Alveolar macrophage metabolic programming via a C-type lectin receptor protects against lipo-toxicity and cell death

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Alveolar macrophages (AM) hold lung homeostasis intact. In addition to the defense against inhaled pathogens and deleterious inflammation, AM also maintain pulmonary surfactant homeostasis, a vital lung function that prevents pulmonary alveolar proteinosis. Signals transmitted between AM and pneumocytes of the pulmonary niche coordinate these specialized functions. However, the mechanisms that guide them metabolichomeostasis of AM remain largely elusive. We show that the NK cell-associated receptor, NKR-P1B, is expressed by AM and is essential for metabolic programming. Nkrp1b−/− mice are vulnerable to pneumococcal infection due to an age-dependent collapse in the number of AM and the formation of lipid-laden AM. The AM of Nkrp1b−/− mice show increased uptake but defective metabolism of surfactant lipids. We identify a physical relay between AM and alveolar type-II pneumocytes that is dependent on pneumocyte Clr-g expression. These findings implicate the NKR-P1B:Clr-g signaling axis in AM-pneumocyte communication as being important for maintaining metabolism in AM.

The innate component of the mammalian immune system is composed of several highly specialized subtypes of macrophages that perform unique tissue-specific functions. Splenic red pulp macrophages, liver Kupffer cells, microglia, and alveolar macrophages are prototypical examples of these specialized tissue-resident macrophage populations. The lungs, which perform gas exchange while being continuously exposed to inhaled pathogens and debris, are host to one such unique population of phagocytes termed alveolar macrophages (AM). AM are positioned on the luminal surface of the alveolar epithelium, where they are uniquely adapted as front-line, sentinel immune cells, aiding debris clearance and surfactant metabolism. Originating from fetal liver monocytes, these precursors seed the embryonic lung and rapidly develop into mature AM post-partum through a burst of proliferation and differentiation that translates into a full complement of AM being present in the lung as early as postnatal day 3 in mice. These AM completely self-renew with very little input from peripheral blood monocytes under steady-state conditions.
conditionsex,6. However, self-renewing tissue-resident AM eventually give way to monocyte-derived AM as they lose their ability to replenish their numbers due to cell senescence.x,6,7.

In immunity defense, AM have been highlighted by studies showing their importance in helping clear pneumococcal infections and controlling inflammation in the lung, and aiding in post-infection recovery.8,9 AM also metabolize pulmonary surfactant that is constantly secreted by type-II pneumocytes in the alveolar epithelium. Pulmonary surfactant is primarily composed of phosphatidylcholine, dipalmitoyl phosphatidylcholine, phosphatidylglycerol, various other free fatty acids, and surfactant proteins.10 This composition of pulmonary surfactant functions to ensure efficient gas exchange and lower surface tension to prevent atelectasis, during expiration.11 While most of this surfactant is taken up and recycled by type-II pneumocytes, about 20% of it is taken up by AM and broken down into cholesterol and other by-products, which are then expelled into the alveoli.12 Pulmonary alveolar proteinosis (PAP) is a surfactant clearance disorder that is caused by an inability or lack of AM to process surfactant, resulting in protein and mucous accumulations in the alveolar space, which without regular intervention eventually leads to death due to gas exchange failure.13 The importance of AM in PAP and the processing of surfactant was highlighted in studies conducted with granulocyte–monocyte colony-stimulating factor-receptor-deficient (Gmcsfr−/−) mice, which manifest classical PAP symptoms, due to a failure in the development of AM, resulting in a very small and dysfunctional population of foam-like AM that are unable to metabolize pulmonary surfactant.x,6

 Peroxisome proliferator-activated receptor-γ (PPAR-γ) induction through constant GM-CSF signaling is necessary for the proper development and inherent metabolic function of AM required for surfactant metabolism.3. Other factors such as PTL, Bcl-2, BileH40/41, mTORC, CEBPβ, and autocrine secretion of TGFβ are critical for proper development, self-renewal, and imprinting of unique metabolic signatures of AM. Experiments from various gene-deficient mice showing the absence of GM-CSF signaling or signaling of the above-mentioned factors result in the formation of foam-like AM with the drastically impaired development and metabolic function that is characterized by either low numbers of AM or their complete absence4,15. Recent reports have highlighted the complex interplay between secreted cytokines, cell-cell interaction, and immune-epithelial communication, all of which can contribute to the acquisition of AM-specific identity16,17. This includes receptor–ligand interactions between AM and cells lining the alveoli. However, the identification of all such surface receptors on AM is incomplete.

The mouse NKR-P1 receptor family comprises the activating receptors, NKR-P1-A, -C, and -F, which stimulate NK cell cytotoxic immune responses18,19, and NKR-P1-B/D (alleles), and -G, which inhibit NK cell responses20,21. The NKR-P1 receptors are inherited together with their ligands Clr-b, -c, -d, and -f as part of the NK gene complex on mouse chromosome 6, and exhibit known associations between NKR-P1/B/D:Clr-b; NKR-P1/F:Clr-c,d,g; and NKR-P1/G:Clr-d,g.22,23 Clr-b engagement by the ITIM-containing receptor results in the recruitment of the phosphatase, SHP-1, which transmits downstream signaling to inhibit NK cell function.24,25 Clr-b interaction acts as an MHC I-independent recognition system that protects potential target cells from NK cell-mediated cytotoxic responses, as well as a regulatory mechanism to protect against immune hyper-reactivity during immune surveillance26,27,28. While the function and distribution of these receptor families are best described on classical NK cells, evidence shows that certain NK cell receptors are present on other immune cell types and perform critical regulatory functions, such as the expression of NKR-P1C (NK cells and ILCs), and Ly49Q (on plasmacytoid dendritic cells and neutrophils).29,30

In this study, we demonstrate that resident AM express the NK cell-associated receptor NKR-P1B, and using Nkrp1b−/− mice, we show that the loss of this receptor has significant functional consequences on the development and metabolism of AM. NKR-P1B loss leads to increased susceptibility to S. pneumoniae infection due to a gradual collapse of tissue-resident AM, followed by a period of peripheral blood monocyte-mediated renewal. The collapse of the population of AM is followed by an accumulation of foam-like AM in the alveolar spaces of NKR-P1B-deficient mice that can be resolved by removing them from the lipid-rich alveolar environment. Similarly, Nkrp1b−/− AM take up exogenous lipid at a higher rate, while also experiencing metabolic and cell-cycle dysregulation, highlighting the importance of NKR-P1B signaling on the lipid processing ability of lung resident AM. We also identify Clr-g expressed on lung epithelial cells (type-II pneumocytes) as a potential ligand for NKR-P1B, whose interaction with NKR-P1B represents a further tissue-specific regulation mechanism that imprints AM with their unique metabolic signature and signaling. Our findings demonstrate tissue-specific signaling through classical ‘NK cell receptors’ that are required for the immune reactivity and homeostatic maintenance of AM. These findings identify potential therapeutic targets and further progress toward understanding how tissue-specific ligands are required for immunosurveillance and immune metabolism.

**Results**

**NKR-P1B-deficient mice exhibit significant mortality and morbidity upon S. pneumoniae infection**

NKR-P1B is expressed broadly on immune cells and is involved in anti-bacterial responses in the gut and potentially in the lung as well.30,31 Based on these observations, we investigated the requirement for NKR-P1B in respiratory immune responses by infecting 6-week-old WT and Nkrp1b−/− littermates with Streptococcus pneumoniae. While ~70% of WT mice were able to clear and survive S. pneumoniae infection, Nkrp1b−/− mice reached humane endpoints by day 4 post-infection resulting in a 100% fatality rate (Fig. 1a). An inability to clear the pneumococcal infection was also observed in 12-week-old Nkrp1b−/− mice infected with S. pneumoniae, with a 24-h delay until the onset of mortality (Fig. 1b). A slight increase in mortality was observed in 12-week-old WT mice upon infection but it is not uncommon to see dysfunctional immune responses in aged mice.31,32 Nkrp1b−/− mice infected at 21 weeks of age show a decrease in mortality compared to infections at earlier ages, yet still showed significantly higher mortality than observed in 21-week-old WT mice (Fig. 1c). To assess the cause of this enhanced mortality, we examined gross lung pathology of Nkrp1b−/− mice post-infection. Nkrp1b−/− mice showed significant alveolar epithelial damage as seen in Fig. 1d compared to WT. Pathological scoring and analysis of lung sections from infected 6 and 12-week-old WT and Nkrp1b−/− littermates also showed an increase in inflammation in Nkrp1b−/− mice (Supplementary Fig. 1a–d). To extrapolate on the pathological findings, we assessed the bacterial burden present in the lungs of WT and Nkrp1b−/− mice 3 days post-infection and we found a significant increase in the bacterial load in lavage fluid of Nkrp1b−/− mice as seen by a three-fold increase in colony-forming unit (CFU) counts shown in Fig. 1e with WT mice showing similar CFU numbers as previously reported with S. pneumoniae infection.33 As NKR-P1B is expressed on NK cells, we assessed whether this reduced survival upon bacterial challenge was NK cell-related. WT mice infected with S. pneumoniae after NK cell depletion (using NK1.1 mAb, PK136) showed that there was no difference in survival compared to mock-depleted WT mice, thus excluding NKR-P1B deficiency on NK cells as a source of this observed survival disparity (Fig. 1f).
The above data indicate that mice deficient in NKR-P1B exhibit dramatic loss of ability to resist pneumococcal infection and increased bacterial burden resulting in significantly elevated mortality, independent of classical NK and NKT cells.

Alveolar macrophages express NKR-P1B and NKR-P1B deficiency results in a gradual loss of AM followed by a CCR2-dependent recovery

The NKR-P1B requirement for clearance of S. pneumoniae led us to explore whether any constituents of the lung immune milieu were detectably altered in Nkrp1b−/− mice at a steady state. This analysis showed a surprising decrease in the number of alveolar macrophages (AM) in Nkrp1b−/− mice, while levels of other major immune cells remained unchanged (Fig. 2a). Resident AM are at the frontline of pulmonary defense against bacterial pathogens and therefore develop very early in mice, becoming terminally differentiated and present in large numbers by 2 weeks of age. Thus, we next determined how NKR-P1B loss affects the population kinetics of AM relative to the age of the mice. As seen in Fig. 2b, AM (gated on live CD45+ SIGLEC-F−, MHC II−, see Supplementary Fig. 10 for full gating strategy) in Nkrp1b−/− mice show a dramatic reduction at 6 weeks of age. Quantification of flow cytometry results show approximately 400,000 AM in the lungs at 2 weeks of age, which then stabilizes to around 250,000 cells/lung up until 21 weeks in WT mice. Conversely, the number of AM in Nkrp1b−/− mouse lungs at 2 weeks of age are similar to WT numbers, but decline significantly with age, with the lowest number of AM detected at 6 weeks of age before the lung is repopulated at 12 weeks (Fig. 2c). In agreement with these observations, anti-SIGLEC-F immunofluorescent staining of mouse lungs showed AM (large, SIGLEC-F− cells) distributed throughout the lungs of WT mice at all ages, while Nkrp1b−/− mice lungs showed a significant decrease in detectable AM at 6 weeks of age followed by subsequent reconstitution (Fig. 2e and f). Conversely, we identified a proportional increase in interstitial macrophages in Nkrp1b−/− mouse lungs (Supplementary Fig. 2c), nor on Kupffer cells, splenic red pulp macrophages, or on either large or small peritoneal macrophages, which also did not show any change in numbers in Nkrp1b−/− mice. We also noticed a corresponding age-associated change in the phenotype of AM in Nkrp1b−/− mice, characterized by a sharp increase in CD11b expression, and a small decrease in F4/80 expression (Supplementary Fig. 2n). These findings demonstrate that NKR-P1B expression is exclusive to lung-resident, fetal-liver monocyte-derived AM and is crucial for their homeostasis.

Resident AM are known to be self-renewing under steady-state conditions, with little input from blood monocytes. This mechanism is GM-CSF-driven and thus we examined the GM-CSF content in alveolar lavage fluid from 2-week and 6-week-old Nkrp1b−/− mice, as well as the levels of GM-CSF receptor on Nkrp1b−/− AM. There were no significant differences observed in lavage fluid GM-CSF content nor GM-CSF receptor expression between WT and Nkrp1b−/− mice, as well as the levels of GM-CSF receptor on Nkrp1b−/− AM. These findings demonstrate that NKR-P1B expression is exclusive to lung-resident, fetal-liver monocyte-derived AM and is crucial for their homeostasis.
Fig. 2 | AM express NKR-P1B and NKR-P1B-deficient mice show a gradual loss of AM followed by a CCR2-dependent recovery. a Number of various immune cells present in the lungs of WT and Nkrp1b−/− mice at 6 weeks of age as determined by flow cytometric phenotyping. Data presented as mean ± SEM. Significance determined by unpaired, two-tailed Student's t-test. **p < 0.001 (n = 3 biologically independent samples). b Flow cytometry dot plots of the numbers of AM from WT and Nkrp1b−/− mice at 2, 6, and 12 weeks of age. Images representative of 7 different experiments. c Quantifications of AM obtained by flow cytometric analysis from WT and Nkrp1b−/− mice at 2, 4, 6, 8, 12, and 21 weeks of age (n = 7 mice per age per genotype). Data presented as mean ± SEM. Statistical significance was determined by two-way ANOVA with Tukey's correction where p = 0.0094, **p < 0.001, and ***p < 0.0001. d Histogram of NKR-P1B surface expression on WT and Nkrp1b−/− AM. e Compiled stack images of WT and Nkrp1b−/− lungs stained with anti-SIGLEC-F antibody (green) and the nuclear stain DRAQ5 (Red). 100 μm scale bars shown. Images are representative of 6 experiments. f Quantifications of AM present in the lungs of WT and Nkrp1b−/− mice as determined by confocal imaging of immunofluorescent stains in (e) (n = 6 mice per age per genotype). Data presented as mean ± SEM. Data presented as a number of SIGLEC-F positive cells/338 μm². Statistics were determined by two-way ANOVA with Tukey’s correction where \( p = 0.0094, **p < 0.001, \) and ***p < 0.0001. g Flow cytometry plots of AM found in the lungs of 12-week-old WT, Nkrp1b−/−, and Nkrp1b−/− Ccr2−/− mice. Images representative of five experiments. h Quantification of AM from 12-week-old WT, Nkrp1b−/− and Nkrp1b−/− Ccr2−/− mice. Data presented as mean ± SEM, n = 7. i and j Quantification of chemokine dot blot array performed on WT and Nkrp1b−/− BALF or serum derived from 6-week-old mice. In all graphs and histograms, WT is blue, Nkrp1b−/− is orange, and Nkrp1b−/− Ccr2−/− is green. Source data are provided as a Source Data file.
mice (Supplementary Fig. 2a and p). To assess if GM-CSF/JAK/STAT signaling was affected in Nkrp1b−/− mice, we measured phosphorylated STAT5 (pSTAT5) levels in AM. However, no significant difference in the level of pSTAT5 of WT and Nkrp1b−/− AM were detected (Supplementary Fig. 2r), suggesting that the observed decline in the number of AM in Nkrp1b−/− was not due to a breakdown in GM-CSF-mediated self-renewal.

Under certain circumstances, such as lower respiratory tract infections, blood monocytes infiltrate the alveolar niche, through a CCR2-mediated mechanism and differentiate into fully functioning AM45,46. To assess the origin of the reconstituted macrophage population, we generated CCR2/NKR-P1B double knockout mice (Nkrp1b−/−Ccr2−/−). The lungs of WT, Nkrp1b−/−Ccr2−/− and Nkrp1b−/−Ccr2−/− littermates were analyzed by flow cytometry. AM have previously been shown to not express CCR2 and do not depend on it for migration, however, peripheral blood monocytes do10. At 12 weeks, the number of AM in the lungs of Nkrp1b−/−Ccr2−/− mice were similar to WT numbers, while the number of AM in the lungs of Nkrp1−/−Ccr2−/− mice remained significantly depleted at 6 weeks and later, with only marginal increases at 21 weeks of age (Fig. 2g and h).

Together, these data show that AM not only express NKR-P1B, but that loss of NKR-P1B leads to a progressive decrease in the number of resident AM beginning at approximately 4 weeks of age, followed by slower CCR2-dependent reconstitution of the alveolar niche by blood monocytes beginning at 5 weeks of age. We directly assessed the signals prompting recruitment and replenishment of AM by measuring chemokines present in lavage fluid and serum of 6-week-old WT and Nkrp1b−/− mice (Fig. 2i and j, and Supplementary Fig. 3a). We detected an increased presence of CCL9/10, Adipsin (CFD), CCL6, SRPSOX (CXCL16), and CCL5 in lavage fluid of Nkrp1b−/− mice compared to WT mice at 6 weeks of age, by staining lavaged AM with amplex-red, using a protocol designed specifically to measure free cholesterol content. We found that Nkrp1b−/− AM exhibited a significant increase in the level of intracellular free cholesterol compared to WT AM (Fig. 3g). CD36 is expressed by AM and is known to be a lipid scavenging receptor and a major determinant in lipid uptake kinetics of AM10,11. Thus, we examined the level of CD36 on Nkrp1b−/− AM and found a slight increase in CD36 expression compared to WT AM (Supplementary Fig. 3b), suggesting a possible mechanism for the increased lipid content of AM. Taken together, the above data indicate that resident Nkrp1b−/− AM accumulate free cholesterol and perhaps other lipids that are present in, or derived from, pulmonary surfactant. As a result, AM of Nkrp1b−/− mice acquire a foam cell phenotype, and the observed increase in cell size and lipid accumulation aligns with the decline in their population numbers, a characteristic reminiscent of cell death caused by lipo-toxicity.

**NKR-P1B-deficient AM experience cell cycle disruption that impairs their ability to self-renew in vivo while showing normal proliferative capacity in vitro**

Since Nkrp1b−/− AM accumulate surfactant, which has the potential to cause lipo-toxicity, we decided to investigate whether AM isolated from the lungs could be induced to proliferate if removed from the lipid-rich alveolar environment. Visual quantification of lavaged AM as seen in Supplementary Fig. 3c showed no proliferation in vitro when GM-CSF is absent from the growth medium, but upon the addition of GM-CSF, AM of 2-week-old Nkrp1b−/−Ccr2−/− and Nkrp1b−/−Ccr2−/− mice showed a similar proliferation rate over the entire 6-day period (Fig. 4a). This was also true for AM extracted from 6-week-old mice (Fig. 4b). Interestingly, while both Nkrp1b−/−Ccr2−/− and Nkrp1b−/−Ccr2−/− lavaged AM were able to survive ex vivo, Nkrp1b−/−Ccr2−/− AM exhibited double nuclei at a frequency that was not observed in WT AM after 6 days of in vitro culture without GM-CSF (Fig. 4c and d) suggesting an inherent cell cycling defect in Nkrp1b−/−Ccr2−/− AM. Flow cytometric BrdU analysis of lavaged AM from Nkrp1b−/−Ccr2−/− mice shows an accumulation of AM in the S-phase of cell division, with Nkrp1b−/−Ccr2−/− mice having almost 20% of cells lingering in S-phase compared to less than 10% for Nkrp1−/−Ccr2−/− AM (Supplementary Fig. 3d). As proliferation of AM in Nkrp1b−/− mice are mostly CCR2-dependent, we proceeded to assess proliferative capacity using Nkrp1b−/−Ccr2−/− mice to exclude any possible monocyte infiltration. At 2 weeks of age Nkrp1b−/−Ccr2−/− AM exhibited lower levels of Ki67, as determined by intracellular flow cytometry than AM of Nkrp1−/−Ccr2−/− mice (Supplementary Fig. 3e). The above data indicate the presence of a defect in cell cycle or cytokinesis in Nkrp1b−/− AM which is resolved when the AM are removed from the lipid-rich alveolar environment and forced to proliferate in vitro.

**RNA-Seq and lipidomic analyses indicate NKR-P1B requirement for metabolic processes that protect AM against the accumulation of toxic cellular lipid species**

Two obvious defects in Nkrp1b−/− AM are apparent: lipid processing and cell cycle. To identify the cellular programs that underlie the observed defects of Nkrp1b−/− AM, we performed RNA-Seq analysis on AM sorted from 2-week-old WT and Nkrp1b−/− mouse lungs. Altogether, 590 genes were significantly dysregulated (Supplementary Fig. 4a). Gene ontology analysis on this data set show a pattern of dysregulation of gene expression characterized by excessive lipid storage and upregulation of various lipid metabolic pathways (Fig. 5a and Supplementary Fig. 4b). As well, kinase activity was found to be dysregulated, as indicated by an apparent increase in factors regulating positive p38-MAPK loops, and a decrease in the ERK cascade and the factors regulating it (Fig. 5b and Supplementary Fig. 4c). The
transcriptional profile of Nkrp1b−/− AM showed altered expression of genes that encode well-characterized components of lipid processing and efflux, such as ABCA1, ABCG1, and DAG-kinas, as well as lipases such as LPCAT2 and PNPLA2 (Fig. 5c). Other dysregulated genes such as Cd36, Lpin2, Apoe, and Cds1 mediate the uptake and conversion of lipids from one species into another (Fig. 5c). We noted the downregulation of Stat5α, Rara, Rarg, and Il4ra and the upregulation of Ppar-γ (Fig. 5d).
These genes have been shown to have significant consequences on the metabolic programming and differentiation of AM. Further, we identified an upregulation in mediators of lipid metabolism in AM. In particular, Nkrp1b, Hilpda, Plin2, Fabp5, and Serpinb6b, were upregulated in Nkrp1b−/− AM, all of which are involved in lipid transition and metabolism, as well as lipid droplet formation. Finally, our RNA-Seq analysis showed significant dysregulation in cell cycling with many critical cell cycle pathways. Combining the transcriptomic data with our lipidomic analysis showed significant dysregulation of lipid metabolic processes in cholesterol, and perhaps LPC and LPG, which together comprise Fig. 5f, the upregulation of CD36 and perhaps the activity of other enzymes such as PLCB2, PNPLA2, and PLPP3, which catalyze the breakdown of TG, PA, and LPA by converting them into DAG and various fatty acids, lipid classes that are respectively diminished and increased in our analysis. This shunt towards DAG accumulation in Nkrp1b−/− AM, combined with a deficiency of DGKG, serves to create an excess of DAG species shunted into lipid droplets along with the canonical lipid storage species of CE, which is also found in excess in Nkrp1b−/− AM. However, the low TG lipid pool, considered to be a safe storage lipid, is indicative that lipid droplets in Nkrp1b−/− AM contain species of DAG, FC, and various fatty acids in excess, which are known to induce lipo-toxicity. The substantial dysregulation in transcriptional programs related to metabolic processes in Nkrp1b−/− AM and the notable changes in the lipid profile of AM in Nkrp1b−/− mice points to an underlying dysregulation of cellular metabolic processes. To test this, we performed a flow cytometry-based profiling of energetic metabolism in AM of WT and Nkrp1b−/− mice using the SCENITH assay. Specifically, AM lavaged from 2-week-old WT and Nkrp1b−/− mice were treated with either an ATP synthase inhibitor, oligomycin-A, or a competitive inhibitor of glycolysis, 2-deoxyglucose, or a combination of both following incubations with puromycin. Flow cytometry measurement of puromycin incorporation into nascent proteins, a reliable measure of protein synthesis only in protein synthesis only in AM treated with 2-deoxyglucose (Fig. 5g and h). However, calculation of the metabolic dependencies and capacities using this data shows a significantly elevated glucose dependency of Nkrp1b−/− AM, and a significantly
**Fig. 5 | RNA-Seq and lipidomics indicate a dysregulation of metabolism in Nkrp1b−/− AM.** a and b Gene ontology pathways enriched (Log2 fold change) in Nkrp1b−/− AM from 2-week-old mice as determined by RNA-Seq analysis. c and d Heatmaps of select lipid metabolic and general metabolic genes in AM. Log2 fold change enrichment presented as Z-scores (n = 3 replicates) for WT and Nkrp1b−/− with a pool of five mice for every replicate. e Comparison between the abundance of an indicated lipid species and internal lipid standards from lipidomic analysis of AM from 6-week-old WT and Nkrp1b−/− mice (n = 4 independent experiments per genotype). LPC (Lysophosphatidylcholine), PC (Phosphatidylcholine), LPG (Lysophosphatidylglycerol), PG (Phosphatidylglycerol), DAG (Diacylglyceride), CE (Cholesterol ester), LPA (Lysophosphaticidic acid), PA (Phosphaticidic acid), TG (Triglyceride). Boxplots indicate median, IQR, maximum, and minimum bars. Large and small data points represent average SD and individual experimental replicates, respectively. f Model combining RNA-seq (c) and lipidomics (d) data showing increased (red) or decreased (blue) lipid species and enzyme/receptor expression in Nkrp1b−/− AM. Dashed arrows indicate lipid shuttling into droplets.

decreased fatty acid and amino acid oxidation capacity compared to WT AM. Additionally, both WT and Nkrp1b−/− AM exhibit a maximal mitochondrial dependency, but minimal glycolytic capacity (Fig. 5I). We further examined the metabolic status of WT and Nkrp1b−/− AM by measuring rate-limiting enzymes across multiple metabolic pathways using the flow cytometry-based Met-Flow antibody panels. From these assays, we observed a significant increase in the glucose uptake transporter, GLUT1, and acetyl-CoA carboxylase alpha (ACCo) the rate-limiting enzyme in de novo long-chain fatty-acid synthesis (Fig. 5J). Together, our
Fig. 6 | NKR-PIB signaling correlates with the rate of surfactant lipid uptake by AM. 

a. RT-qPCR of select metabolic genes from WT and Nkrp1b−/− AM from 2-week-old mice. Data normalized to WT levels.
b. RT-qPCR analysis of WT AM crosslinked with 2D12 or isotype antibody. Data normalized to isotype control.
c. Fluorescence of WT and Nkrp1b−/− AM after 40 min NBD-PC incubation. MFI of WT and Nkrp1b−/− AM after 0, 20, 40, and 60 min NBD-PC incubation.
d. Fluorescence of WT and Nkrp1b−/− AM after 20 and 60 min incubation with NBD-PG.
e. Fluorescence of WT and Nkrp1b−/− AM after 40 min NBD-PC incubation in the presence of GM-CSF or vehicle control. MFI of NBD-PC uptake in (k).
f. MFI of NBD-PC uptake in (g).
g. Images of WT and Nkrp1b−/− AM after 0 or 6 days of in vitro culture post-ORO staining. 100 μm scale bars shown. ORO-staining indicated (arrows).
h. Quantification of ORO-positive AM/700 μm² shown in (m). Data in m and n represent (n = 2; day 0) or (n = 3; day 6) independent experiments. Data in a, b, c, e, f, g, i, l, n show mean ± SEM. Statistics for d and f represent an unpaired, two-tailed Student’s t-test of data prior to normalization where *p < 0.05 and **p < 0.01. Statistics for h, j, l, and n represent a two-way ANOVA with Tukey’s correction where **p < 0.01, ***p < 0.001, and ****p < 0.0001. All graphs and histograms indicate WT in blue and Nkrp1b−/− in orange. Source data are provided as a Source Data file.
metabolic profiling data indicates a shift in the metabolism of \( \text{Nkrp1b}^{-/-} \) AM towards elevated glycolysis and possibly lipogenesis, and a decrease in lipid catabolism.

**NKR-P1B signaling regulates critical metabolic genes responsible for lipid turnover**

To verify the transcriptional dysregulation of specific lipid uptake and metabolic mediators, we performed qPCR analysis on AM of 2-week-old WT and \( \text{Nkrp1b}^{-/-} \) mice for the lipid uptake receptor, \( \text{Cd}36 \), the cholesterol efflux cassettes, \( \text{Abca1}, \text{Abcg1}, \) and the transcription factor, \( \text{Rara} \), all of which have been highlighted by RNA-seq as being dysregulated. The differential expression results for all the genes selected for qPCR analysis, except for \( \text{Ldlr} \), corresponded with the observed changes identified by RNA-seq (Fig. 6a). We postulated that if a lack of NKR-P1B results in dysregulation in the genes examined in Fig. 6a, cross-linking the receptor using an anti-NKR-P1B antibody (clone 2D12), thereby forcing signaling through its ITIM, should produce the opposite effect in WT AM. Indeed, cross-linking of NKR-P1B on WT AM resulted in increases of \( \text{Cd}36, \text{Cd}63, \text{Ldlr}, \text{Abca1}, \) and \( \text{Rara} \) were similar to that of NKR-P1B-deficient AM indicating a secondary effect of NKR-P1B loss. We assayed the p38 MAPK phosphorylation status in \( \text{Nkrp1b}^{-/-} \) AM, as the p38 MAPK cascade is a known transcriptional inducer of various scavenger receptors and processes associated with lipid foam cell formation. We observed elevated phosphorylation of p38 MAPK in \( \text{Nkrp1b}^{-/-} \) AM (Supplementary Fig. 4). Taken together, the RNA-seq and lipidomic data highlight a fundamental dysregulation of lipid metabolism, which biases NKR-P1B-deficient AM to store toxic lipid species. The immediate modulation of \( \text{Nceh}, \text{Abcg1}, \text{Serpinb6}, \text{Fabps}, \text{Apoe}, \text{Psap}, \text{and Rara} \) expression after NKR-P1B activation, points to an interaction between the regulation of lipid processing in AM and NKR-P1B.

**NKR-P1B negatively regulates lipid uptake by AM**

While previous data points to an apparent defect in lipid metabolism and storage, the modulation of scavenger receptors by NKR-P1B led us to examine whether NKR-P1B is also required for phagocytic process in AM. Lavaged AM from \( \text{Nkrp1b}^{-/-} \) mice did not exhibit notable alteration in phagocytic capacity compared to WT AM (Supplementary Fig. 5a and b). We, therefore, measured lipid uptake kinetics in AM of WT and \( \text{Nkrp1b}^{-/-} \) mice. Lavage \( \text{Nkrp1b}^{-/-} \) AM showed an increase in both PC and PG uptake in vitro compared to their WT counterparts (Fig. 6c and e) when incubated with NBD-labeled PC or PG for 20, 40, and 60 min. Quantifications show a trend of increased PC and PG uptake in \( \text{Nkrp1b}^{-/-} \) AM, which is primarily evident at 40- and 60-min time points and 20- and 60-min time points, respectively (Fig. 6d and f). On this basis, we hypothesized that cross-linking (via 2D12 mAb) of the NKR-P1B receptor on WT AM should produce the opposite effect. Indeed, cross-linking of NKR-P1B on AM derived from WT mice prior to co-incubation with PC resulted in significantly decreased PC uptake (Fig. 6g and h). Decreases in lipid uptake were not observed when cross-linking AM with an isotype control antibody, nor when using AM derived from \( \text{Nkrp1b}^{-/-} \) mice (Fig. 6g and h). Likewise, cross-linking of WT AM prior to co-incubation with PG also resulted in decreased lipid uptake (Fig. 6i and j).

The GM-CSF signaling axis is an established regulator of lipid metabolic processes that could affect lipid uptake in AM. However, our findings in Supplementary Fig. 2o, p, and r showed no significant differences observed in GM-CSF content of lavage fluid, nor differences in GM-CSF receptor expression or pSTAT5 levels between WT and \( \text{Nkrp1b}^{-/-} \) AM. Nevertheless, we tested whether changes in lipid uptake could be observed in AM with or without NKR-P1B receptor cross-linking both in the presence or absence of GM-CSF (Fig. 6k and l). We found that while lipid uptake does increase globally in the presence of GM-CSF, it does not appear to have any particular effect when the receptor is activated, suggesting the GM-CSF pathway-mediated alteration of lipid metabolism in AM is separate from NKR-P1B.

The engulfed lipids by AM during routine phagocytic turnover and clearance of apoptotic debris in the lung, generate ligands for nuclear receptors that modulate lipid metabolism and phagolysosome processing. Given the observed changes in both lipid uptake and expression of transcriptional activators of lipid metabolism in \( \text{Nkrp1b}^{-/-} \) AM, we assessed if NKR-P1B altered phagolysosome formation. The lavaged AM from 6-week-old \( \text{Nkrp1b}^{-/-} \) mice showed no significant changes in the distribution or number of phagolysosomes in AM (Supplementary Fig. 5c and d).

Given that \( \text{Nkrp1b}^{-/-} \) AM show elevated lipid uptake within a lipid-rich environment we assessed if the lipid-laden phenotype of \( \text{Nkrp1b}^{-/-} \) AM could be resolved if removed from the lipid-rich alveolar environment. To test this, we cultured lavaged AM in vitro for 6 days, then stained them with ORO and analyzed them by microscopy side-by-side with freshly lavaged AM as a positive ORO control. As can be seen in images in Fig. 6m, WT AM have little to no ORO positivity either when freshly lavaged or after 6 days in vitro. Conversely, \( \text{Nkrp1b}^{-/-} \) AM shows a dramatic decrease in ORO-positive cells after 6 days in vitro compared to freshly lavaged AM from \( \text{Nkrp1b}^{-/-} \) mice, with no correlation, observed between remaining ORO-positivity and a polyploid phenotype. Quantifying these observations confirmed a clear significant reduction in ORO-positive \( \text{Nkrp1b}^{-/-} \) AM following 6 days of in vitro culture (Fig. 6n).

Due to a recent publication showing the potential of statin therapy to alleviate metabolic defects in AM in mice with severe PAP67, we decided to apply the same treatment to \( \text{Nkrp1b}^{-/-} \) mice. After 2 weeks of pravastatin administration, we collected the lungs of WT and \( \text{Nkrp1b}^{-/-} \) mice and stained them with ORO. A significant reduction in ORO-positive AM of statin-treated compared to vehicle-treated \( \text{Nkrp1b}^{-/-} \) mice was observed (Supplementary Fig. 6a and b). Since \( \text{Nkrp1b}^{-/-} \) AM are rescued from their lipid burden by removing them from the surfactant-rich alveolar environment and culturing them in vitro, while lipid burden of \( \text{Nkrp1b}^{-/-} \) AM appeared to be reversed by in vivo statin administration, indicating the loss of \( \text{Nkrp1b}^{-/-} \) AM metabolic activity can be modulated with the administration of pharmaceuticals. The observations that several \( \text{Nkrp1b}^{-/-} \) AM phenotypes were reversible, led us to examine whether \( \text{Nkrp1b}^{-/-} \) AM exhibited any intrinsic changes in metabolism. We found the \( \text{Nkrp1b}^{-/-} \) AM had a higher glucose uptake than WT AM (Supplementary Fig. 7a), which supports our finding that AMs have elevated glycolytic metabolism (Fig. 5g–j). Also, analysis of \( \text{Nkrp1b}^{-/-} \) oxygen consumption rate by AM showed an elevated rate of extracellular oxygen consumption compared to WT AM (Supplementary Fig. 7b). We also detected a modest decrease in mitochondrial membrane potential by TMRE, and mitochondrial mass by MitoTracker staining (Supplementary Fig. 7c and d). To assess if these differences altered oxidative stress, we measured cellular levels of ROS in \( \text{Nkrp1b}^{-/-} \) AM and measured ROS-2 levels (Supplementary Fig. 7e and f) and found decreased ROS and slightly elevated NOS-2 in \( \text{Nkrp1b}^{-/-} \) AM. Together, these findings are indicative of an intrinsic change in the cellular respiration, mitochondrial status, and potentially glycolysis of AM in \( \text{Nkrp1b}^{-/-} \) mice.

**NKR-P1B tetramers bind to Clr-g-expressing type-II pneumocytes in an infection-sensitive manner**

The only known ligand for NKR-P1B that has been identified to date is Clr-b, but surprisingly, Clr-b deficient mice do not replicate the phenotype observed in \( \text{Nkrp1b}^{-/-} \) AM neither in terms of cell numbers

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**Article**

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nor *S. pneumoniae* challenge at 6-weeks of age (Supplementary Fig. 8a and b). Quantifications of AM isolated from Clr-b<sup>−/−</sup> mouse lungs show normal levels of AM present throughout all time points analyzed (Supplementary Fig. 8c). We have also verified that Clr-b deficiency does not disrupt NKR-P1B expression on AM (Supplementary Fig. 8d). The failure of Clr-b<sup>−/−</sup> AM to phenocopy Nkrp1B<sup>−/−</sup> AM implies that there must be another lung-specific, interacting partner for NKR-P1B present. Our previous study found high levels of Clr-g mRNA present in the lung epithelium<sup>30</sup> as demonstrated by in situ hybridization and qPCR. The alveolar epithelium is made up of type-I and type-II pneumocytes, and given the intimate relationship between the surfactant-secreting type-II pneumocytes and AM<sup>58</sup>, we decided to probe whether these cells express a ligand that our NKR-P1B tetramer could bind to and whether it can serve as a potential interacting partner for NKR-P1B expressed on AM. Staining lung tissue sections of WT and Clr-b<sup>−/−</sup> mice with pro-surfactant protein C (P-SPC) antibody (a marker exclusive for type-II pneumocytes) and biotinylated NKR-P1B tetramers (which faithfully stain Clr-b-expressing lymphocytes in mouse tissues as seen in Supplementary Fig. 9e and f), we were able to show co-localization of P-SPC and NKR-P1B tetramer in WT and Clr-b<sup>−/−</sup> mouse lungs (Fig. 7a). This is indicative of a type-II pneumocyte-restricted interaction between NKR-P1B and another ligand, with Clr-g being the best candidate based on previous evidence. AM respond to infection with their immune and metabolic programming controlled in part through interactions with pathogens and the alveolar epithelium<sup>62</sup>. As such, we wanted to explore whether this potential ligand responds to infection. Confocal imaging of *S. pneumoniae* (Fig. 7b) or influenza A virus-infected (Supplementary Fig. 9) WT and Clr-b<sup>−/−</sup> mouse lung sections shows a reduction in NKR-P1B tetramer staining when compared to vehicle controls, thus indicating that type-II pneumocytes respond by downregulating a ligand from the surface of type-II pneumocytes. Finally, to confirm this interaction between AM and type-II pneumocytes, we cultured MLE-12 cells (derived from type-II pneumocytes) on coverslips and transiently transfected them with a construct expressing Clr-g and GFP. Using NKR-P1B tetramers, we were able to confirm the colocalization of tetramers to GFP<sup>+</sup> MLE-12 cells transfected with the Clr-g expression construct, while no colocalization occurred in the MLE-12 cells transfected with the empty vector (Fig. 7c). To confirm that Clr-g was the specific Clr expressed in type-II pneumocytes, we assayed lung type-II pneumocytes isolated by FACs sorting of WT and Clr-b<sup>−/−</sup> lungs and performed RT-PCR analysis for transcript expression of both Clr-b and Clr-g transcripts, the two Clr family members previously found to be expressed in the lung. Our RT-PCR analysis identified Clr-g transcript in type-II pneumocytes, while Clr-b was absent (Fig. 7d). Together, the above data indicate that type-II pneumocytes express a potential ligand for NKR-P1B on their surface as determined by NKR-P1B tetramer binding and the detection of this ligand on the cell surface of pneumocytes is lost during infection. NKR-P1B tetramers can bind to this ligand, which in all likelihood is Clr-g expressed on type-II pneumocytes, as seen in vivo and recapitated in vitro, thus establishing a tissue-specific binding partner for NKR-P1B expressed on AM.

**Discussion**

The conserved NK cell receptor, NKR-P1B is known to exhibit inhibitory functions on NK cells and to act as a mediator for MHC-I-independent NK recognition<sup>25,26</sup>. The discovery that mouse AM rely on NKR-P1B to function and survive, extends both our understanding of this lectin-like receptor and how the alveolar niche supports resident AM. The regulation of resident immune cell activity through the modulation of tissue-specific signals is a hallmark feature of tissue microenvironments. As such, NKR-P1B receptor sensing of dynamically regulated ligands present in alveoli may represent a flexible line of communication between the alveolar epithelium and AM that functions in parallel with other reported AM–epithelial interactions. Previous work has shown that communication between the alveolar epithelium and the lung immune complement is necessary for proper immune functioning. In particular, the interaction between CD200 expressed on AM and CD200R expressed on the alveolar epithelium is necessary for myeloid restraint and aid in AM-mediated inflammation resolution post-infection. Lack of this interaction leads to extended inflammatory processes and increased morbidity and mortality due to run-away inflammation<sup>31</sup>. Similar research conducted by Metzger found that the presence of IFN<sub>γ</sub>, during pulmonary infection has a suppressive effect on both ILC2<sup>−/−</sup> and AM<sup>−/−</sup> leading to extended inflammatory processes and poor prognosis during post-IAV bacterial super-infections. Our findings here indicate that the communication between pneumocytes and AM which supports lung immune surveillance, the maintenance of lung AM, and metabolic functions of AM also relies on NKR-P1B receptor expression on AM. The interconnections between innate immune detection and response mechanisms and lipid metabolism are unique attributes of the lung alveolar environment. Surfactant proteins, which are C-type lectin proteins, are intimately involved in both lipid metabolism and lung immunity. Specifically, the surfactant proteins SP-D and SP-A, are able to bind numerous viruses and bacteria, while also having diverse functions in the regulation of innate and adaptive immune responses<sup>64</sup>. The surfactant proteins not only exhibit broad binding capabilities to lipids, carbohydrates, and proteins but also differential immune regulatory functions depending on the level of multimerization. A similar multi-purpose system could have likewise evolved for C-type lectin proteins on AMs. Such a system would permit the ability for pathogen detection, and activation of AM, and permit the induction of a more glycolytic state instead of an anti-inflammatory state without the need for multiple receptors. Notably, the unique function that NKR-P1B performs in resident AM may reflect characteristics that are partly unique to the alveolar niche and partly lineage-specific, as monocyte-derived AM do develop, but do not exhibit synonymous functional attributes observed in the fetal liver-derived resident population.

AM have several well-defined functions in the protection of the lungs from pathogens, such as *S. pneumoniae*, and in the resolution of inflammation following pathogenic insult<sup>65,66</sup>. Therefore, taking in mind that Nkrp1b<sup>−/−</sup> mice suffer from a collapse in the number of AM, it follows that they also succumb to the pneumococcal challenge. It is important to note that while Nkrp1b<sup>−/−</sup> AM reconstitute almost the entire alveolar niche in Nkrp1b<sup>−/−</sup> mice they exhibit both phenotypic and immunological defects which may impair their ability to clear *S. pneumoniae* infection despite their number. This finding led us to originally hypothesize that NKR-P1B in AM operates through interaction with Clr-b, the prototypical inhibitory ligand for the NKR-P1B receptor on NK cells. Clr-b expression is downregulated upon infection and cellular stress<sup>67,68</sup>, a signal that guides NK cell immune surveillance of peripheral tissues. However, Clr-b does not exhibit a comparable function in the regulation of AM similar to NKR-P1B. While Clr-b transcripts are also present in the lung, their expression has been validated only in lymphocytes. Rather, our data support an alternate binding partner for NKR-P1B in the lung. One potential binding partner, Clr-g, is expressed in type-II pneumocytes and shares similarities with Clr-b<sup>11,31</sup>. Although our evidence here supports Clr-g as an NKR-P1B ligand in the lung, the generation of a Clr-g mutant mouse is needed to establish the definitive nature of this interaction. It is also plausible that a much broader range of receptor/ligand combinations exist between the NKR-P1 and Clr proteins, as the interactions defined to date were largely understood through interaction assays such as BWZ reporter assays, tetramers, and crystallography in over-expression studies<sup>5</sup>, which may not account for optimal post-translational modifications or glycosylation (such as on type-II...
**Fig. 7** | **Infection downregulates NKR-P1B ligand expression.** a Confocal image stacks of WT and Clr-\( b^- \) frozen lung sections stained with CD45 (Green), NKR-P1B tetramer (Red), DAPI (Blue), and pro-surfactant protein C (White). b Confocal image stacks of WT and Clr-\( b^- \) frozen lung sections post-infection with *S. pneumoniae* harvested 3 days post-infection. Sections are stained with CD45 (Green), NKR-P1B tetramer (Red), DAPI (Blue), and pro-surfactant protein C (White). c MLE-12 cells transiently transfected with either empty vector or Clr-g expressing vector. Vector contains a separate GFP message for verification of transfection. Cells were grown on coverslips and stained 48 h post-transfection. d RT-PCR amplification of Clr-b and Clr-g transcripts in type-II pneumocytes sorted from WT and Clr-\( b^- \) lungs. No reverse transcriptase (−RT) controls and WT total lung controls are shown. All data presented are representative of \( n = 3 \) independent experiments. Source data are provided as a Source Data file.
pneumocytes), which may affect C-type lectin and perhaps lectin-like receptor binding\textsuperscript{23,27}. Moreover, the observed downregulation of the NKR-P1B binding partner in the lung during infection has interesting implications for epithelial interactions by AM through NKR-P1B. This downregulation may serve as a signal of epithelial damage instructing AM to shift from surfactant metabolism and homeostasis to an immune active phenotype. Further studies utilizing infection models and cytokine profiling of WT and NKR-P1B-deficient AM could be used to elucidate the specific immunomodulatory impact of NKR-P1B:Clr-g interactions.

The slow progressive decline in the number of AM in Nkrp1b\textsuperscript{−/−} mice, which occurs over 4–6 weeks, contrasts reports of developmental failures of AM in GM-CSF receptor-, PPAR-γ, and TGF-β-deficient mice, in which AM are not present in any significant numbers from birth, or they collapse very shortly (7–14 days) post-partum\textsuperscript{13,20}. Similar findings have also been reported in studies of VHL, mTORC, and BACH2\textsuperscript{46,74}. Conversely, the knockout of the Bhlhe40/Bhlhe81 transcriptional regulators did not alter the number of AM at steady state, despite affecting surfactant metabolism and impairing colonization of the alveolar niche by AM in competitive mixed bone-marrow chimeras\textsuperscript{17}. The disruptions in cytokine receptors or transcription factors that do affect the numbers of AM, were largely lethal due to PAP, whereby the residual foamy, lipid-laden AM in the lungs were unable to clear surfactant, leading to mortality through gas exchange interruption. While we report that Nkrp1b\textsuperscript{−/−} mice accrue a similar foamy phenotype in AM, AM appear to develop normally during the first two critical weeks post-partum before their decline in the following 4 weeks, and mice are otherwise completely healthy and viable. Moreover, while Nkrp1b\textsuperscript{−/−} AM tend to have a foamy, lipid-laden phenotype, which is most often accompanied by alveolar mucous/protein accumulation and easily detected through PAS staining\textsuperscript{13,25}, Nkrp1b\textsuperscript{−/−} mice have no significant mucous/protein in the alveolar space at all. This is very likely due to the peripheral blood monocytes infiltrating the alveolar niche and differentiating into AM, thus providing the necessary metabolic capacity to prevent PAP development.

The CCR2-dependent replenishment of AM from the blood monocyte pool is consistent with previous reports that peripheral monocytes replace AM when resident AM are subjected to damage that impairs their ability to self-renew\textsuperscript{23,75}. There also exist non-CCR2-mediated pathways of monocyte egress and entry into tissues, which likely contribute to the slight increase in the number of AM observed between the 12- and 21-week timepoint in Nkrp1b\textsuperscript{−/−} Ccr2\textsuperscript{−/−} mice, such as through CX3CR1 and/or SIRPs\textsuperscript{52}. While the alveolar niche supports incoming monocyte differentiation into AM\textsuperscript{13,20}, subtle differences remain when compared to liver monocyte-derived tissue-resident AM\textsuperscript{13}. These key differences in origin may account for the ability of Nkrp1b\textsuperscript{−/−} mice to survive the loss of tissue-resident AM. The incoming monocyte-derived AM, which are then able to carry out the necessary surfactant metabolism to prevent PAP-related mortality, do not appear to undergo full differentiation, exhibiting instead a subtle variation in CD11b, Siglec-F, and MHC II levels. Also, the presence of some lipid-laden cells in the lung at 12 weeks of age in Nkrp1b\textsuperscript{−/−} mice makes the function of NKR-P1B in monocyte-derived AM unclear. Given the subtlety and long manifestation time of the NKR-P1B-deficient AM phenotype, it is possible that upon reconstitution, AM undergo a constant cycle of cell death due to lipotoxicity and continuous replacement that would account for the longevity of the Nkrp1b\textsuperscript{−/−} mouse, as well as the small aberrations found in AM extracted from 12-week-old Nkrp1b\textsuperscript{−/−} mice.

The ability at steady state to self-renew with very little input from the peripheral blood monocyte population is a hallmark of AM. The disruption in NKR-P1B-mediated metabolic programming is a likely contributor, but not a sole cause of, self-renewal defects of AM. The observation that AM extracted from Nkrp1b\textsuperscript{−/−} mice were able to survive and proliferate ex vivo shows that the impairment in self-renewal in Nkrp1b\textsuperscript{−/−} AM. However, the significant dysregulation of cell cycle genes in 2-week-old Nkrp1b\textsuperscript{−/−} mouse AM, as well as the decrease in Ki67 and characteristic hallmarks of S-phase arrest in BrdU staining in Nkrp1b\textsuperscript{−/−} AM, implies a potentially fundamental involvement of NKR-P1B in the renewal of AM\textsuperscript{43,44}. This is further supported by the appearance of double nuclei in Nkrp1b\textsuperscript{−/−} AM cultured in vitro, which may reflect the consequence of persistent inflammation and DNA damage that is known to result in the formation of polyploid macrophages without cell-to-cell fusion\textsuperscript{46,86}. Lack of NKR-P1B signaling resulting in the accumulation of toxic lipid species may be the trigger that induces chronic stress on the AM, some of which resort to a polyploid phenotype to preserve genomic stability or it could potentially be caused by a defect in machinery responsible for cytokinesis due to metabolic stress, thus leading to failure of cell division.

Nkrp1b\textsuperscript{−/−} AM exhibit a metabolic profile characterized by increased lipid uptake, storage, and processing pathways. The concomitant increase in the expression of Apoe, CD36, and various lipid processing enzymes, with the downregulation of cholesterol efflux pumps (Abca1, Abcg1), could explain the accumulation of lipid droplets in Nkrp1b\textsuperscript{−/−} AM, as reported for metabolically impaired AM\textsuperscript{56,87}. Although GM-CSF receptor levels, phospho-STAT3 levels, and secreted alveolar GM-CSF levels remain unchanged in Nkrp1b\textsuperscript{−/−} mice, NKR-P1B activity may regulate the metabolic responses through Rara and Rarg and parts of the Ras pathway, which are downregulated in Nkrp1b\textsuperscript{−/−} AM and are necessary for metabolic and GM-CSF signaling, respectively\textsuperscript{60}. If the dysregulation in this lipid uptake and processing pathways underlies the Nkrp1b\textsuperscript{−/−} phenotype, then the dramatic increase in PC, PG, LPC, and LPG in lipid-laden AM at 6 weeks of age is not entirely surprising, since these are the main constituents of pulmonary surfactant\textsuperscript{88}, and therefore reflect a potentially increased uptake, reduced metabolism (or both) causing a backlog of unprocessed lipid. It is also possible that accelerated fatty-acid biosynthesis contributes to the accumulation of lipids in Nkrp1b\textsuperscript{−/−} AM, as the level of ACCI of significantly increased in these cells compared to WT. TG and CE are known to be safe lipid storage molecules\textsuperscript{90}, while excess accumulation of free cholesterol, free fatty acids, and DAG induces lipotoxicity\textsuperscript{91,92}, exacerbated in Nkrp1b\textsuperscript{−/−} AM. While the mechanism by which NKR-P1B regulates lipid uptake requires further study, it may function through CD36\textsuperscript{90}, which contains a signaling domain whose gene expression is enhanced by the binding of its ligand\textsuperscript{93} and is important for DPPC uptake by AM\textsuperscript{94}. Likewise, our qPCR screen of NKR-P1B cross-linking on AM showed that transcription levels of Serpinb6, Fabp5, and Apoe, which were downregulated in Nkrp1b\textsuperscript{−/−} AM, were upregulated upon NKR-P1B engagement, indicating a direct involvement of NKR-P1B signaling in the transcriptional control of these genes. These findings paint a picture in which NKR-P1B loss contributes to both increased surfactant uptake and the dysregulation in lipid metabolism that leads to the storage of toxic lipid species that slowly impacts the viability of AM.

In sum, this report demonstrates the presence of lung-specific function for the NKR-P1B receptor in the control of key components of the homeostatic maintenance pathways and pulmonary surfactant clearance functions in AM, thus providing another piece of the puzzle of tissue-specific determinants governing resident macrophage identity factors.

Methods

Ethics statement

All animal experiments were conducted in accordance with protocols 19–135 and 20–084 approved by the Dalhousie University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals.

Study design

The study aimed to investigate the contribution of NKR-P1B to the lipid metabolism of AM since many of the tissue-specific determinants of macrophage metabolic programming are still unknown. We took
advantage of NKR-P1B and Clr-b-deficient mice as well as soluble NKR-P1B tetramers to explore this relationship. The lack of phenocopying of Clr-b-deficient mice led us to explore potential alternatives for a tissue-specific NKR-P1B ligand which led us to utilize a mouse type-II pneumocyte cell line and Clr-g expression system to show the possibility of this ligand–receptor interaction in this model system.

**Experimental model and mouse details**

C57BL/6 (Jackson laboratories), Nkrp1b−/− (previously described)29, and Clr-b−/− (a generous gift from Dr. Matthew T. Gillespie)30 mice were bred in-house. CCR2-deficient mice were purchased from Jackson Laboratories and bred in-house. Generation of Ccr2−/− Nkrp1b−/− double mutant mice entailed crossing CCR2-deficient and NKR-P1B-deficient mice. Mice produced from the second generation of this pair were screened by PCR to establish the Ccr2−/− Nkrp1b−/− mouse which was subsequently bred in-house. Genotyping primers were Common forward (5′-GAACATCTCTCATCCGTGTC-3′), reverse deletion (5′-GCTGTTAAGATTCCAG-3′), reverse intron (5′-CACCTGTGTACCTTTGCA-3′). Animals were housed under specific pathogen-free conditions. All mice were maintained under a 12 h light/12 h dark cycle with free access to food and water in a temperature (22 ± 1 °C) and humidity (50 ± 5%) controlled room. Mice were age and gender-matched for experiments.

**Tissue processing and flow cytometry**

Mice were euthanized, their lungs excised, and homogenized with a razor blade. The homogenate was incubated at 37 °C with 5% CO2 in the presence of 5 mg/mL collagenase D (Worthington Biochemical Corporation Cat# LS04188) for 1 h. The resulting homogenate was then passed through a 70 μm cell strainer (Falcon Cat#352350). The cell suspension was then subjected to ACK lysis (previously described)29, double mutant sections. After cutting sections were fixed in acetone for 13 min and stained with Mayer’s hematoxylin to highlight nuclei, and then mounted using aqueous mounting media (Vectorlabs Cat#H-5501). Sections were blocked with normal horse serum containing 5% neonatal bovine serum albumin and 1% BSA for 1 h and then incubated with anti-SIGLEC-F antibody (Invitrogen Cat#A11114; 1:200), and in some experiments with anti-CD11c-FITC Cat#107635; 1:200), anti-SIGLEC-F-PerCP-eFluor 710 (Invitrogen Cat#A451327; 1:200), and anti-CD11c-APC (BioLegend Cat#103112; 1:200), anti-MHC II-PerCP (BioLegend Cat#1067635; 1:200), anti-SIGLEC-F-PerCP-eFluor 710 (Invitrogen Cat#46-1702-82; 1:200), and in some experiments with anti-CD11c-FITC (eBioscience Cat#11-0114-85; 1:200), anti-NKR-P1B at 1:100 (a generous gift from Dr. Koho Iizuka, University of Minnesota), anti-Clr-b antibodies at 1:100 as described previously30. After gating out doublets, AM were identified as CD45+MHC II+SIGLEC-F+CD11c+ cells with high forward and side scatter characteristics (see Supplementary Fig. I1a for gating strategy). In lavage experiments, mice were anesthetized by CO2 asphyxiation, the trachea exposed and then cannulated using a 20-gauge syringe wrapped in surgical tubing. Up to 1 mL of cold PBS was then placed into a sterile 15 mL tube. This was repeated three more times. The lavaged AM were then used for downstream analysis. MFIs were used to analyze 2-NDBG uptake. For tetramethyl rhodamine ethyl ester (TMRE) staining 1 mM TMRE (Thermo Fisher Cat#T669) stock solutions were dissolved in DMSO. AM obtained from 2-week-old WT and Nkrp1b−/− mice were stained with 50 nM TMRE for 15 min and then immediately acquired on a flow cytometer. For mitochondrial mass analysis, lavaged AM obtained from 2-week-old WT and Nkrp1b−/− mice were stained using Mitotracker Red CMXRsos from Thermo Fisher (Cat#M7512) as per manufacturer’s instructions. Cellular ROS was analyzed on lavaged AM obtained from 2-week-old WT and Nkrp1b−/− mice using DCFDA/H2DCFDA cellular ROS assay kit from Abcam (Cat#ab113851) as per manufacturer’s instructions. FlowJo 10.0.6 software was used to analyze FACS data.
microscope, and images captured using a Hamamatsu ORCA-HR digital camera.

Lipid uptake assays and NKR-P1B cross-linking
Lipid uptake assays were performed on AM from 2-week-old WT and \( \text{Nkrp1b}^- \) mice by lavage as described above. AM from three mice was pooled together for each replicate per independent experiment. Nitrobenzofurazan-labeled dipalmitoylphosphatidylcholine (NBD-PC) and phosphatidylglycerol (NBD-PG) (Avanti BioLipids Cat\# 81031, 810164 respectively). NBD-PC and NBD-PG were resuspended in 90% ethanol to a concentration of 4 mg/mL. For the assay, a final lipid concentration of 20 μg was applied to lavaged AM resuspended in 100 μL of DMEM with no supplements and then allowed to incubate for 10, 20, and 40 min. Cells were then washed once with FACS buffer, stained for viability, and immediately acquired on a flow cytometer without fixation. MFIs were used to analyze lipid uptake. In some experiments, lavaged AM were first blocked using normal mouse serum, stained with biotinylated anti-NKR-P1B (0.05 mg/mL) antibody, and then cross-linked in suspension with streptavidin (0.02 mg/mL). In some experiments, AM obtained from 2-week-old WT and \( \text{Nkrp1b}^- \) mice were then washed once with FACS buffer, stained for viability, and left to incubate with the AM for 20, 40, and 60 min. Cells were then washed once with FACS buffer, stained for viability, and immediately acquired on a flow cytometer without fixation. MFIs were used to analyze lipid uptake.

Glucose uptake assay
For glucose uptake analysis, 2-[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino]-2-Deoxyglucose (2-NBDG) from Cayman Chemical (Cat\# 11046) was prepared as a 30 mM stock solution by dissolving in DMSO. 2- NBDG or vehicle was added to a concentration of 25 μM to AM obtained from 2-week-old WT and \( \text{Nkrp1b}^- \) mice and left to incubate with the AM for 20, 40, and 60 min. Cells were then washed once with FACS buffer, stained for viability, and immediately acquired on a flow cytometer without fixation. MFIs were used to analyze 2-NBDG uptake.

Extracellular oxygen consumption assay
AM lavaged from 2-week-old WT and \( \text{Nkrp1b}^- \) were analyzed for O\(_2\) consumption kinetics by the extracellular oxygen consumption assay kit (Abcam Cat\# ab977243) as per the manufacturer’s instructions. Oxygen consumption data were acquired on a CLARIOstar high-performance plate reader (BMG Labtech). Glucose uptake analysis, 2-[(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino]-2-Deoxyglucose (2-NBDG) from Cayman Chemical (Cat\# 11046) was prepared as a 30 mM stock solution by dissolving in DMSO. 2- NBDG or vehicle was added to a final concentration of 25 μM to AM obtained from 2-week-old WT and \( \text{Nkrp1b}^- \) mice and left to incubate with the AM for 20, 40, and 60 min. Cells were then washed once with FACS buffer, stained for viability, and immediately acquired on a flow cytometer without fixation. MFIs were used to analyze 2-NBDG uptake.

In situ hybridization
In situ hybridization was performed using the Roche DIG RNA labeling kit SP6/T7 (Roche Cat\# Roche75029910) as described previously by our research group36, with some minor modifications. The hybridization temperature of the Cgr-g probe during the overnight hybridization step was 56 °C. The slides were then scanned on a Leica Aperio slide scanner and visualized using the Leica Aperio-ImageScope software.

In situ tetramer staining
NKR-P1B Tetramer assembly was conducted by the addition of PE-labeled streptavidin at a molecular ratio of 4:1 biotin monomer:streptavidin. 1/10th of the PE-labeled streptavidin was added at a 15-min interval at 4 °C. NKR-P1B tetramers were generated as described previously100. Frozen lung sections were cut at 10 μm and left to air-dry for at least 3 h. Native biotin was blocked using the BioLegend avidin-biotin blocking system (Cat\# 927301) as per the manufacturer’s instructions. Sections were subsequently blocked using rodent M block (ThermoFisher Cat\# 5083262) for 2 h. Primary antibodies against CD45 (1:100) (BioLegend Cat\# 103101; 1:200) and PSPTC (1:500) (Abcam Cat\# ab90716) and L1.1 μg of NKR-P1B tetramers were applied to the sections and incubated overnight at 4 °C. The next day, sections were washed once in PBS and fixed using 4% paraformaldehyde (PFA) for 12 min and washed three times in PBS. The sections were then
incubated for 2 h with 1:100 anti-PE antibody (BioLegend Cat#408101;1:200). The sections were washed and incubated with anti-rat IgG-AlexaFluor 488 (Invitrogen Cat#A-11001; 1:200), anti-mouse IgG-AlexaFluor 555 (BioLegend Cat#405324; 1:200), and anti-goat IgG-Cy5 (Invitrogen Cat#A-10523; 1:200) secondary antibodies for 1 h at room temperature. Sections were then washed three times in PBS and mounted using Vectashield (Vector Labs Cat#H-1200-10). DAPI containing fluorescent mounting medium. Sections were imaged on a Leica SP8 confocal microscope.

S. pneumoniae infections
Two, six, and 12-week-old mice age and sex-matched mice were infected intranasally with 730 CFU/g bodyweights of S. pneumoniae encapsulated serotype III (ATCC 6303). Mice were monitored for up to 7 days and sacrificed when reached appropriate endpoints which included either 20% weight loss or a combination of external observable factors (labored breathing, low response to stimulus, ruffled fur, hunched posture, etc.). For colony-forming unit (CFU) counts, mice were sacrificed at day 3 post-infection and lavaged as described previously. The lavage fluid was then plated onto fresh sheep blood agar plates and the alpha-hemolytic colonies, indicating S. pneumoniae, were counted and CFUs determined.

In vivo BrdU assay
Two-week-old male and female mice were injected with 1 mg of BrdU intraperitoneally. 24 h later mice were sacrificed and lavaged as described previously to obtain the AM. The cells were then processed using the BioLegend Phase-Flow kit (Cat#370704) as per the manufacturer’s instruction with no modification. The samples were then run on a BD-Fortessa flow cytometer and analyzed for cell cycle status.

Pravastatin treatment
Three-week-old Nkrp1b+/−Ccr2+/− and Nkrp1b+/−Ccr2−/− male or female mice were given either regular water or water supplemented with pravastatin calculated to deliver 10 μg/g bodyweight of the drug each day. The mice were treated for two weeks after which the lungs were extracted and frozen at −80 °C and processed for ORO staining as described above.

NK cell depletion
NK cell depletion was achieved by intraperitoneally injecting 6-week-old male or female WT mice with either 200 μg of anti-NK1.1 antibody, derived from the PK136 hybridoma or mock saline injection (n = 8 per treatment group). Depletion was maintained through injection of 100 μg of anti-NK1.1 mAb every 48 h. Throughout the anti-NK1.1 depletion treatment, mice were infected with S. pneumoniae as described above.

Cell lines and transfections
The full coding sequence for NKR-P1B was isolated from mouse cDNA and inserted into the pLJM1 vector (Addgene 91980). Lentiviral assembly was conducted in HEK293T cells (ATCC CRL3216) by PEI transfection with the pLJM1 vector containing the NKR-P1B sequence or an empty pLJM1 vector, pSfPA2 viral packaging vector, and the pMD2G viral envelope vector. Cell supernatants were harvested 48 h post-transfection and lentiviral stocks were kept at −80 °C until further use. MLE-12 cells obtained from ATCC (CRL-2110) were grown on coverslips in HITES medium. MLE-12 cells were then transfected with either an empty PcDH vector (Addgene 72299) or PcDH vector containing the sequence for C57BL/6 mouse Crl-g gene using FuGene (Promega Cat# E2311). Transfection efficiency was verified by GFP expression as observed on an immunofluorescent microscope. After 48 h the media and transfection reagent was removed, and cells were fixed with 4% PFA for 12 min then stained with NKR-P1B tetramers as described earlier.

RNA sequencing
AM were extracted from 2-week-old mouse lungs as described above and sorted on a BD FACS ARIA II cell sorter achieving an average of 98% purity. RNA from the sorted AM was extracted using Ribozol (VWR). The RT-PCR primers for Crl-b RT-PCR were Crl-b forward (5'-ACTCAGCTCTCAGCTCTGA-3') and Crl-b reverse (5'-GGCTAAAGCGCTCTCTG-3'), and the primers for Crl-g RT-PCR were: Crl-g forward (5'-AGATTGCTTGAGACAGAG-3') and Crl-g reverse (5'-GAGAGGTTCTGGAACAG-3').

RT-qPCR
AM were extracted from 2-week-old WT and Nkrp1B+/− mice, or WT AM following NKR-P1B crosslinking as described, and total RNA was isolated using Ribozol (VWR Cat#97064-952) in conjunction with a RNA clean-up and concentrator kit (Zymo Research; cat # R1016). cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific; cat # K1622). SYBR-green-based (qPCR) was performed on 0.1−0.5 μg of cDNA using NEB Luna Universal qPCR Master Mix (NEB; cat # M3003S/LX/E). Data was acquired on a BioRad CFX96 Real-Time PCR analyzer. Primers used to amplify target genes C3D6, C3D5, Ldlr, Nceh, ABCA1, ABCG1, Serpinb6, Rara, Fabps, Apoe and Psap are listed in Table 1 (see Supplementary Data 1).

ELISA
ELISA for GM-CSF was obtained from BioLegend (Cat#432207). Two-week-old mice were lavaged as described above with minor modifications. The lungs were lavaged once using 500 μl of PBS. Samples were processed as per the manufacturer’s instruction.

Phospho-STAT5 Assay
AM isolated from 2-week-old mouse lavage fluid as described earlier were fixed with 2% PFA for 25 min at 4 °C and permeabilized using 100% ice-cold methanol for 45 min on ice. Finally, AM were stained for Tyro3−phosphorylated STAT5 (pSTAT5) with an anti-pSTAT5-PE antibody (Invitrogen, Cat#12-9010-42; 1:50) or IgG1, k-PE Isotype Antibody (Invitrogen, Cat#12-9010-42; 1:50) antibodies into Ribozol (VWR). The RT-PCR primers for Crl-b RT-PCR were Crl-b forward (5'-ACTCAGCTCTCAGCTCTGA-3') and Crl-b reverse (5'-GGCTAAAGCGCTCTCTG-3').

Chemokine dot plot
500 μl of BAL and 200 μl of serum from 6-week-old WT and Nkrp1B+/− mice were applied to a Proteome Profiler Mouse Chemokine Array Kit
from R&D Systems (Cat#ARY020) and processed according to manufacturer’s directions.

Lipidomic analysis
AM were collected by lavage from 6-week-old mice as described above. AM from 5 mice was pooled to represent one biological replicate. The lavage fluid was spun, the supernatant removed, and the cell pellet frozen at −80 °C for analysis. Analysis was performed at the Laboratory of Genetic Metabolic Diseases, the University of Amsterdam as previously described.

Statistical analysis
Statistical significance was determined by Student’s t-test where applicable, with a cut-off p-value of 0.05. Other experiments, where appropriate utilized a two-way ANOVA or one-way ANOVA analysis with Tukey’s or Šidák post hoc correction for multiple comparisons. Significance in survival experiments was determined using the log-rank approach. Source data are provided with this paper.

Graphs and statistics were generated using GraphPad Prism v9.

Data availability
The RNA-seq data generated in this study have been deposited in the NCBI Gene Expression Omnibus database under primary accession GSE14867. All other data are available in the article and its Supplementary files or from the corresponding author upon request. Source data are provided with this paper.

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Author contributions

M.S., S.D., F.A.I.S., M.M.A.R., B.D.P., and A.P.M. designed and performed all experiments included in the manuscript and worked as a team. D.M.-L., G.G. analyzed and evaluated cytometric data. A.V. performed the experiments that initiated the study. J.R.C. and O.A.A. oversaw and coordinated the entire study and helped M.S. and B.D.P. write the manuscript.

Competing interests

The authors declare no competing interests.
