Influenza-induced activation of recruited alveolar macrophages during the early inflammatory phase drives lung injury and lethality

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Abstract (155 words)

During infection with respiratory viruses, disease severity is linked to a hyperinflammatory endotype, characterized, in part, by increased levels of circulating cytokines and worse clinical outcomes. In this study, we identify gene expression patterns associated with three distinct phases (Infiltrating, Early Inflammatory, Late Inflammatory) in the influenza A virus response of recruited alveolar macrophages. In the Early Inflammatory phase, recruited macrophages begin expressing pro-inflammatory genes, driving cytokine-mediated lung damage and contributing to the morbidity observed in influenza-infected wild-type mice. In mice lacking vimentin (*Vim*^{-/-}), genes associated with the Early Inflammatory phase are suppressed, while the expression of the Infiltrating Phase genes is maintained, which is associated with protection from influenza-induced injury. Using a bone-marrow chimera mouse model, we validate that *Vim*^{-/-} recruited alveolar macrophages are sufficient to confer protection from influenza-induced mortality. Our results define the temporal dynamics of gene expression in recruited alveolar macrophages, which shape the host response to infection with respiratory viruses.

Introduction

Recovery from viral pneumonia is a clinically important yet understudied process. A recent collaborative study from our group and others determined that viruses are the most common pathogens identified in patients with community-acquired pneumonia¹. Severe viral pneumonia due to influenza A virus (IAV) or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic agent of coronavirus disease 2019 (COVID-19), damages the lower respiratory tract to cause acute respiratory distress syndrome (ARDS)². Unremitting respiratory failure in patients with ARDS is a consequence of persistent inflammation and the failure of normal mechanisms of inflammation resolution and lung tissue repair. This hypothesis is clinically supported by a recent analysis of patients enrolled in the ARDSnet, in which patients with a hyperinflammatory endotype of ARDS, characterized in part by increased levels of circulating cytokines and chemokines, had worse clinical outcomes³. This points to the importance of a balanced and regulated immune response, where infection can be controlled while keeping tissue damage to a minimum.

Macrophages are the lung's primary cells that initiate and orchestrate the immune response after IAV infection⁴. Shortly after IAV or SARS-CoV-2 infection, tissue-resident alveolar macrophages (TRAMs) are depleted, and monocyte-derived alveolar macrophages (MoAMs) are recruited into the lung^{2,5,6}. MoAM recruitment has been associated with excessive or dysregulated cytokine and immune cell responses in humans and mice^{2,3,5,7}. Upon entering the infected lung, MoAMs express genes driving an inflammatory phenotype that contributes to persistent inflammation during the course of viral pneumonia resulting in

acute lung injury. Preventing the recruitment of these macrophages into the lung by deleting CCR2 or its ligand reduces the severity of IAV infection without affecting viral clearance⁵. Viral infections are sensed in host cells by pattern recognition receptors (PRR), such as the NOD-like receptor family, pyrin domain containing 3 (NLRP3). A crucial step in the immune response to IAV is the activation of the NLRP3 inflammasome and the subsequent secretion of inflammatory cytokines, IL-1 β , and IL-18^{5,8-10}. Inhibition of the NLRP3 inflammasome early in IAV infection impairs viral clearance and increases morbidity and mortality in mice¹¹. In contrast, inhibition of the NLRP3 inflammasome post-viral clearance reduces inflammation-induced tissue damage and promotes lung repair^{10,11}. As a result, therapies that target these macrophages may be of interest^{5,12,13}; however, no study has yet investigated the time-dependent functions of MoAMs in response to influenza A virus infection.

Vimentin, a type III intermediate filament, is ubiquitously expressed in macrophages and plays a role in several critical immune response processes. Vimentin suppresses inflammation in a mouse model of colitis¹⁴ and alters inflammatory gene expression in microglia lacking vimentin in a murine model of Alzheimer's disease¹⁵. Vimentin has been described as a ligand for some PRRs, such as the nucleotide-binding oligomerization domain-containing protein 2 (NOD2), and this interaction is required for subsequent NF- κ B activation¹⁶. We, and others, showed that vimentin is required for assembly and activation of the NLRP3 inflammasome^{17,18} and that vimentin-null (*Vim*^{-/-}) mice are protected from LPS-induced lung injury by suppressing NLRP3 pro-inflammatory cytokines¹⁷. These

studies support the notion that vimentin may play a role in macrophage immune response to IAV infection.

In this study, we use a mouse model of IAV to demonstrate that *vimentin-null* (*Vim*^{-/-}) mice are protected from the lung damage and mortality associated with severe viral pneumonia. Furthermore, we perform RNA-seq on MoAMs over a 6-day time-course following IAV infection to characterize the immune response. We define temporal gene expression patterns associated with three phases of IAV response in which MoAMs exhibit distinct biological functions. Next, we compare these results to the same RNA-seq time course in *Vim*^{-/-} MoAMs to determine which gene expression patterns and biological pathways contribute to the observed protection. Finally, we generate *Vim*^{-/-} bone marrow chimeras to test the role of MoAMs in response to IAV infection. Together, our results indicate that the activation of MoAMs in the Early Inflammatory phase of IAV response drives lung damage and lethality.

Results

Vimentin null mice are protected from influenza-induced lung injury and mortality

Our group have recently reported that persistent inflammation in patients with severe SARS-CoV-2 pneumonia is sustained via stable circuits between alveolar macrophages and T cells². Using single-cell RNA-seq, four distinct clusters of monocytes-derived alveolar macrophages (MoAMs) isolated from bronchoalveolar lavage fluid collected from patients with severe SARS-CoV-2 pneumonia were identified (**Figure 1a, Supplemental Figure 1a**)². By querying this dataset, we found that *VIM* was among the genes differentially

expressed between MoAM1 and MoAM3: with highest expression in MoAM1 and decreasing in MoAM3 (**Figure 1b,c**). MoAM1 cluster was characterized by high expression of pro-inflammatory cytokine genes, such as *IL1B*, *CXCL2*, and *CCL3* and low expression of macrophage markers *MRC1* and *C1QA* (**Figure 1c,d**). In contrast, cluster MoAM3 exhibited the highest expression of *MRC1*, *C1QA*, and *MERTK*. *MERTK* encodes a receptor necessary for phagocytosis of apoptotic cells – a process which has been mechanistically linked to a change in macrophage phenotype from pro-inflammatory to pro-resolving^{19,20} (**Figure 1c, d**). *VIM* expression did not vary between the two clusters annotated as tissue-resident alveolar macrophages (TRAMs) (**Supplemental Figure 1b**). This data from patients with severe SARS-CoV-2 pneumonia suggests that a higher level of *VIM* expression in MoAMs is associated with a pro-inflammatory phenotype in viral pneumonia, while MoAM with a pro-resolving phenotype was characterized by decreased *VIM* expression.

Next, we used a clinically relevant murine model of viral pneumonia induced by the influenza A virus to examine whether the loss of vimentin dampens the host immune response. Wild-type (WT) and *Vim*^{-/-} mice were infected with a lethal dose of IAV (Strain A/WSN/1933 (H1N1), 200 pfu) and assessed for morbidity and mortality. WT mice had a 100% mortality rate, whereas <17% of the *Vim*^{-/-} mice died by 10 days post-infection (p.i.) (**Figure 1e**). By 14 days p.i., the 50% of surviving *Vim*^{-/-} mice had recovered from mild lethargy, coat ruffling, febrile shaking, and eye-watering. *Vim*^{-/-} mice exhibited a lower wet-to-dry lung weight ratio and decreased total protein concentration in bronchoalveolar lavage fluid (BALF) than wild-type controls infected with IAV, indicating less damage to the

Figure 1

Figure 1. Vimentin-null mice are protected from influenza-induced lung injury and mortality. **a)** Uniform Manifold Approximation and Projection (UMAP) of 40,248 cells annotated as monocyte-derived alveolar macrophages (MoAMs) obtained from 10 patients with severe COVID-19 disease within 48 hours after intubation. **b)** Fraction of cells expressing *VIM* and mean expression in each MoAM cluster. **c)** Visualization of *IL1-B*, *CCL3*, *CXCL2*, *C1QA*, and *MERTK* expression in MoAM cells using density projection plots. Scale bar represents expression averaged within hexagonal areas on UMAP. **d)** Fraction of cells expressing the representative genes and their mean expression in each MoAM cluster. **e)** Survival of wild-type (WT) and *Vim*^{-/-} mice intratracheally infected with a lethal dose of influenza A virus (IAV) (200pfu) was measured and compared. Survival curves were analyzed using log-rank test, p-value =0.001. Data shown are n=12 per group. **f)** Lung wet-to-dry ratio measured at day 3 and 5 p.i. (n = 8-11/timepoint) **g)** Bronchoalveolar lavage fluid (BALF) protein levels measured by Bradford assay, n=5/timepoint. In **f** and **g**, differences between genotypes on each day were assessed by t-test. p-values were 0.0015 and <0.0001, for 3 and 5 days p.i., respectively. **h)** Representative images of murine lung architecture assessed by H&E staining, scale bar 100 um. **i)** Viral titer assessed by plaque assay. Difference between genotypes was statistically not significant. n=5-9/timepoint. **j)** Flow cytometric analysis of tissue resident (TRAMs) and infiltrating monocyte-derived alveolar macrophages (MoAMs) out of the total lung CD45⁺ cell population normalized to body weight during the IAV response, n=4/timepoint. **k)** Levels of inflammatory cytokines in BALF were assessed using a custom multiplex ELISA. n=3-4/timepoint. Bar graphs represent mean, error bars represent SD, differences between genotypes on each day were assessed by t-test. * p<0.05, ** p<0.001, *** p<0.0001, **** p<0.00001.

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Supplemental Figure 1. **a)** UMAP of 77,146 cells annotated by cell type obtained from 10 patients with severe COVID-19 disease within 48 hours after intubation. **b)** Fraction of cells expressing the representative genes and their mean expression in each TRAM cluster. **c)** Quantification of H1N1 viral RNA molecules detected among RNA isolated from MoAMs, normalized to the number of reads uniquely aligned to mouse genome (mm10) for each sample. Bar graphs represent mean, error bars represent SD. **d)** Gating strategy used for flow cytometry analysis of neutrophils, TRAMs, interstitial macrophages (IM) and MoAMs. **e)** Flow cytometric analysis demonstrated no differences in number of neutrophils out of the total lung CD45+ cell population normalized to body weight between *Vim*^{-/-} and WT mice during the IAV response.

alveolar-capillary barrier (**Figure 1f-g**). Histopathological examination of IAV-infected *Vim*^{-/-} mice lungs also demonstrated less severe alveolar damage, characterized by reduced alveolar edema and lower lung tissue damage than in IAV-infected WT mice (**Figure 1h**). Vimentin-deficiency did not interfere with IAV replication or clearance, as evidenced by comparable viral titers (**Figure 1i**) and similar levels of the H1N1 viral RNA molecules, such as matrix protein 2, nucleoprotein, and neuraminidase, which were detected in macrophages from WT and *Vim*^{-/-} mice by RNA-seq (**Supplemental Figure 1c**). These results indicate that *Vim*^{-/-} mice have decreased lung injury and improved survival rates following IAV infection independent of viral load.

The influx of immune cells into the airspace during IAV infection and the resulting production of pro-inflammatory cytokines is a major driver of lung injury. To characterize the immune cell recruitment into the lungs, we performed a systematic flow cytometric analysis of whole-lung lysates (**Supplemental figure 1d**). The number of TRAMs rapidly decreased after IAV infection at a similar rate in both genotypes, with a more than 80% reduction by 6 days p.i. (**Figure 1j**). MoAMs were deficient in the alveolar compartment at steady-state (e.g., 0 days p.i.) and recruited into the lung parenchyma following infection with IAV⁶. WT and *Vim*^{-/-} IAV infected lungs recruited comparably increasing numbers of MoAMs from 2 days p.i. onward (**Figure 1j**). Similarly, neutrophils peaked on day 3 p.i. in both genotypes (**Supplemental Figure 1e**). Despite similar immune cell recruitment, *Vim*^{-/-} mice had decreased levels of pro-inflammatory cytokines and chemokines, such as IL-1 β , TNF α , RANTES, MIP-2, MCP-1, and Type I interferons IFN α/β , compared to WT mice following IAV infection (**Figure 1k**). In summary, wild-type mice, but not *Vim*^{-/-} mice,

exhibited a hyperinflammatory phenotype in response to IAV. As there was no difference in MoAM recruitment between genotypes, these data implicate a vimentin-dependent change in MoAM function.

Three distinct temporal patterns characterize the transcriptional response of MoAMs to influenza A virus

To investigate the response of WT MoAMs to IAV infection, we performed RNA-seq on flow-sorted MoAMs to assess global gene expression profiles on 2, 3, 4, and 6 days post-IAV infection (**Supplemental Figure 2a, 2b**). We identified 2623 differentially expressed genes over this time course and, using k-means clustering, defined three clusters that correspond temporally to three phases of the IAV response: Infiltrating (2 days p.i.), Early Inflammatory (3-4 days p.i.), and Late Inflammatory (6 days p.i.) (**Figure 2a**).

The first cluster is characterized by genes with the highest expression in the **Infiltrating Phase** at 2 days p.i. (**Figure 2a-b, Supplemental Figure 2c**). Through Gene Ontology (GO) analysis, we find this cluster is enriched for genes associated with *cell migration* and *cell adhesion*, such as *Cx3cr1*, *Rock1*, *Tln1*, and *Cxcl14* (**Figure 2c-d**), likely involved in transendothelial migration of leukocytes²¹⁻²⁴. These results are consistent with the requirement for MoAMs to leave the vasculature and migrate across the endothelium to reach the alveolar space soon after IAV infection. We also observed genes in this cluster associated with the recruitment of lymphocytes, such as *Cd86*, MHC Class II gene *H2-Aa*, and *Ccl8* (**Figure 2c,d, Supplemental Figure 2d**). Furthermore, our results suggest that MoAM may be proliferating during this phase due to the enrichment of GO processes, such

Figure 2

Figure 2. Wild-type monocyte-derived alveolar macrophages exhibit gene expression patterns associated with three phases of influenza A viral infection. **a)** Heatmap of normalized expression of 2,623 differentially expressed genes (ANOVA adjusted p-value <0.05). Z-score normalization for each row was applied to gene expression (FPKM) data. K-means clustering (k=3) identified three clusters; Infiltrating (1131 genes), Early Inflammatory (440 genes), Late Inflammatory (1052 genes). **b)** Mean z-scores calculated across genes for each cluster **c)** Gene Ontology (GO) term enrichment for each phase. **d)** Bar graphs of gene expression (FPKM) for representative genes in each phase. Error bars represent SD. (ANOVA adjusted p-value <0.05). **e)** Bubble plot representing the proportion of genes (%) within each process that had an expression above the row mean. Color gradient represents mean z-score of expression across genes. **f)** Enrichment of gene sets in each cluster as defined in A. Asterisk represents significantly enriched overlaps (hypergeometric p-value <0.0018, FWER = 0.05). Dotted line indicates expected enrichment score of 1. n=3-4 per group. Ox Phos = Oxidative phosphorylation.

Supplemental Figure 2

Supplemental Figure 2. a) Schematic of experimental design and workflow. WT or *Vim*^{-/-} mice were infected with a lethal dose of influenza A virus (200 pfu). MoAMs were sorted by flow cytometry (FACS) from lungs on 2, 3, 4, and 6 days p.i. Total RNA was isolated, mRNA was enriched, and bulk RNA-seq was performed. Principal component analysis (PCA) was used to visualize replicates. **b)** Sequential gating strategy used for FACS collection of TRAMs and MoAMs **c)** Violin plots of log₂(FPKM+1) counts data for all genes in each cluster. Solid lines represent median, dotted lines represent quartiles. **d)** Bar graphs of gene expression (FPKM) in MoAM for representative genes in each phase. Error bars represent SD. (ANOVA adjusted p-value <0.05).

as *DNA replication initiation*, and the expression of cell cycle genes, such as *Mcm5*, in this cluster (**Figures 2c-f**). Taken together, these data show that the primary role of MoAMs in the Infiltrating Phase of the IAV response is to enter the lung parenchyma and promote the recruitment of immune cells.

The second cluster is characterized by genes that peak in expression during the **Early Inflammatory Phase** at 3 days p.i. (**Figure 2a-b, Supplemental Figure 2c**). This cluster is enriched for genes associated with *inflammatory response* and *regulation of cytokine secretion* (**Figure 2c**) – such as *Tlr2*, *Il1b*, and *Il1r2* (**Figure 2d**). These gene expression changes correspond with the secretion of inflammatory cytokines, such as IL-1 β and TNF α , beginning on Day 3 of IAV response (**Figure 1k**) and binding of viral PAMPs through pattern recognition receptors (PRRs), including TLR2²⁵. The expression of genes associated with *chemotaxis*, such as *Ccl3*, *Ccl2*, and *Cxcl2*, also peak during the Early Inflammatory phase (**Figure 2c-d**) and corresponds with the secretion of their respective proteins MCP-1, MIP1- α and MIP-2 measured in the BALF (**Figure 1k**). These data collectively suggest that WT MoAMs adopt a pro-inflammatory state during the Early Inflammatory Phase of the IAV response.

The third cluster is characterized by genes that display a continuous increase in expression until the **Late Inflammatory Phase** at 6 days p.i. (**Figure 2a-b, Supplemental Figure 2c**). This cluster is enriched for genes associated with the GO processes of *cell differentiation* and *adaptive immune response* – such as *Irf8*, *Mafb*, *Ifng*, *Ctsh*, *Ccr1* and, *Arg2* (**Figure 2d, Supplemental Figure 2d**). The increasing expression of *Irf8* and *Mafb* are in line with the transition from a monocyte to macrophage-like state²⁶. Although GO analysis implicated

metabolic processes in this cluster (**Figure 2c**), and macrophage activation has been associated with cellular respiration changes²⁷⁻²⁹, we did not observe preferential expression of genes in the glycolysis or oxidative phosphorylation pathways at any time point (**Figure 2e-f**). Interestingly, the gene encoding vimentin increased in expression over the time course, suggesting that vimentin may play a role throughout the IAV response (**Figure 2d**). Overall, these data suggest that WT MoAMs initiate a shift to a more adaptive immune response by the Late Inflammatory phase.

Since interferon pathways are an essential element of the viral immune response, we investigated Type I and Type II interferon genes' temporal expression. We found Type I *IFN α* response genes peaked in expression on day 2, which coincided with their enrichment in the Infiltrating cluster (**Figure 2e-f**). In contrast, Type II *IFN γ* response genes did not reach their highest expression level until 6 days p.i. and were enriched in the Late Inflammatory cluster (**Figure 2e-f**). This data supports Type I interferon's role as an early effector of the immune response, while Type II interferon is involved in the later adaptive immune response.

While GO analysis can be informative, many of the terms, such as *inflammatory response* and *innate immune response*, lack cell-type-specificity. We took advantage of published studies of *in vitro* stimulated MoAMs, polarized towards a pro-inflammatory or alternatively-activated phenotype through the administration of LPS or IL-4, respectively³⁰⁻³³. To determine whether similar states were observed during our IAV time course, we generated

annotations for these responses by integrating the gene sets resulting from several published studies on LPS ($M_{(LPS)}$) and IL-4 ($M_{(IL-4)}$) stimulation in macrophages (see Methods). We found that $M_{(IL-4)}$ genes peaked on day 2 p.i. and were enriched in the Infiltrating cluster (**Figure 2e-f**). Furthermore, we found that $M_{(LPS)}$ genes peaked on day 3 p.i. in WT MoAMs (**Figure 2e**) and were enriched in the Early Inflammatory cluster (**Figure 2f**). $M_{(LPS)}$ genes were also enriched to a lesser degree in the Late Inflammatory cluster (**Figure 2f**). It is worth noting that although we observe these general trends, the *in vivo* state of MoAMs does not entirely reflect the responses defined by our *in vitro* derived annotated gene lists. Taken together, our results support a critical switch in WT MoAM function as they transition from their initial transcriptional state in the Infiltrating phase to a more pro-inflammatory phenotype during the Early and Late Inflammatory phases.

At least two phenotypically distinct macrophages are present in the lung at steady-state: TRAMs and interstitial macrophages (IMs). In steady-state conditions, IMs are found at two-fold lower frequencies than TRAMs in mouse lung tissue³⁴. Interstitial macrophage numbers expand more rapidly in response to PAMPs and DAMPs compared with TRAMs³⁴, and are preferentially replenished from blood monocytes compared with TRAMs³⁵. To determine whether MoAMs resembled steady-state lung macrophages during the response to IAV, we defined TRAM- and IM-specific gene sets by performing RNA-seq on flow-sorted TRAM and IM cells from uninfected WT animals (**Figure 3a**). We observed that WT MoAMs decrease expression of IM-specific genes over the course of IAV infection, while they increase expression TRAM-specific genes (**Fig 3b,c**). For example, *Cx3cr1* and *Lyve1* exhibit highest expression on day 2 p.i., while *Il17ra* and *Csf2rb*

Figure 3

Figure 3. Wild-type monocyte-derived alveolar macrophages resemble interstitial macrophages during the infiltrating phase and exhibit increased expression of alveolar macrophages genes over time. **a)** Volcano plot of differentially expressed genes (edgeR $p\text{-value} < 0.05$, and edgeR reported $\log_2\text{FC} > |1.0|$) between TRAM and IM at steady-state (0 dpi). The number of genes increased (red) and decreased (blue) in TRAMs compared to IMs are shown. **b)** Mean z-scores calculated for IM- and TRAM-specific gene sets over the course of IAV infection in MoAMs **c)** Plots representing % of genes at each time point expressed above the mean. Each dot represents a replicate. Lines represent median. **d)** Bar graphs of gene expression (FPKM) for representative genes in IM- and TRAM-specific gene sets. Error bars represent SD. **e)** Enrichment of IM- and TRAM-cell specific gene signatures in MoAMs for each phase. Asterisk represents significantly enriched overlaps (hypergeometric $p\text{-value} < 0.006$, FWER = 0.05).

progressively increase in expression until day 6 p.i. (**Figure 3d**). Similarly, the IM gene signature is significantly enriched in the Infiltrating phase, whereas and the TRAM gene signature is significantly enriched in both the Early and Late Inflammatory clusters (**Figure 3e**). These data suggest that WT MoAMs resemble steady-state IMs transcriptionally upon extravasation, but become more like steady-state TRAMs as IAV infection progresses.

***Vim*^{-/-} MoAMs exhibit increased expression of Infiltrating phase genes and decreased expression of Early Inflammatory phase genes**

To gain a better understanding of the conferred protection from IAV infection in *Vim*^{-/-} mice (**Figure 1**), we performed RNA-seq on flow-sorted MoAMs from *Vim*^{-/-} mice at days 2, 3, 4, and 6 following IAV infection (**Supplemental Figures 2a, 2b, 4a**). Using the prior clustering of 2623 genes across the three phases of the IAV response (**Figure 2a**), we compared expression between MoAMs in WT and *Vim*^{-/-} mice (**Figure 4a**). While *Vim*^{-/-} MoAMs exhibited the same overall gene expression patterns, we found that genes in the Infiltrating cluster tended to be increased in expression while genes in the Early Inflammatory cluster tended to be decreased (**Figure 4b-d, Supplemental Figure 4b**). In contrast, genes in the Late Inflammatory cluster were less changed between genotypes (**Figure 4b-d, Supplemental Figure 4b**). Interestingly, day 3 p.i. represented the largest difference between genotypes in any cluster. In line with these observations, day 2 and day 3 *Vim*^{-/-} MoAMs were globally more similar to each other than their WT counterparts, and the change in expression associated with the transition from 2 to 3 days p.i. was reduced (**Supplemental Figure 4c,d**). Steady-state *Vim*^{-/-} TRAM and IM gene expression from uninfected mice were mostly unchanged from WT cells, suggesting that vimentin-deficiency

Figure 4. *Vim*^{-/-} MoAMs exhibit increased expression of Infiltrating phase genes and decreased expression of Early Inflammatory phase genes. **a)** Heatmap of normalized expression of 2,623 genes as defined in Figure 2a. *Vim*^{-/-} data was normalized to the WT mean and standard deviation (adapted z-score). **b)** Mean adapted z-scores were calculated across genes for each cluster in WT MoAMs and *Vim*^{-/-} MoAMs. Shading represents WT MoAMs inter-quartile range (IQR) **c)** Bar graphs representing the fold change in gene expression (FPKM) in *Vim*^{-/-} MoAMs compared to the WT MoAMs mean for representative genes. (n=3-4 per group). **d)** % genes in each cluster that exhibit decreased or increased expression in *Vim*^{-/-} MoAMs compared to WT MoAMs (n=3-4 per group). Decreased genes defined as $\log_2\text{FC} < -0.58$ (FC of 1.5); increased genes defined as $\log_2\text{FC} > 0.58$.

Supplemental Figure 4

Supplemental Figure 4. **a)** Principal component analysis (PCA) of *Vim*^{-/-} MoAMs replicates. **b)** Violin plots representing the log₂FC between *Vim*^{-/-} and WT for each gene by cluster in MoAMs. Solid lines represent median, dotted lines represent quartiles. **c)** Heatmap showing pairwise Pearson correlation of mean gene expression across replicates between each time point in WT and *Vim*^{-/-} MoAMs. Pearson correlation coefficient values shown within each square. Scale bar represents Pearson correlation coefficient. **d)** Violin plots representing the log₂FC between days p.i. for each gene by cluster in WT and *Vim*^{-/-} MoAMs. Solid lines represent median, dotted lines represent quartiles. **e)** Violin plots representing the log₂FC between *Vim*^{-/-} and WT in TRAMs and IMs at steady-state (0 dpi) for each gene by cluster. Solid lines represent median, dotted lines represent quartiles. **f)** Heatmap of normalized expression of 2,623 differentially expressed genes in *Vim*^{-/-} MoAMs (ANOVA adjusted p-value <0.05). Adapted Z-score normalization for each row was applied to gene expression (FPKM) data using *Vim*^{-/-} MoAMs mean and standard deviation. K-means clustering (k=3) was performed on *Vim*^{-/-} MoAMs expression and WT MoAMs data was visualized for the same genes **g)** Mean adapted z-scores were calculated across genes for each cluster in *Vim*^{-/-} and WT MoAMs. Shading represents *Vim*^{-/-} MoAMs IQR.

has a larger effect during the response to IAV-infection than in steady-state (**Supplemental Figure 4e**).

Additionally, we performed a reverse analysis where we first clustered on differentially expressed genes across IAV infection in *Vim*^{-/-} MoAMs and then compared with the expression of these 2623 genes in WT MoAMs (**Supplemental Figure 4f,g**). Although the gene sets did not entirely overlap, the resulting gene expression patterns were generally conserved, and no new GO processes were identified. These results support an altered function of *Vim*^{-/-} MoAMs in response to IAV that favors the maintenance of Infiltrating phase genes and the suppression of Early Inflammatory phase genes to confer protection from influenza-induced lung injury.

Identification of cellular processes impacted in *Vim*^{-/-} MoAMs response to IAV.

To further investigate the pathways influenced by vimentin-deficiency in IAV infection, we evaluated global gene expression between genotypes on each day p.i. (**Figure 5a**). Compared to WT MoAMs, we found that *Vim*^{-/-} MoAMs exhibit decreased expression of genes involved in the inflammatory response and stress response throughout IAV infection and increased expression of genes involved in cell cycle and extracellular matrix organization (**Figure 5b**). Furthermore, we found that gene sets associated with inflammation – including M(LPS), Type I Interferon, and Type II Interferon – were significantly decreased in expression in *Vim*^{-/-} MoAMs on every day p.i.; however, these responses peaked at different days of IAV infection in wild-type mice (**Figure 5c**, **Supplemental Figure 5a-c**). In contrast, *Cell Cycle* and *M(IL-4)* associated genes were

Figure 5

Figure 5. Inflammatory pathways are consistently decreased in *Vim*^{-/-} MoAMs compared to WT MoAMs during influenza A infection. **a)** Volcano plots of differentially expressed genes (edgeR p-value<0.05, and edgeR reported log₂FC>|1.0|) between *Vim*^{-/-} and WT MoAMs on each day post-infection. The number of genes increased (red) and decreased (blue) in *Vim*^{-/-} compared to WT MoAMs are shown. **b)** GO term enrichment across days p.i. in the increased (red) and decreased (blue) genes in *Vim*^{-/-} compared to WT MoAMs. Scale represents -log₁₀ p-value. **c)** Violin plots representing the log₂FC for each process between *Vim*^{-/-} and WT MoAMs. Solid lines represent median, dotted lines represent quartiles. **d)** Scatter plots of z-score normalized gene expression across genes in M_(IL-4) vs Cell Cycle. Pearson correlation coefficients (r) across WT or *Vim*^{-/-} MoAM replicates and associated p-values are shown. **e)** Heatmap matrix of Pearson correlation between processes, asterisk indicate p-value <0.05.

Supplemental Figure 5

Supplemental Figure 5. **a)** Plots representing % of genes at each time point expressed above their WT mean. Each dot represents a replicate. Lines represent median. **b, c)** Gene Set Enrichment Analysis (GSEA) enrichment plots. GSEA was performed on gene lists ranked by edgeR reported fold-change between *Vim*^{-/-} and WT MoAMs at each time point. Normalized enrichment scores (NES) are shown. Asterisks indicate statistically significant adjusted p-values <0.05. **d)** Violin plots representing the log₂FC between *Vim*^{-/-} and WT TRAMs and IMs at steady-state (0 dpi). Solid lines represent medians, dashed lines represent quartiles.

increased in expression in *Vim*^{-/-} MoAMs compared to WT MoAMs (**Figure 5c, Supplemental Figure 5a-c**). In this case, the difference in expression between genotypes appears to be driven by the failure of *Vim*^{-/-} MoAMs to reduce the expression of *Cell Cycle* and *M(IL-4)* associated genes during the course of IAV infection as observed in WT mice. None of these gene sets were significantly different in expression in steady-state alveolar macrophages (TRAMs and IMs) **Supplemental Figure 5d**).

We next asked whether any of the individual gene sets correlated with each other. We observed that *M(IL-4)* and Cell cycle gene expression are highly correlated in both genotypes, suggesting they may be part of a more extensive network of co-regulated processes (**Figure 5d-e**). Taken together, these data suggest that MoAM activation following IAV infection is affected by the loss of vimentin through the precise temporal regulation of specific pathways.

***Vim*^{-/-} MoAMs fail to activate core pro-inflammatory genes in the early response to IAV**

Thus far, our results suggest that the suppression of pro-inflammatory genes across IAV infection, particularly in the Early Inflammatory phase, is responsible for the protection conferred by vimentin-deficiency. To identify specific genes involved in this process, we focused on the *M(LPS)* response, which was associated with an increased expression on day 3 p.i. in WT mice. In MoAMs from *Vim*^{-/-} mice, fewer *M(LPS)* genes exhibited peak expression on day 3 p.i. (**Figure 6a**). Moreover, *M(LPS)* genes were enriched among those genes exhibiting the greatest expression decrease in *Vim*^{-/-} MoAMs compared with WT

Figure 6

Figure 6. Protective phenotype of *Vim*^{-/-} mice is associated with the failure of *Vim*^{-/-} MoAMs to activate core pro-inflammatory genes in the early response to IAV. **a)** Percent (%) $M_{(LPS)}$ genes peaking in expression on each day post-infection in WT and *Vim*^{-/-} MoAMs **b)** Bar graphs of gene expression (FPKM) for representative $M_{(LPS)}$ genes present in the leading edge of at least 1 day from GSEA. Error bars represent SD. **c)** Heatmap of normalized expression of 84 $M_{(LPS)}$ genes present in the GSEA leading edge on at least 1 day (indicated by color-coded bar on left). Adapted Z-score normalization using WT mean and standard deviation for each row was applied to gene expression (FPKM) data. **d)** Histogram representing the distribution of $\log_2FC$ between *Vim*^{-/-} and WT MoAMs on 3 dpi of the Early Inflammatory phase (440 genes). Dashed line represents mean of the distribution. Number of genes less than (186) and greater than (254) the mean are shown. Genes with $\log_2FC$ between *Vim*^{-/-} and WT MoAMs less than the mean represent those genes that are most changed between *Vim*^{-/-} and WT MoAMs. **e)** Mean adapted z-scores were calculated across genes for most (186) and least (254) changed genes on 3 dpi in the Early Inflammatory Phase in WT and *Vim*^{-/-} MoAMs. **f)** HOMER known motif analysis using genomic background of most changed genes on 3 dpi in the Early Inflammatory Phase (186 genes). Motif logos are shown below motif names. $-\log_{10}p$ values of enrichment in most or least changed gene set is shown. $\log_2(FPKM+1)$ average gene expression of transcription factor family members are shown for WT TRAMs and WT IMs at steady-state (0 dpi) and 2-6 dpi for WT MoAMs. $\log_2FC$ between *Vim*^{-/-} and WT MoAM gene expression of transcription factor family members on each dpi. (n=3-4)

Supplemental Figure 6

Supplemental Figure 6. **a)** Mean adapted z-scores for $M_{(LPS)}$ and $M_{(IL-4)}$ genes present in the leading edge of at least 1 day from GSEA in WT and *Vim*^{-/-} MoAMs. **b)** Heatmap of normalized expression of $M_{(IL-4)}$ genes present in the leading edge of at least 1 day from GSEA. Adapted Z-score normalization for each row was applied to gene expression (FPKM) data. Bar graphs of gene expression (FPKM) for representative $M_{(IL-4)}$ genes present in the leading edge of at least 1 day from GSEA. Error bars represent SD. **c)** Heatmap of normalized expression of 186 most changed, and 254 least changed genes on 3dpi of the Early Inflammatory Phase. Adapted Z-score normalization for each row was applied to gene expression (FPKM) data. **d)** HOMER known motif analysis using genomic background of least changed genes on 3 dpi in the Early Inflammatory Phase (254 genes). Motif logos are shown below motif names. $\log_2(FPKM+1)$ average gene expression of transcription factor family members are shown for WT TRAMs and WT IMs at steady-state (0 dpi) and 2-6 dpi for WT MoAMs. $\log_2FC$ between *Vim*^{-/-} and WT MoAM gene expression of transcription factor family members on each dpi. (n=3-4). **e)** Motif logos are shown below motif names. $-\log_{10}p$ values of enrichment in most or least changed gene set is shown.

during the course of IAV response (**Supplemental Figure 5b**). Of the $M_{(LPS)}$ genes that were most decreased on at least one day, we find that the majority failed to intensify in expression on day 3 p.i. in $Vim^{-/-}$ MoAM compared with WT MoAM (**Figure 6b-c, Supplemental Figure 6a**). This core set of pro-inflammatory genes includes interferon-stimulated genes (ISGs), such as *Ifit1*, *Irgm1*, and *Rsad2*, genes associated with chemokine pathways, including *Ccr12* and *Cxcl2*, and genes associated with pathogen sensing, *Fcgr1*, and *Tlr2* (**Figure 6b-c**). In contrast, $M_{(IL-4)}$ genes, such as *Cd36*, *Cd11c*, and *Cd83*, exhibited higher expression on all days post IAV infection in $Vim^{-/-}$ MoAMs compared with WT MoAMs (**Supplemental Figures 5b, c, 6b**). Collectively, these data suggest $Vim^{-/-}$ MoAMs fail to activate core pro-inflammatory genes associated with the Early Inflammatory phase in response to IAV.

Prior work has demonstrated that macrophage activation is largely defined by the activity of cell-type-specific transcription factors (TF) that poise regulatory elements for activation by stimulus-specific TFs³⁶⁻³⁸. To better characterize the impact on gene regulation observed in $Vim^{-/-}$ MoAMs, we searched for TF binding sites enriched in the promoters of vimentin-dependent genes associated with the Early Inflammatory phase. First, we classified the 440 Early Inflammatory genes into those “most changed” and “least changed” in $Vim^{-/-}$ MoAMs based on their fold-change compared with WT MoAMs (**Figure 6d-e, Supplemental Figure 6c**). Next, we performed motif-finding on each gene set using a known sequence motifs database (see Methods). We found that TF families previously implicated in an inflammatory response, such as *Jun-AP1* and *Rela*, were significantly enriched among the most changed but not the least changed genes (**Figure 6f**). Some *Irf*

family motifs were differentially enriched between the two gene sets, while others were shared (**Figure 6f**). As supported by induction of gene expression in IAV and the decrease observed in *Vim*^{-/-}, those associated with ISGs (*Irf1/7/9*) were more likely to be affected by vimentin-deficiency (**Figure 6f**), while *Irf8*, which is more generally involved in macrophage function, exhibited similar enrichment in both groups (**Supplemental Figure 6e**). Furthermore, the CEBP motif was associated with the most changed genes in *Vim*^{-/-} MoAMs and *Cebpb* was expressed highly at both baseline and throughout IAV infection (**Figure 6f**). This observation is consistent with *Cebpb*, a known macrophage TF which can also act in combination with AP1 factors (**Figure 6f**), specifying poised regulatory elements in macrophages that activate genes on stimulation^{39,40}. On the other hand, ETS was the main TF family with motifs enriched in the least changed but not the most changed genes (**Supplemental Figure 6d**). This family marginally includes PU.1, a well-known myeloid lineage factor, but PU.1 is unlikely to have a specific role in IAV response (**Supplemental Figure 6e**). Instead, the expression of *Elf1* and *Elf4* suggests that they may have an interferon-independent antiviral role in influenza as previously reported⁴¹, that is not impacted by vimentin-deficiency. In summary, vimentin-deficiency impedes the stimulus-dependent regulation of pro-inflammatory genes in MoAMs during the Early Inflammatory phase of IAV infection. Although this effect leads to subtle changes in overall gene expression, it appears to be sufficient to alter MoAM function.

Vimentin-deficient MoAMs are sufficient to confer protection from IAV-induced mortality

To determine whether vimentin-deficiency in recruited macrophages alone decreases lung injury following IAV infection, bone marrow from either WT or *Vim*^{-/-} donor mice (CD45.2) were transferred into irradiated WT recipient mice (CD45.1), allowing for the specific reconstitution of MoAMs with a WT or *Vim*^{-/-} genotype (**Figure 7a**). All mice showed reconstitution of donor hematopoietic cells in peripheral blood above 85% (**Supplementary Figure 7a**). Following confirmed engraftment and recovery, mice were infected with a lethal dose of IAV (**Figure 7a**). We found that IAV-infected WT mice who received *Vim*^{-/-} donor bone marrow exhibited significantly lower total protein concentration in the BALF and lower wet-to-dry lung weight ratio than mice that received WT donor bone marrow, indicating less severe alveolar damage (**Figure 7b-c**). Mice showed no difference in IAV replication or clearance regardless of donor genotype (**Figure 7d**). As in *Vim*^{-/-} mice (**Figure 1**), WT mice with *Vim*^{-/-} MoAMs exhibited lower levels of pro-inflammatory cytokines and chemokines (**Figure 7e**). Taken together, these data support the notion that vimentin-deficiency is sufficient to alter MoAM function and provide protection to the host in response to IAV infection. Thus, it follows that MoAMs that express a pro-inflammatory phenotype drive lung injury following IAV infection.

Discussion

We used a clinically-relevant murine model of severe viral pneumonia to identify recruited alveolar macrophage-specific gene expression patterns associated with three distinct phases: Infiltrating, Early Inflammatory, and Late Inflammatory. WT MoAMs began expressing pro-inflammatory genes and secreting tissue-damaging pro-inflammatory cytokines in the Early Inflammatory phase, resulting in increased morbidity and mortality in

Figure 7

Figure 7. Vimentin-deficiency in the MoAM compartment is sufficient to protect from influenza A virus-induced lung damage and mortality. **a)** Schematic of the experimental design. Recipient mice (CD45.1) were irradiated and reconstituted with bone marrow cells from either WT or *Vim*^{-/-} donor mice (CD45.2). After a 30-day recovery period, chimeric mice were intratracheally (i.t.) infected with a lethal dose of IAV at 5 days p.i. and subjected to analyses. **b)** Bronchoalveolar lavage fluid (BALF) protein levels measured by Bradford assay, n=3-4 **c)** Lung wet-to-dry ratio measured at day 5 p.i. n=7-10. **d)** Viral titer assessed by plaque assay. n=11-14. **e)** Levels of inflammatory cytokines in BALF assessed using a custom multiplex ELISA. n=4-9. Bar graphs represent mean, error bars represent SD. Differences between genotypes were assessed by t-test. * p<0.05, ** p<0.001, *** p<0.0001, **** p<0.00001.

Supplemental Figure 7

Supplemental Figure 7. Representative flow cytometry dot plots demonstrate successful engraftment of WT and *Vim*^{-/-} CD45.2 donor cells into CD45.1 recipient mice.

influenza-infected wild-type mice. Conversely, *Vim*^{-/-} mice suppressed the expression of pro-inflammatory genes and the secretion of pro-inflammatory cytokines conferring protection to the host. By leveraging *Vim*^{-/-} mouse's protective phenotype, we identified a core set of proinflammatory genes that drive lung injury and mortality. Although prior studies have examined macrophage gene expression at steady-state or single time points following IAV infection^{4,42}, by analyzing gene expression over a time course post-IAV infection, our results identified critical temporal dynamics that shape the function of recruited alveolar macrophages in response to respiratory infections.

In vitro studies have established vimentin's role in essential intracellular processes, such as cell motility, cell signaling, protein complex scaffolding, and mechano-sensing through the dynamic reorganization of vimentin intermediate filaments (IFs). In morphological studies of circulating lymphocytes, lymphocyte extravasation was associated with the pronounced reorganization of the vimentin IF network. Retinoic acid-inducible gene I, a cytosolic PRR that detects viral RNA and mediates induction of type I interferon expression, leads to increased vimentin expression⁴³. Our data support this previous observation, as type I interferon genes peaked on day 2 p.i., preceding the increase in vimentin gene expression in WT MoAMs. We confirmed previous reports that *vimentin-deficient* mice fail to secrete mature IL-1 β , as assembly, and activation of the NLRP3 inflammasome requires vimentin^{17,18}. Complementary to our findings, vimentin's interaction with NOD2, a cytoplasmic PRR, activated NF- κ B and induced a proinflammatory immune response in an inflammatory bowel disease model¹⁶. Collectively, these and our studies suggest that vimentin mediates the innate immune response(s) through the dynamic reorganization of

its IF network, protein:protein interactions with PRRs, and regulating the expression of pro-inflammatory genes. Our findings show that depleting vimentin in MoAMs modulates the proinflammatory response and protects the host. It serves as a proof-of-principle to support the development of pharmacological agents to target vimentin selectively. The tools may be clinically beneficial in modulating the hyperinflammatory microenvironment of the virus-infected lung.

We used a well-established bone-marrow chimera mouse model to demonstrate that *Vim*^{-/-} MoAMs were sufficient to confer protection from IAV-induced lung injury and mortality. However, by generating a *Vim*^{fl/fl}*Cx3cr1*^{CreERT2}*zsGreen* transgenic mouse, we could selectively delete vimentin and track the recruitment of *GFP*⁺*Vim*^{-/-} alveolar macrophages during IAV infection. Regrettably, the vimentin-floxed mouse is not yet available. Second, our RNA-seq data identified distinct transcriptional phases of the IAV response in MoAMs; however, it is unclear whether different MoAM subpopulations, as suggested by prior work², entered the lung at different time points from a single compartment. For example, MoAMs may differentiate directly from circulating monocytes that extravasate from the pulmonary capillaries into the alveolar space. Alternatively, MoAMs may stem from interstitial macrophages already residing in the lung at steady-state. Pulsed lineage tracing using the *Vim*^{fl/fl}*Cx3cr1*^{CreERT2}*zsGreen* mouse would address these important questions.

In summary, we identified three distinct temporal patterns that characterize the transcriptional response of MoAMs to IAV infection. Leveraging the protective phenotype observed in *Vim*^{-/-} mice, we identified a core set of pro-inflammatory genes whose

upregulation during the early response to IAV drives lung injury and mortality. We show that vimentin-deficient MoAMs exhibit a dampened inflammatory response in a cell-autonomous manner, sufficient to confer protection from IAV-induced mortality. Our results define the temporal, transcriptional dynamics in recruited alveolar macrophages that shape the response to respiratory infection and demonstrate that vimentin plays an essential role in modifying macrophage phenotype and function.

Materials & Methods

In silico single cell RNA-seq data analysis. We queried a single cell RNA-seq transcriptomic data set derived from bronchoalveolar lavage samples from 10 patients with severe SARS-CoV-2 viral pneumonia². We downloaded normalized counts tables and visualized the pre-determined cell population clusters using Scanpy⁴⁴. 40,000 single cells representing the four monocyte-derived alveolar macrophages (MoAMs) populations were visualized as an UMAP. Mean expression data of individual genes per cell population cluster were visualized as dot plots in Scanpy.

Mice. All experiments were performed using wild-type or vimentin-null (*Vim*^{-/-}) 129S/V mice, age 8-12 weeks. Wild-type mice were purchased from Taconic, *Vim*^{-/-} mice were a gift from Albee Messing (University of Wisconsin, Madison, WI). All animals were kept under pathogen-free conditions, and all experiments were approved in accordance with Northwestern University's Institutional Animal Care and Use Committee (IACUC) guidelines.

Bone marrow chimeras. 8-12 week old recipient mice (Ptpcr^a/Pep-Boy, CD45.1) were lethally irradiated with a single dose of 1,000 cGy γ-radiation using a Cs-137–based

Gammacell-40 irradiator (Nordion). Within 24 hours, fresh bone marrow cells (2×10^6) from either WT donor mice or *Vim*^{-/-} donor mice (CD45.2) were injected via retro-orbital injection. Mice were housed together in individually ventilated cages and were given *ad libitum* access to food and water supplemented with Trimetoprim/Sulfamethoxazole (TMP/SMX 40/8 mg formula, Hi-Tech Pharmacal) for 4 weeks and then switched to standard housing regimen. Reconstituted mice were used for influenza infection experiments ~4 weeks after transplantation. Engraftment was assessed after two weeks, using flow cytometry to measure the percentage of CD45.1+ and CD45.2+ cells in peripheral blood collected from the facial vein. All mice used for experiments showed reconstitution of the donor (CD45.2) circulating leukocytes in peripheral blood above 85%.

Virus. H1N1 Influenza virus strain A/WSN/1933 (WSN) was propagated in fertile chicken eggs (Sunnyside Hatchery) as previously described^{5,6}. Viral titers were determined by measurement of plaque-forming units (pfu) in Madin-Darby canine kidney epithelial cells (ATCC).

Plaque Assay. Confluent monolayers of Madin-Darby canine kidney epithelial cells were infected with stock WSN virus in 1% BSA DMEM for 2h at 37°C. Plates were washed with PBS and an overlay of 50% 2x replacement media (2x DMEM, 0.12M NaHCO₃, 2% penicillin-streptomycin, and 1% HEPES), 50% Avicel (2.35%), and N-acetyl trypsin (1.5µg/mL) remained on the cells for 72h at 37°C. Plates were washed with PBS and fixed with 0.2% PFA before staining the monolayers with naphthalene blue-black stain.

In vivo influenza virus infection. 8-12 week old WT and *Vim*^{-/-} 129S/V male mice were anesthetized using isoflurane and intratracheally (i.t.) infected with 200 pfu WSN. Mice were monitored daily, and weight was recorded every 24 hours.

Histology. Mice were euthanized, after which a 20-gauge angiocath was sutured into the trachea. Heart and lungs were removed *en bloc*, and lungs were inflated with 0.8mL of 4% paraformaldehyde (PFA) at a pressure not exceeding 16 cm H₂O. Tissue was fixed and embedded in paraffin. 5 µm sections were stained with H&E by the Mouse Histology Phenotyping Laboratory (Northwestern University, Chicago, IL). Tissue sections were visualized using TissueGnostics, a Tissue/Cell High Throughput Imaging Analysis Systems (Vienna, Austria) and captured using TissueFAXS software (TissueGnostics, Los Angeles, CA) at the Northwestern University Cell Imaging Facility (Northwestern University, Chicago, IL).

Bronchoalveolar Lavage. A 20-gauge angiocath was ligated into the trachea, and 1mL of sterile PBS was instilled into the lungs, then removed through the angiocath. This process was repeated three times. Bronchoalveolar lavage fluid (BALF) was centrifuged at 500 x g for 10 min. Protein levels in the supernatant were measured by Bradford assay (Bio-Rad), and cytokine levels were measured using a custom multiplex ELISA (ThermoFisher Scientific).

Wet-to-dry weight ratio. Mice were euthanized, and lungs were surgically removed *en bloc*. The left lung was ligated, excised, and weighed in a tared container. The left lung was dried at 70°C in a Speed-Vac SC100 evaporator (Thermo Scientific, Waltham, MA) until a constant weight was obtained, and the wet-to-dry weight ratio was calculated.

Flow cytometry analysis of lung cell populations. Mice were euthanized, and lungs were perfused with 10mL HBSS with Ca²⁺ and Mg²⁺. Lung lobes were removed and inflated with enzyme solution (5mL of 0.2 mg/mL DNase I and 2 mg/mL collagenase D in HBSS with Ca²⁺ and Mg²⁺) using a 30-gauge needle. Tissue was minced and processed in

gentleMACS dissociator tubes according to the manufacturer's instructions (Miltenyi Biotec). Processed lungs were passed through a 40 μ m cell strained, and RBCs were lysed using BD PharmLyse (BD Biosciences, San Jose, CA). The remaining cells were counted using a Countess cell counter (Invitrogen, Grand Island, NY). CD45 microbeads were added, and cells were eluted according to the Miltenyi manufacturer's instructions. Cells were stained with viability dye Aqua (Invitrogen), and non-specific antibody (Ab) binding was inhibited by adding Fc Block (553142, clone 2.4G2; BD Pharmingen). Blocked cells were stained with a mixture of fluorochrome-conjugated Abs (see **Online Supplemental Table 1** for list of Abs). Data were acquired on a BD LSR II flow cytometer using BD FACSDiva software (BD Biosciences), and data analyses were performed using FlowJo software (Tree Star, Ashland, OR). Cell populations were identified using a sequential gating strategy (**Supplemental Figure 1b**). Cell number was normalized to body weight.

Macrophage harvesting and flow sort. Mice were euthanized and perfused with 5 mL sterile PBS by cardiac perfusion through the right ventricle. A 20-gauge angiocath was ligated into the trachea, and the lung and trachea were removed *en bloc*. Lungs were instilled with 1 mL dispase (Corning) through the angiocath, and a second suture was tied around the trachea to avoid leakage. Lungs were placed in 50 mL tubes (Falcon) containing 750 μ L cold dispase and rocked for 45 min at RT. Trachea and angiocath were removed, and lungs were minced using curved-edge scissors. Digested tissue was poured into 50 mL tubes containing 7 mL DMEM (Corning) and 14 μ L DNase I (50 mg/mL, Sigma). Tubes were rocked for 10 minutes at RT. Using serological pipettes, tissue was gently further disrupted and subsequently filtered through 70 μ m cell strainers and 40 μ m strainers (Corning). Tubes were rinsed with DMEM + 5% FBS and filtered through the same

strainers. Cell suspensions were centrifuged for 10 min at 1300 rpm, 4°C and pellets were resuspended in 350 μ L MACS buffer (Milteny). 100 μ L CD11c magnetic microbeads (Milteny) were added, and cells were eluted according to the Miltenyi manufacturer's instructions. Cells were stained for CD11b (Biolegend), Siglec F (BD), Ly6C (eBioscience), CD45 (eBioscience), MHCII (Biolegend), CD64 (Biolegend), Ly6G (BD), and NK1.1(BD). Viability dye (eFluor506, eBioscience) was used to sort living cells only (**Online Supplemental Table 1**). Using sequential gating (**Supplemental Figure 2b**), recruited alveolar macrophages (MoAMs) and tissue-resident alveolar macrophages (TRAMs) were flow-sorted using a BD FACSaria III 5-laser cell sorter and 100 μ m nozzle (BD Biosciences). Of note, MoAMs were collected in the same gate as interstitial macrophages (Ly6C-, CD64+); however, by 2 days p.i., interstitial macrophages were no longer present in this gate (see **Online Supplemental Figure 2**). Flow-sorted cells were collected into 1.5 mL Eppendorf tubes containing 200 μ L MACS buffer and centrifuged at 5,000 rpm for 5 min at RT. Pellets were resuspended and lysed in RLT lysis buffer (Qiagen) and stored at -80°C until all time points were collected.

RNA sequencing (RNA-seq). Samples collected from infected mice that at flow sort appeared uninfected (had no infiltrating MoAM) were excluded from RNA isolation. RNA was isolated in all samples at the same time to minimize the batch effect. All libraries for a given cell type were prepared as a single batch for sequencing. Total RNA was isolated using RNeasy Plus Mini kits (Qiagen). RNA quality (RIN) and quantity were assessed using a TapeStation 4200 (Agilent Technologies). RIN scores ranged from 7.2 to 9.4, with an average of 8.6. Using poly(A) mRNA magnetic enrichment kits from NEBNext (New England Biolabs), mRNA was isolated. cDNA libraries were prepared using NEBNext Ultra

DNA library kits (New England BioLabs) for Illumina. Libraries were sequenced using high-output 75-cycle single-end kits on an Illumina NextSeq 500 sequencer to an average depth of 4.1 million reads per sample. Reads were demultiplexed (bcl2fastq), trimmed, and aligned to reference genome mm10 using TopHat2⁴⁵. Aligned reads were mapped to genes using HTSeq with an Ensembl annotation (version 78)⁴⁶.

RNA-seq analysis. For k-means clustering, we used gene counts normalized to FPKM (Fragments per Kilobase of transcript per Million mapped reads). Low counts were removed, with a cut-off set as row sum >27 (# of samples used for analysis), yielding a gene counts table of 9,169 expressed genes. One-way ANOVA with Benjamini-Hochberg (BH) FDR correction was used to define differentially expressed genes over time. Genes with an FDR <0.05 were considered differentially expressed, resulting in 2,623 DEGs. Clustering was performed on the 2,623 DEGs using k-means clustering (GENE-E, <https://software.broadinstitute.org/GENE-E/index.html>).

For pairwise comparison, low counts were trimmed if CPM <2 in all replicates in the smallest group of the comparison. EdgeR was used to define pairwise differentially expressed genes WT and Vim-/- MoAMs^{47,48}. Principal Component analysis (PCA) plots were generated using *pca* function in R. Pearson correlation matrix, and heatmaps were generated using GENE-E. Gene ontology (GO) analysis was run using Gorilla^{49,50}. Expressed genes, filtered for low counts (9,169) were used as background. Gene set enrichment analysis (GSEA) was run using the weighted pre-ranked setting based on the log₂FC reported by edgeR^{51,52}. Gene sets associated with specific processes were downloaded from KEGG (Cell Cycle) or Hallmark pathways (Glycolysis, Oxidative Phosphorylation, IFN alpha (Type I interferon), IFNγ (Type II Interferon) with the exception of the custom gene sets described

below. The 85 overlapping genes between the IFN α and IFN γ Hallmark pathways were excluded from the IFN γ gene set. Overlaps between gene lists were visualized using Venny (<https://bioinfogp.cnb.csic.es/tools/venny/index.html>).

Custom gene set generation. Custom gene sets for LPS/IFN γ and IL-4 stimulated were generated by querying primary published data available in relevant experimental models. The final curated gene sets, $M_{(LPS/IFN\gamma)}$ and $M_{(IL-4)}$, were derived from four primary publications³⁰⁻³³. All four publications used mouse bone-marrow-derived macrophages (BMDM) and stimulated them *in vitro* with either LPS+IFN γ or IL-4. Methodologies used included LC-ESI-MS/MS mass spectrometry, microarray, and RNA-seq. A summary of the experimental approach, methods, and results for all publications can be found in **Online Supplemental Table 2**. We included all genes that were upregulated in at least one data set and could be converted into a unique Ensembl ID. Three genes were found in both $M_{(LPS/IFN\gamma)}$ and $M_{(IL-4)}$ and were removed to avoid any overlap between gene sets. These trimming steps resulted in two annotated gene sets of 110 LPS/IFN γ stimulated genes and 101 IL-4 stimulated genes (**Online Supplemental Table 2**).

Motif finding. We generated two gene sets of the 186 most and 254 least changed genes in *Vim*^{-/-} MoAM by splitting the 440 genes of the Early Inflammatory cluster (Figure 2) based on whether their $\log_2(Vim^{-/-}/WT)$ expression on day 3 p.i. was above or below the mean of -0.23. (**Figure 6d,e**). To identify motifs that were enriched in either of these gene sets, we ran findmotifs.pl from the HOMER package with default parameters and the mouse mm10 genome as background³⁹. We identified significantly enriched motifs as those with p-value < 0.05 in the known motif results.

Viral reads. To identify viral reads within libraries, unmapped reads were aligned to the H1N1 reference genome. Reads for the H1N1 matrix protein 2 (M2), nucleoprotein (NP), and neuraminidase (NA) were normalized by sample to the number of uniquely aligned (mm10) reads.

Statistics. Analyses for cytokine levels, BALF protein, viral titer, and wet-to-dry ratio were performed using GraphPad Prism (GraphPad Software). P values were calculated using a two-tailed unpaired t-test assuming equal variance.

Acknowledgments

Histology services were provided by the Northwestern University Mouse Histology and Phenotyping Laboratory, which is supported by NCI P30-CA060553 awarded to the Robert H Lurie Comprehensive Cancer Center. Flow Cytometry was supported by the Northwestern University RHLCCC Flow Cytometry Facility and a Cancer Center Support Grant (NCI CA060553). This research was supported in part through the computational resources and staff contributions provided by the Genomics Compute Cluster which is jointly supported by the Feinberg School of Medicine, the Center for Genetic Medicine, and Feinberg's Department of Biochemistry and Molecular Genetics, the Office of the Provost, the Office for Research, and Northwestern Information Technology. The Genomics Compute Cluster is part of Quest, Northwestern University's high performance computing facility, with the purpose to advance research in genomics. Graphic design was created with BioRender.com. Kishore R. Anekalla was supported by NIH T32 HL076139. Shang-Yang Chen was supported by a pre-doctoral AHA award (19PRE34380200). Alexander V. Misharin was supported by N.I.H. grants U19AI135964, P01AG049665, R01HL153312,

and NUCATS COVID-19 Rapid Response Grant. Karen M. Ridge was supported by N.I.H. grants P01AG049665 and P01GM096971. Bria M. Coates was supported by funds provided by the Manne Research Institute COVID-19 Springboard Exploratory Research Award and N.I.H. grant K08HL143127. Deborah R. Winter was supported by the American Lung Association, Arthritis National Research Foundation, American Thoracic Society, Scleroderma Foundation, American Federation for Aging Research, American Heart Association 18CDA34110224, and NIH/NIAID U19AI135964. G.R.S.B. was supported by NIH grants U19AI135964, P01AG049665, P01AG04966506S1, R01HL147575 and Veterans Affairs grant I01CX001777.

Author contributions: C.M.K., K.R.A., Y.H., B.M.C., A.V.M., D.R.W., K.M.R., contributed to the acquisition, analysis, and interpretation of the data and drafting the manuscript. M.C., G.G., S.C., M.T., Y.C., J.M.D., contributed to the acquisition and interpretation of data. C.M.K., K.R.A., Y.H., B.M.C., H.A.V., P.A.R., A.V.M., G.R.S.B., D.R.W., K.M.R. contributed to the conception and design of the study and interpretation of the data. All authors critically revised and approved the final version of the manuscript.

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Figure Legends

Figure 1. Vimentin-null mice are protected from influenza-induced lung injury and mortality. **a)** Uniform Manifold Approximation and Projection (UMAP) of 40,248 cells annotated as monocyte-derived alveolar macrophages (MoAMs) obtained from 10 patients with severe COVID-19 disease within 48 hours after intubation. **b)** Fraction of cells expressing *VIM* and mean expression in each MoAM cluster. **c)** Visualization of *IL1-B*, *CCL3*, *CXCL2*, *C1QA*, and *MERTK* expression in MoAM cells using density projection plots. Scale bar represents expression averaged within hexagonal areas on UMAP. **d)** Fraction of cells expressing the representative genes and their mean expression in each MoAM cluster. **e)** Survival of wild-type (WT) and *Vim*^{-/-} mice intratracheally infected with a lethal dose of influenza A virus (IAV) (200pfu) was measured and compared. Survival curves were analyzed using log-rank test, p-value = 0.001. Data shown are n=12 per group. **f)** Lung wet-to-dry ratio measured at day 3 and 5 p.i. (n = 8-11/timepoint) **g)** Bronchoalveolar lavage fluid (BALF) protein levels measured by Bradford assay, n=5/timepoint. In **f** and **g**, differences between genotypes on each day were assessed by t-test. p-values were 0.0015 and <0.0001, for 3 and 5 days p.i., respectively. **h)** Representative images of murine lung architecture assessed by H&E staining, scale bar 100 um. **i)** Viral titer assessed by plaque assay. Difference between genotypes was statistically not significant. n=5-9/timepoint. **j)** Flow cytometric analysis of tissue resident (TRAMs) and infiltrating monocyte-derived alveolar macrophages (MoAMs) out of the total lung CD45⁺ cell population normalized to body weight during the IAV response, n=4/timepoint. **k)** Levels of inflammatory cytokines in BALF were assessed using a custom multiplex ELISA. n=3-4/timepoint. Bar graphs represent mean, error bars represent SD, differences between genotypes on each day were assessed by t-test. * p<0.05, ** p<0.001, *** p<0.0001, **** p<0.00001.

Supplemental Figure 1. **a)** UMAP of 77,146 cells annotated by cell type obtained from 10 patients with severe COVID-19 disease within 48 hours after intubation. **b)** Fraction of cells expressing the representative genes and their mean expression in each TRAM cluster. **c)** Quantification of H1N1 viral RNA molecules detected among RNA isolated from MoAMs, normalized to the number of reads uniquely aligned to mouse genome (mm10) for each sample. Bar graphs represent mean, error bars represent SD. **d)** Gating strategy used for flow cytometry analysis of neutrophils, TRAMs, interstitial macrophages (IM) and MoAMs. **e)** Flow cytometric analysis demonstrated no differences in number of neutrophils out of the total lung CD45⁺ cell population normalized to body weight between *Vim*^{-/-} and WT mice during the IAV response.

Figure 2. Wild-type monocyte-derived alveolar macrophages exhibit gene expression patterns associated with three phases of influenza A viral infection. **a)** Heatmap of normalized expression of 2,623 differentially expressed genes (ANOVA adjusted p-value <0.05). Z-score normalization for each row was applied to gene expression (FPKM) data. K-means clustering (k=3) identified three clusters; Infiltrating (1131 genes), Early Inflammatory (440 genes), Late Inflammatory (1052 genes). **b)** Mean z-scores calculated across genes for each cluster **c)** Gene Ontology (GO) term enrichment for each phase. **d)** Bar graphs of gene expression (FPKM) for representative genes in each

phase. Error bars represent SD. (ANOVA adjusted p-value <0.05). **e)** Bubble plot representing the proportion of genes (%) within each process that had an expression above the row mean. Color gradient represents mean z-score of expression across genes. **f)** Enrichment of gene sets in each cluster as defined in A. Asterisk represents significantly enriched overlaps (hypergeometric p-value <0.0018, FWER = 0.05). Dotted line indicates expected enrichment score of 1. n=3-4 per group. Ox Phos = Oxidative phosphorylation.

Supplemental Figure 2. a) Schematic of experimental design and workflow. WT or *Vim*^{-/-} mice were infected with a lethal dose of influenza A virus (200 pfu). MoAMs were sorted by flow cytometry (FACS) from lungs on 2, 3, 4, and 6 days p.i. Total RNA was isolated, mRNA was enriched, and bulk RNA-seq was performed. Principal component analysis (PCA) was used to visualize replicates. **b)** Sequential gating strategy used for FACS collection of TRAMs and MoAMs **c)** Violin plots of log₂(FPKM+1) counts data for all genes in each cluster. Solid lines represent median, dotted lines represent quartiles. **d)** Bar graphs of gene expression (FPKM) in MoAM for representative genes in each phase. Error bars represent SD. (ANOVA adjusted p-value <0.05).

Figure 3. Wild-type monocyte-derived alveolar macrophages resemble interstitial macrophages during the infiltrating phase and exhibit increased expression of alveolar macrophages genes over time. a) Volcano plot of differentially expressed genes (edgeR p-value<0.05, and edgeR reported log₂FC>|1.0|) between TRAM and IM at steady-state (0 dpi). The number of genes increased (red) and decreased (blue) in TRAMs compared to IMs are shown. **b)** Mean z-scores calculated for IM- and TRAM-specific gene sets over the course of IAV infection in MoAMs **c)** Plots representing % of genes at each time point expressed above the mean. Each dot represents a replicate. Lines represent median. **d)** Bar graphs of gene expression (FPKM) for representative genes in IM- and TRAM-specific gene sets. Error bars represent SD. **e)** Enrichment of IM- and TRAM-cell specific gene signatures in MoAMs for each phase. Asterisk represents significantly enriched overlaps (hypergeometric p-value <0.006, FWER = 0.05).

Figure 4. *Vim*^{-/-} MoAMs exhibit increased expression of Infiltrating phase genes and decreased expression of Early Inflammatory phase genes. a) Heatmap of normalized expression of 2,623 genes as defined in Figure 2a. *Vim*^{-/-} data was normalized to the WT mean and standard deviation (adapted z-score). **b)** Mean adapted z-scores were calculated across genes for each cluster in WT MoAMs and *Vim*^{-/-} MoAMs. Shading represents WT MoAMs inter-quartile range (IQR) **c)** Bar graphs representing the fold change in gene expression (FPKM) in *Vim*^{-/-} MoAMs compared to the WT MoAMs mean for representative genes. (n=3-4 per group). **d)** % genes in each cluster that exhibit decreased or increased expression in *Vim*^{-/-} MoAMs compared to WT MoAMs (n=3-4 per group). Decreased genes defined as log₂FC < -0.58 (FC of 1.5); increased genes defined as log₂FC>0.58.

Supplemental Figure 4. a) Principal component analysis (PCA) of *Vim*^{-/-} MoAMs replicates. **b)** Violin plots representing the log₂FC between *Vim*^{-/-} and WT for each gene by cluster in MoAMs. Solid lines represent median, dotted lines represent quartiles. **c)** Heatmap showing pairwise Pearson correlation of mean gene expression across replicates between each time point in WT and *Vim*^{-/-} MoAMs. Pearson correlation coefficient values

shown within each square. Scale bar represents Pearson correlation coefficient. **d)** Violin plots representing the log₂FC between days p.i. for each gene by cluster in WT and *Vim*^{-/-} MoAMs. Solid lines represent median, dotted lines represent quartiles. **e)** Violin plots representing the log₂FC between *Vim*^{-/-} and WT in TRAMs and IMs at steady-state (0 dpi) for each gene by cluster. Solid lines represent median, dotted lines represent quartiles. **f)** Heatmap of normalized expression of 2,623 differentially expressed genes in *Vim*^{-/-} MoAMs (ANOVA adjusted p-value <0.05). Adapted Z-score normalization for each row was applied to gene expression (FPKM) data using *Vim*^{-/-} MoAMs mean and standard deviation. K-means clustering (k=3) was performed on *Vim*^{-/-} MoAMs expression and WT MoAMs data was visualized for the same genes **g)** Mean adapted z-scores were calculated across genes for each cluster in *Vim*^{-/-} and WT MoAMs. Shading represents *Vim*^{-/-} MoAMs IQR.

Figure 5. Inflammatory pathways are consistently decreased in *Vim*^{-/-} MoAMs compared to WT MoAMs during influenza A infection. **a)** Volcano plots of differentially expressed genes (edgeR p-value<0.05, and edgeR reported log₂FC>|1.0|) between *Vim*^{-/-} and WT MoAMs on each day post-infection. The number of genes increased (red) and decreased (blue) in *Vim*^{-/-} compared to WT MoAMs are shown. **b)** GO term enrichment across days p.i. in the increased (red) and decreased (blue) genes in *Vim*^{-/-} compared to WT MoAMs. Scale represents -log₁₀ p-value. **c)** Violin plots representing the log₂FC for each process between *Vim*^{-/-} and WT MoAMs. Solid lines represent median, dotted lines represent quartiles. **d)** Scatter plots of z-score normalized gene expression across genes in M(IL-4) vs Cell Cycle. Pearson correlation coefficients (r) across WT or *Vim*^{-/-} MoAM replicates and associated p-values are shown. **e)** Heatmap matrix of Pearson correlation between processes, asterisk indicate p-value <0.05.

Supplemental Figure 5. **a)** Plots representing % of genes at each time point expressed above their WT mean. Each dot represents a replicate. Lines represent median. **b, c)** Gene Set Enrichment Analysis (GSEA) enrichment plots. GSEA was performed on gene lists ranked by edgeR reported fold-change between *Vim*^{-/-} and WT MoAMs at each time point. Normalized enrichment scores (NES) are shown. Asterisks indicate statistically significant adjusted p-values <0.05. **d)** Violin plots representing the log₂FC between *Vim*^{-/-} and WT TRAMs and IMs at steady-state (0 dpi). Solid lines represent medians, dashed lines represent quartiles.

Figure 6. Protective phenotype of *Vim*^{-/-} mice is associated with the failure of *Vim*^{-/-} MoAMs to activate core pro-inflammatory genes in the early response to IAV. **a)** Percent (%) M(LPS) genes peaking in expression on each day post-infection in WT and *Vim*^{-/-} MoAMs **b)** Bar graphs of gene expression (FPKM) for representative M(LPS) genes present in the leading edge of at least 1 day from GSEA. Error bars represent SD. **c)** Heatmap of normalized expression of 84 M(LPS) genes present in the GSEA leading edge on at least 1 day (indicated by color-coded bar on left). Adapted Z-score normalization using WT mean and standard deviation for each row was applied to gene expression (FPKM) data. **d)** Histogram representing the distribution of log₂FC between *Vim*^{-/-} and WT MoAMs on 3 dpi of the Early Inflammatory phase (440 genes). Dashed line represents mean of the distribution. Number of genes less than (186) and greater than (254) the mean are shown. Genes with log₂FC between *Vim*^{-/-} and WT MoAMs less than the mean represent those

genes that are most changed between *Vim*^{-/-} and WT MoAMs. **e)** Mean adapted z-scores were calculated across genes for most (186) and least (254) changed genes on 3 dpi in the Early Inflammatory Phase in WT and *Vim*^{-/-} MoAMs. **f)** HOMER known motif analysis using genomic background of most changed genes on 3 dpi in the Early Inflammatory Phase (186 genes). Motif logos are shown below motif names. -log₁₀pvalues of enrichment in most or least changed gene set is shown. Log₂(FPKM+1) average gene expression of transcription factor family members are shown for WT TRAMs and WT IMs at steady-state (0 dpi) and 2-6 dpi for WT MoAMs. log₂FC between *Vim*^{-/-} and WT MoAM gene expression of transcription factor family members on each dpi. (n=3-4)

Supplemental Figure 6. a) Mean adapted z-scores for M_(LPS) and M_(IL-4) genes present in the leading edge of at least 1 day from GSEA in WT and *Vim*^{-/-} MoAMs. **b)** Heatmap of normalized expression of M_(IL-4) genes present in the leading edge of at least 1 day from GSEA. Adapted Z-score normalization for each row was applied to gene expression (FPKM) data. Bar graphs of gene expression (FPKM) for representative M_(IL-4) genes present in the leading edge of at least 1 day from GSEA. Error bars represent SD. **c)** Heatmap of normalized expression of 186 most changed, and 254 least changed genes on 3dpi of the Early Inflammatory Phase. Adapted Z-score normalization for each row was applied to gene expression (FPKM) data. **d)** HOMER known motif analysis using genomic background of least changed genes on 3 dpi in the Early Inflammatory Phase (254 genes). Motif logos are shown below motif names. Log₂(FPKM+1) average gene expression of transcription factor family members are shown for WT TRAMs and WT IMs at steady-state (0 dpi) and 2-6 dpi for WT MoAMs. log₂FC between *Vim*^{-/-} and WT MoAM gene expression of transcription factor family members on each dpi. (n=3-4). **e)** Motif logos are shown below motif names. -log₁₀pvalues of enrichment in most or least changed gene set is shown.

Figure 7. Vimentin-deficiency in the MoAM compartment is sufficient to protect from influenza A virus-induced lung damage and mortality. a) Schematic of the experimental design. Recipient mice (CD45.1) were irradiated and reconstituted with bone marrow cells from either WT or *Vim*^{-/-} donor mice (CD45.2). After a 30-day recovery period, chimeric mice were intratracheally (i.t.) infected with a lethal dose of IAV at 5 days p.i. and subjected to analyses. **b)** Bronchoalveolar lavage fluid (BALF) protein levels measured by Bradford assay, n=3-4 **c)** Lung wet-to-dry ratio measured at day 5 p.i. n=7-10. **d)** Viral titer assessed by plaque assay. n=11-14. **e)** Levels of inflammatory cytokines in BALF assessed using a custom multiplex ELISA. n=4-9. Bar graphs represent mean, error bars represent SD. Differences between genotypes were assessed by t-test. * p<0.05, ** p<0.001, *** p<0.0001, **** p<0.00001.

Supplemental Figure 7. Representative flow cytometry dot plots demonstrate successful engraftment of WT and *Vim*^{-/-} CD45.2 donor cells into CD45.1 recipient mice.