
28. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
29. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
30. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
31. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
32. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
33. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
34. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.
35. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, et al. (2001) Recognition of viral hemagglutinins by NKp44 but not by NKp30. J Exp Med 31(9): 2680–2689.