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Denny K. Gao, … , Kathryn A. Wikenheiser-Brokamp, Grant S. Schulert


In-Press Preview

Macrophage activation syndrome (MAS) is a life-threatening cytokine storm complicating systemic juvenile idiopathic arthritis (SJIA) driven by IFNγ. SJIA and MAS are associated with an unexplained emerging lung disease (SJIA-LD), with our recent work supporting pulmonary activation of IFNγ pathways pathologically linking SJIA-LD and MAS. Our objective was to mechanistically define the novel observation of pulmonary inflammation in the TLR9 mouse model of MAS. In acute MAS, lungs exhibit mild but diffuse CD4-predominant, perivascular interstitial inflammation with elevated IFNγ, IFN-induced chemokines, and AMΦ expression of IFNγ-induced genes. Single-cell RNA-sequencing confirmed IFN-driven transcriptional changes across lung cell types with myeloid expansion and detection of MAS-specific macrophage populations. Systemic MAS resolution was associated with increased AMΦ and interstitial lymphocytic infiltration. AMΦ transcriptomic analysis confirmed IFNγ-induced proinflammatory polarization during acute MAS, which switches towards an anti-inflammatory phenotype after systemic MAS resolution. Interestingly, recurrent MAS led to increased alveolar inflammation and lung injury, and reset AMΦ polarization towards a proinflammatory state. Furthermore, in mice bearing macrophages insensitive to IFNγ, both systemic feature of MAS and pulmonary inflammation were attenuated. These findings demonstrate that experimental MAS induces IFNγ-driven pulmonary inflammation replicating key features of SJIA-LD, and provides a model system for testing novel treatments directed towards SJIA-LD.

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IFNγ is essential for alveolar macrophage driven pulmonary inflammation in macrophage activation syndrome

Denny Gao¹, Nathan Salomonis²,³, Maggie Henderlight¹, Christopher Woods⁴, Kairavee Thakkar³, Alexei A. Grom¹,², Sherry Thornton¹,², Michael B. Jordan²,⁵, Kathryn A. Wikenheiser-Brokamp⁴,⁶, Grant S. Schulert¹,²

Divisions of ¹Rheumatology, ²Biomedical Informatics, ⁴Pathology & Laboratory Medicine, and ⁵Immunobiology, Cincinnati Children’s Hospital Medical Center and Departments of ²Pediatrics and ⁶Pathology & Laboratory Medicine, University of Cincinnati College of Medicine, Cincinnati, OH, USA

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Brief summary: Experimental MAS displays an IFNγ-driven alveolar macrophage and pulmonary inflammation reflecting high-fatality lung disease in children with systemic JIA and MAS.

Address for Correspondence:
Grant S. Schulert, MD PhD
Division of Rheumatology, Cincinnati Children’s Hospital Medical Center
Department of Pediatrics, University of Cincinnati College of Medicine
MLC 4010
3333 Burnet Ave
Cincinnati, Ohio 45229
Grant.schulert@cchmc.org
ABSTRACT

Macrophage activation syndrome (MAS) is a life-threatening cytokine storm complicating systemic juvenile idiopathic arthritis (SJIA) driven by IFNγ. SJIA and MAS are also associated with an unexplained emerging inflammatory lung disease (SJIA-LD), with our recent work supporting pulmonary activation of IFNγ pathways pathologically linking SJIA-LD and MAS. Our objective was to mechanistically define the potentially novel observation of pulmonary inflammation in the TLR9 mouse model of MAS. In acute MAS, lungs exhibit mild but diffuse CD4-predominant, perivascular interstitial inflammation with elevated IFNγ, IFN-induced chemokines, and AMϕ expression of IFNγ-induced genes. Single-cell RNA-sequencing confirmed IFN-driven transcriptional changes across lung cell types with myeloid expansion and detection of MAS-specific macrophage populations. Systemic MAS resolution was associated with increased AMϕ and interstitial lymphocytic infiltration. AMϕ transcriptomic analysis confirmed IFNγ-induced proinflammatory polarization during acute MAS, which switches towards an anti-inflammatory phenotype after systemic MAS resolution. Interestingly, recurrent MAS led to increased alveolar inflammation and lung injury, and reset AMϕ polarization towards a proinflammatory state. Furthermore, in mice bearing macrophages insensitive to IFNγ, both systemic feature of MAS and pulmonary inflammation were attenuated. These findings demonstrate that experimental MAS induces IFNγ-driven pulmonary inflammation replicating key features of SJIA-LD, and provides a model system for testing novel treatments directed towards SJIA-LD.
Introduction

Macrophage activation syndrome (MAS) is a life-threatening “cytokine storm” with clinical similarity to hemophagocytic lymphohistiocytosis (HLH) (1–3). MAS occurs most commonly in the setting of systemic juvenile idiopathic arthritis (SJIA), a distinct subtype of JIA with features of autoinflammation (4). The pathogenesis of familial HLH is dependent on IFNγ, and neutralization of IFNγ improves clinical and immunologic features of the disease (5–8). Similarly, recent findings by us and others have found that SJIA-associated MAS is also distinguished by a surge in IFNγ and IFN-induced chemokines (9, 10).

In contrast to familial HLH, the genetic basis of MAS remains unclear, but several animal models replicate the immunopathology of MAS. The best characterized model was developed by Behrens and colleagues (11), wherein repeated stimulation of mice with the TLR9 agonist CpG induced features reflecting the early or subclinical MAS that affects at least one-third of children with SJIA. These animals also developed peripheral monocytosis sustaining the systemic hyperinflammation, although the precise contribution of myeloid-lineage cells remains undefined (12). Full cytokine storm in this model was dependent on IFNγ (11, 13, 14).

Along with MAS, an emerging cause of mortality in children with SJIA is chronic lung disease (SJIA-LD) (15), with pulmonary and alveolar inflammation including patchy but extensive lymphoplasmacytic interstitial infiltrates and mixed features of pulmonary alveolar proteinosis (PAP) and endogenous lipid pneumonia (16). The etiology of SJIA-LD is unknown but several risk factors and triggers have been proposed. Increasing use of anti-cytokine therapy for SJIA has been temporally linked to incidence of SJIA-LD (15, 17), with possible allergic reactions as a trigger (17). On the other hand, several large studies have linked SJIA-LD to recurrent episodes of MAS (15–17), and our recent work identified IFNγ pathway activation in the lungs of children with SJIA-LD (16). Notably, the various causes of PAP are linked through alterations in alveolar macrophage (AMφ) biology, including impaired functional maturation and
polarization (18–21). Together, this raises the intriguing possibility of a connection between MAS, AM\(\phi\) biology, and SJIA-LD.

Here, we provide the first direct evidence that mice with MAS exhibit pulmonary inflammation that reflects key features of SJIA-LD, including IFN\(\gamma\) activation. Single-cell RNA sequencing (scRNA-seq) reveals marked expansion of the monocyte and macrophage compartment in the lungs, including emergence of MAS-specific macrophage populations. Higher levels of pulmonary inflammation were seen after systemic MAS resolution, while recurrent MAS demonstrated a reprogramming of AM\(\phi\) towards proinflammatory polarization. Finally, mice with macrophages insensitive to IFN\(\gamma\) showed attenuated lung inflammation. These findings support a model whereby MAS induces IFN\(\gamma\)-driven pulmonary inflammation and dynamic changes in AM\(\phi\) polarization, contributing to development of SJIA-LD.
RESULTS

Pulmonary inflammation in the TLR9 model of MAS

The best described mouse model of MAS involves repeated administration of the TLR9 agonist CpG, leading to clinical features which closely mimics the “subclinical” MAS observed in nearly one-third of SJIA patients (Figure S1) (11), including anemia, lymphopenia, and thrombocytopenia, massive splenomegaly and hyperferritinemia (Figure S2). MAS was also associated with increased serum cytokine and chemokine levels consistent with developing cytokine storm. In particular, mice demonstrated increased serum IL-1β, IL-6, IL-10, IL-12p70, IL-17a, TNF, IL-18, and IFNγ, along with the IFN-induced chemokines CXCL9 and CXCL10 (Figure S3).

While the above model has become the system of choice for experimental study of MAS, pulmonary involvement in these animals has not been previously reported. Here, we find that lung tissue sections from mice with MAS showed mildly increased mononuclear lymphocyte predominant interstitial inflammation that was primarily perivascular but also involving alveolar septa (Figure 1A). In agreement with this, flow cytometry of dissociated lung tissue cells demonstrated a significant increase in CD3+ and CD3+CD4+ T lymphocytes in mice with acute MAS compared to control (PBS treated) mice (Figure 1B). Homogenized whole lung tissue in acute MAS also showed significantly increased levels of IFNγ, the IFN-induced chemokines CXCL9 and CXCL10, as well as RANTES and CXCL1 (Figure 1C). Interestingly, other proinflammatory cytokines that were significantly elevated in serum (including IL-1β, IL-6, and IL-10) were unchanged in lung tissue (Figure S4A), suggesting that increases in IFNγ and IFN-induced chemokines may not simply reflect systemic levels or contamination from pulmonary vasculature. Together, we provide the first direct demonstration that mice with acute MAS have a mild lymphocyte-predominant pulmonary interstitial inflammation associated with increased IFNγ and IFN-induced chemokines.
Alveolar macrophage activation in acute MAS

Given the histology seen in SJIA-LD, and the link between PAP and AMϕ dysfunction, we performed bronchoalveolar lavage (BAL) on mice with acute MAS to assess for signs of airway inflammation. While mice with CpG-induced MAS did not show increased total alveolar cells (data not shown), there was a small but significant decrease in the AMϕ proportion and increase in T cells and CD45- epithelial cells during acute MAS (Figure 2A). In addition, BAL fluid showed a significant increase in IL-18, IFNγ, and CXCL9 and CXCL10 (Figure 2B), similar to that found in SJIA-LD patients (16). In contrast, levels of other cytokines including IL-1β, IL-6, IL-10, IL-12, and TNF were unchanged (Figure S4B). We also found that CD11c+CD11b+CD64+ AMϕ had significantly increased surface expression of MHC class II markers, reflecting classical activation, without any significant changes in M2 markers such as CD206 (Figure 2C-D). In contrast, there were no significant changes in surface marker expression in CD11c-CD11b+ macrophages. AMϕ also showed significant upregulation in IFNγ-induced, proinflammatory genes including IL12A and CXCL9, without changes in other polarization markers such as HMOX1 and TGFB (Figure 2F). AMϕ also showed significant upregulation of KLF13 mRNA, which is essential for M1 polarization in mice (22). MicroRNA are increasingly recognized as key regulators of macrophage polarization (23). Here, we find increased expression of the proinflammatory miR-146a, but no significant changes in the key regulatory microRNA miR-125a-5p, which directly targets KLF13 (Figure 2G). Finally, we found that AMϕ from mice with acute MAS secreted significantly increased levels of CXCL9 and CXCL10, along with IL-12p70 and Mip-1α when cultured ex vivo compared to cells from control mice (Figure 2E). Taken together, we find that during MAS AMϕ demonstrate changes in surface MHC class II and gene expression reflecting proinflammatory polarization and IFNγ activation, and secrete IFN-induced chemokines into BAL fluid. Notably, the phenotype of AMϕ in MAS does not simply reflect that of mouse peripheral blood monocytes, which have distinct gene expression signatures including failure to increase IL12A or KLF13, and decreased expression of TGFB and IL10 (Figure S5).
These divergent transcriptional responses may reflect differential and tissue-specific effects of IFN\(\gamma\), along with inherent properties of AM\(\phi\), and support distinct effects of MAS in the lungs.

**Broad interferon-induced transcriptional responses and myeloid expansion and diversification revealed with scRNA-seq**

To better identify these cellular and molecular impacts of MAS across lung cell populations, we performed scRNA-Seq using the 10x Genomics platform to interrogate of ~15,000 single-cells from wild-type (PBS treated) and MAS lung tissue (Table S1). Using the unsupervised single-cell population discovery and annotation pipeline ICGS2, we identified 33 transcriptionally unique cell clusters in the combined wild-type and MAS captures (Figure 3A-B and Table S2-3). Two of these clusters were excluded based on their broad distribution within the produced UMAP graph and lack of population specific protein-coding marker genes, which were predicted as unknown cell-types by ICGS2. We aligned the cell-type prediction names to the literature and confirmed their specificity based on the expression of prior defined markers (Figure 3C). As expected, distinct monocytic, macrophage, neutrophil and T- and B-cell subsets were enriched in MAS, with less frequent detection of epithelial and endothelial cell populations. When normalized to the most frequently detected endothelial cell population in both captures, we find the most significant increases during MAS in Classical Monocytes (the dominant cell population in MAS), Extravasating Interstitial Mono-Mac cells, and Inflammatory Monocytes, as well as two MAS specific Macrophage clusters (c17 and c30) absent in wild-type lung tissue (Figure 3D). Genes uniquely expressed in cluster c17 macrophages were most enriched in pathways for complement activation and apoptotic clearance, and expressed markers of recruited airway macrophages (24) including CD14, MAFB, and CCR5 (Figure 3E, S6; Table S4,5). Compared to classical monocytes (c36), c17 also downregulated NFKB1, an NF-\(\kappa\)B repressor (25), and upregulated many of its targets including IL18 expression. In contrast, uniquely expressed genes in macrophage cluster c30 were most enriched in
response to interferon-gamma, leukocyte migration, and chemokine receptor binding pathways, including CXCL9, CXCL10, and TNF. (Figure 3E, S6 and Table S4, S5).

To more broadly assess gene expression differences in each pair of MAS and wild-type cell populations, we performed an exhaustive comparison analysis using the cellHarmony workflow with the cell labels assigned from ICGS2. This analysis highlighted close to 1,900 confidently differentially expressed genes across 21 out of 31 compared cell populations, in which sufficient cell numbers were present for comparison (Figure 4A and Table S6). Most notably, genes associated with the adaptive immune system were highly and broadly and consistently up-regulated across the majority of cell populations in MAS, whereas hypoxia genes were consistently down-regulated. Among genes broadly up-regulated across cell populations were those associated with interferon signaling, including a number of proteasomal components in addition to interferon receptors (Figure 4B). In addition, highly specific gene programs were impacted in each of the Monocyte and Macrophage cell populations, with the largest transcriptomic impact in Classical Monocytes (Figure 4A). While Classical Monocytes up-regulated genes were selectively enriched in energy metabolism and TCA genes, cellHarmony predicted regulation of a core Stat1 transcriptional network in these cells, in combination with Irf1 and Rel responsive transcripts, by virtue of prior experimentally evidenced direct targets (Figure 4C). Finally, T cell populations (c42) showed gene signatures reflecting activation including IL-2-mediated signaling events (adjusted p=0.001). Taken together, these findings demonstrate that experimental MAS induced a broad interferon-induced transcriptional response throughout the lung, along with massive expansion and emergence of potentially novel monocyte and macrophage populations.

**Persistent pulmonary inflammation but shift in AMΦ phenotype after systemic MAS resolution**

Most patients with SJIA-LD develop pulmonary complications not at systemic disease onset but in the subsequent months (16, 17). Therefore, we have further developed this TLR9 model system by for
the first time characterizing the resolution phase of MAS (Figure S1). We find that 3 weeks after MAS induction, CpG-treated mice have largely resolved their systemic cytokine storm including cytopenias (Figure S7A), and had markedly reduced spleen size compared to acute MAS (though still slightly larger than control mice) (Figure S7B-C). In addition, the serum cytokine profile including IFNγ and IL-6 had largely normalized at this later time point, with the notable exception of persistent IL-18 elevation (Figure S7D). Interestingly, IL-18 remains chronically elevated in patients with SJIA for many months despite achieving clinically inactive disease (26).

Mice after systemic MAS resolution continue to have pulmonary interstitial inflammation (Figure 5). Histologic examination demonstrated lymphocyte predominant, chronic mononuclear perivascular and peribronchiolar infiltrates, which was variable among mice but diffuse involving multiple lung lobes with focal lymphocyte aggregates (Figure 5A). Analysis of BAL fluid after systemic MAS resolution revealed a modest but significant increase in total number of alveolar cells as well as the proportion of AMϕ, not seen during acute MAS, providing evidence of disease progression (Figure 5B). In order to quantify the scattered interstitial inflammation, we developed an Aperio-based staining algorithm on digitized slides to quantify positively stained cells (Figure S8). Mice with resolving MAS showed a nearly 50% increase in the number of CD3+ cells/mm² of lung compared with untreated control mice (average 47.5 vs 32.2 positive cells/mm², respectively). Consistent with the serum cytokines, BAL chemokine levels had normalized by this stage (Figure 5C), and cultured AMϕ did not secrete significant levels of chemokines (Figure 5G). However, CD11c⁺CD11b⁻CD64⁺ AMϕ continued to have a small but significant increase in MHCII expression, without any difference in CD206 expression (Figure 5D). Targeted gene expression analysis demonstrated a shift in AMϕ polarization phenotypes during MAS recovery. While expression of IFN-induced genes CXCL9 and IL12A, as well as the “M1”-associated microRNA miR-146a, had normalized, other polarization markers were significantly altered including downregulation of HMOX1 and upregulation of TGFB (Figure 5F-G). In addition, miR-125a-5p
levels were significantly reduced, while its target *KLF13* remained elevated. In total, we find that during the resolution phase of TLR9-induced MAS, mice demonstrate 1) persistent interstitial inflammation, 2) increased alveolar macrophages, and 3) further alterations in AMφ gene and microRNA expression suggesting a shift in macrophage polarization.

**Transcriptional profiling of AMφ shows shift in polarization phenotypes between acute MAS and resolution**

Genome-wide transcriptional profiles using microarray were generated of adherence-purified AMφ from control mice (PBS treated) or mice with acute MAS or after systemic MAS resolution ([Figure S1](#)). Principle component analysis showed that samples from the three treatment groups were clearly distinct ([Figure 6A](#)). Comparing acute MAS to control AMφ, we identified 94 differentially expressed genes (DEG; fold change >2, p<0.05) including 64 upregulated and 30 downregulated genes ([Figure 6B; Table S7-S8](#)). Pathway analysis on these DEGs identified cellular response to interferon-gamma (GO:0071346) as the most significantly enriched pathway among upregulated genes (z-score 25.0, adjusted p=1.47x10^{-8}). Other enriched GO pathways are shown in [Figure 6C](#) and support a proinflammatory phenotype, while downregulated genes did not show any significantly enriched GO pathways.

Interestingly, transcriptional profiles of AMφ in the resolution phase of MAS displayed much more extensive transcriptional changes than during acute MAS, with 316 upregulated and 307 downregulated genes compared to control AMφ ([Figure 6D-E, Table S9](#)). The most enriched GO pathways of upregulated genes in resolution phase AMφ included those involved in regulation of metabolic processes (GO:0080090, z-score 6.6, adjusted p=1.5x10^{-5}; [Figure 6F](#)). Specific upregulated genes include several transcription factors of the Kruppel-like factor (KLF) family (Table S10), including *KLF13*, with key roles in cellular metabolism and macrophage polarization, including regulatory and anti-
inflammatory phenotypes (27–29). There was no evidence of an IFN-induced signature in resolution phase AMφ. On the other hand, upregulated genes did include a STAT6-regulated cluster (z-score 5.0, adjusted p=0.03), which is induced by cytokines that drive M2 polarization (Figure S8) (30). In contrast, downregulated gene pathways in MAS resolution AMφ encompassed cellular and immune processes and response to cytokine stimulation (Figure 6G), including those regulated by IFN-activated STAT1 (z-score 4.1, adjusted p=0.05) and by Bach1 (z-score 6.1, adjusted p=0.01) (Figure S9). Bach1 is notable as loss of this activity in mice may exacerbate certain forms of PAP (20). Taken together, we find that AMφ after systemic MAS resolution exhibit large-scale transcriptional changes impacting inflammatory and metabolic pathways towards a more anti-inflammatory and regulatory polarization state.

Augmented pulmonary inflammation and lung injury and AMφ reprogramming in recurrent MAS

Many patients with SJIA-LD suffer from repeated episodes of overt or subclinical MAS. As such, we have for the first time treated mice with either PBS or CpG (round 1) followed by retreatment with CpG (round 2), yielding controls (PBS) as well as mice with one (PBS-CpG) or two (CpG-CpG) episodes of MAS (Figure S1). Overall, most systemic features of MAS were similar between PBS-CpG and CpG-CpG, including anemia and thrombocytopenia (Figure 7A), splenomegaly (Figure 7B), and hyperferritinemia (Figure 7C), although mice with recurrent MAS did not show significant lymphopenia (Figure 7A). While serum cytokine profiles showed largely similar increases between PBS-CpG and CpG-CpG (Figure 7D), serum IL-18 levels showed a trend towards increased levels in mice with recurrent MAS (p=0.09), in agreement with elevated IL-18 in patients with history of MAS (26).

We next examined whether recurrent MAS altered features of pulmonary inflammation and AMφ polarization. Mice with recurrent MAS continued to show significantly higher numbers of AMφ (Figure 8A) and significantly higher BAL levels of CXCL9 and CXCL10 compared to those with a single MAS episode (Figure 8B). This is likely related to the enhanced number of AMφ, as ex vivo chemokine
release was similar between AMϕ from PBS-CpG and CpG-CpG mice (Figure 8C). Histologically, mice with recurrent MAS demonstrated similar findings of increased chronic lymphocytic perivascular infiltrates; however, inflammation was more extensive and more diffuse throughout lung lobes than in acute MAS including presence of focal aggregates rarely seen after a single acute treatment (Figure 8D). Isolated AMϕ in recurrent MAS showed a reprogramming from the anti-inflammatory phenotype seen after systemic MAS resolution towards a similar proinflammatory phenotype seen in acute MAS with activation of IFNγ-induced genes such as CXCL9 and IL12A (Figure 8E), along with decreases in TGFB and HMOX1 (Figure 8E), and more pronounced changes in miR-146a and miR-125a-5p (Figure 8F). Finally, we quantified levels of IgM protein in BAL fluid as a marker of epithelial permeability and lung injury (31). While BAL protein concentration was unchanged from control in acute MAS, recurrent MAS showed a significant increase in IgM concentration (Figure 8G). Overall, mice with recurrent TLR9-induced MAS showed more pronounced lung inflammation and lung injury than seen in acute MAS and a reprogramming of AMϕ phenotypes from resolution towards proinflammatory polarization.

Blockade of IFNγ limits features of pulmonary inflammation

Previous work has shown that signs of IFNγ-pathway activation are also observed in the lungs of children with SJIA-LD (16). As blockade of IFNγ reduces the systemic cytokine storm in the TLR9 model of MAS (11, 13, 14), we investigated whether IFNγ blockade could similarly limit pulmonary and airway inflammation. Administration of IFNγ neutralizing antibodies concomitant with CpG treatment as described (11) significantly reduced some systemic features of MAS – most notably anemia and splenomegaly (Figure 9A-B). IFNγ blockade also attenuated the elevation of several serum cytokines and chemokines, including IL-6, IL-10, TNF, CXCL9, and CXCL10, although others such as IL-12 and RANTES appeared relatively unaffected, possibly reflecting the incomplete nature of systemic IFNγ blockade using the monoclonal antibody (Figure 9C).
In the lungs, IFN\(\gamma\) blockade significantly reduced BAL chemokine levels including CXCL9 (Figure 9D). This was accompanied by a reduced ability of AM\(\phi\) to secrete CXCL9 ex vivo (Figure 9E). In addition, IFN\(\gamma\) blockade reduced expression of MHC class II activation markers specifically on CD11c\(^+\)CD11b\(^{var}\)CD64\(^+\) AM\(\phi\) (Figure 9F). IFN\(\gamma\) blockade also reduced expression of CXCL9 by AM\(\phi\); however, IL12A and miR-146a expression was largely unchanged (Figure 9G-H). However, there were no clear histological differences in the lungs when mice were given anti-IFN\(\gamma\) antibody concomitant with CpG along (data not shown). These findings show that AM\(\phi\) activation in the TLR9 model of MAS is at least partially IFN\(\gamma\) dependent.

Macrophage lineage responses to IFN\(\gamma\) is essential for full systemic cytokine storm and alveolar inflammation

To further define the role of systemic and alveolar macrophage responses to IFN\(\gamma\) in MAS, we utilized MIIG (macrophages insensitive to IFN\(\gamma\)) mice (32), expressing a dominant-negative IFNGR1 in CD68\(^+\) cells, with tissue macrophages including AM\(\phi\) unresponsive to IFN\(\gamma\) stimulation (32). Compared to WT, MIIG mice showed significant reductions in key clinical features of MAS including splenomegaly, anemia, and thrombocytopenia (Figure 10A and 10B), while others such as lymphopenia and hyperferritinemia were largely unchanged (Figure 10B and 10C). MIIG mice treated with CpG also showed less of an increase in a broad array of serum cytokines and chemokines implicated in cytokine storm, including TNF, IL-10, IL-18, IFN\(\gamma\), CXCL9 and CXCL10 (Figure 10D), while other cytokines such as IL-6, GM-CSF showed similar upregulation. Together, we find that many systemic clinical features including cytokine production in MAS specifically requires macrophage responsiveness to IFN\(\gamma\), while others including IL-6 induction and lymphopenia were unaltered.

We then examined whether macrophage responsiveness to IFN\(\gamma\) was required for pulmonary and airway inflammation. MIIG mice treated with CpG did not show any increase in IL-18, CXCL9, or
CXCL10 in BAL fluid (Figure 10E). AMφ isolated from MIIG mice treated with CpG to induce MAS and then cultured ex vivo failed to secrete CXCL9 after MAS induction (Figure 10F). Finally, AMφ from MIIG mice showed reduced upregulation of several genes and microRNA associated with proinflammatory polarization including CXCL9, HMOX1, IL12A, and miR-146a (Figure 10G). Taken together, experiments with IFNγ blockade and MIIG mice demonstrate that macrophage responses to IFNγ are required for pulmonary and airway inflammation in the CpG model of MAS, and further supports that systemic MAS induces pulmonary inflammation reflecting key features of SJIA-LD in children.
Discussion

The risk factors and underlying mechanisms driving often fatal SJIA-LD are unclear, but have been proposed to include introduction of anti-cytokine therapies, allergic reactions, and recurrent MAS (15–17). This knowledge gap represents a key barrier to progress towards treating and preventing the condition. Here, our previously unreported findings of significant pulmonary and airway inflammation in a well-characterized mouse model of MAS contribute to improved understanding of SJIA-LD and potential therapeutic effects. Utilizing scRNA-seq, we find features of IFN-pathway transcriptional activation throughout the lung during acute MAS, along with massive expansion of the myeloid compartment and emergence of MAS-specific macrophage populations including recruited airway macrophages and a distinct IFNγ-induced chemokine producing population. Histologic lung infiltrates are present acutely during MAS, but becomes much more prominent after systemic MAS resolution. This is accompanied by large-scale changes in AMΦ activation and polarization from an M1 to an M2 phenotype. In contrast, with recurrent episodes of MAS pulmonary inflammation in mice is exacerbated, and associated with a reprogramming of AMΦ polarization from an anti-inflammatory/resolution phenotype to proinflammatory. Finally, pulmonary inflammation was ameliorated by direct IFNγ blockade, and requires macrophage responsiveness to IFNγ. Together, our findings support CpG-induced MAS as a powerful model system for the study of SJIA-LD.

There are striking similarities between SJIA-LD and the findings presented herein (16). First, mice with MAS display chronic lymphocytic perivascular and peribronchiolar interstitial inflammation that is patchy and multifocal, resembling the interstitial component of SJIA-LD. Second, pulmonary inflammation in mice was more extensive after both systemic MAS resolution and during recurrent MAS, in agreement with patients developing signs of SJIA-LD in the months after disease onset. Third, both murine MAS and children with SJIA-LD display high levels of IL-18 in both serum and BAL. Fourth, BAL fluid in mice with MAS similarly displayed high levels of IFN-induced chemokines, which was also seen in
some children with SJIA-LD. Finally and most strikingly, both pulmonary gene expression in murine MAS and in SJIA-LD reflected IFNγ activation, a key pathogenic mediator of MAS. These findings support a shared pathogenic link between MAS and SJIA-LD.

Our gene expression profiling demonstrates large-scale reprogramming of AMφ polarization phenotypes both during acute MAS and resolution. AMφ in acute MAS activated gene pathways consistent with proinflammatory “M1” polarization including response to IFNγ. In contrast, resolution phase AMφ displayed more extensive transcriptional changes reflecting alternative activation and pro-resolution phenotypes, including increased TGFβ mRNA, STAT6 activation, and decreased expression of STAT1 targets (associated with IFNγ) (33, 34). Interestingly recurrent MAS rapidly reprogrammed AMφ back to a proinflammatory polarization phenotype. Such alterations in AMφ polarization are notable given the association of SJIA-LD with PAP, caused by defects in AMφ homeostatic functions and polarization (21, 35, 36). Systemic MAS resolution phase AMφ also downregulated genes regulated by Bach1, a transcriptional repressor with key roles in limiting “M2” phenotypes (37). Bach1 deficiency has been shown to worsen some forms of PAP, raising the intriguing possibility that failure to properly activate pro-resolution programs such as Bach1 during chronic/recurrent MAS could contribute to PAP (20).

Our findings also support a central role for IFNγ in a broad array of inflammatory features in the lungs during MAS. There is increasing evidence in both animal models and patients for a key role of IFNγ broadly in MAS pathogenesis (3) including the TLR9 mouse model (11, 13, 14). Our findings in MIIG mice extend these observations by showing for the first time that specifically macrophage responses to IFNγ are required for full MAS features, including more pronounced reductions in some serum cytokines than seen with anti-IFNγ monoclonal antibody treatment. We also demonstrate broad transcriptional responses to interferon throughout the lungs, including emergence of MAS-specific macrophage populations with phenotypes suggesting tissue hemophagocytes including apoptotic cell removal and
response to IFNγ. Notably these phenotypes are similar to those we have recently reported in the bone marrow of a patient with early MAS (38). The present study also complements our recent findings in children with SJIA-LD, and suggests that IFNγ may also have a key role in lung disease pathogenesis (16). An anti-IFNγ monoclonal antibody has been recently approved for familial HLH and is currently being investigated for treatment of MAS (NCT03311854). It remains to be seen whether such IFN-targeted therapy may be beneficial in SJIA-LD.

Our findings demonstrate chronic lymphocytic interstitial inflammation and IFN-driven transcriptional response in the lungs of mice with experimental MAS. However, despite signs of AMϕ activation, we did not identify features of PAP, which is a key pathologic feature of SJIA-LD (16). This suggests that MAS alone is not sufficient to cause SJIA-LD. Our findings would support a model where the cytokine milieu of MAS – including highly elevated IL-18 and IFNγ – is a necessary but not sufficient risk factor for SJIA-LD development. Many SJIA-LD patients experience recurrent MAS or chronic, subclinical MAS. Given our above findings regarding reprogramming of AMϕ with recurrent MAS, could SJIA-LD patients fail to activate resolution AMϕ phenotypes (including Bach1) that affect surfactant metabolism? Additionally, a crucial unknown regarding SJIA-LD is the possible role of cytokine-blocking biologic medications (anti-IL-1 and IL-6), the introduction of which is temporally associated with emergence of SJIA-LD (17). IL-1 blockade can potentiate IFN responses in children with SJIA (39, 40); however, the effects of biologics on pulmonary inflammation remain unknown. As such, further work is urgently needed to define the pathogenic mechanisms connecting SJIA, MAS, and chronic lung disease.
Methods

Murine MAS induction. Female C57BL/6J mice 6 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME). Macrophage insensitive to IFNγ (MIIG) mice were obtained from Dr. Michael Jordan (CCHMC, Cincinnati, OH (32). Mice were treated with five intraperitoneal doses of vehicle (PBS) or 50 μg CpG 1826 as described (Figure S1) (11). For IFNγ blockade, mice given IP injections of isotype control or XMG1.2 in conjunction with CpG as described (11). Further details on mice, sample collection, histology, and immunohistochemistry are in the supplemental methods.

Flow Cytometry, cytokine determination, quantitative PCR, and microarrays. Full experimental procedures are in the supplemental methods.

Single-cell RNA sequencing. scRNA-seq experimental protocol and analysis are described in the supplemental methods.

Statistical Analysis. Data are presented as the mean ± standard error. PBS vs. CpG comparisons were performed with two-tailed student t-test; comparison between multiple groups compared using one-way ANOVA with follow-up Dunnett’s multiple comparisons test. For all tests a P value less than 0.05 was considered significant.

Study Approval. All studies were approved by the Cincinnati Children’s Hospital Medical Center Institutional Animal Care and Use Committee (IACUC; Cincinnati, Ohio).
Author contributions: GSS designed the study. DG, MH, ST, and GSS performed experiments. DG, NS, CW, KT, AAG, ST, MBJ, KWB, and GSS analyzed the data. GSS and NS wrote the first draft of the manuscript. All authors contributed to, reviewed and approved the final version of the manuscript.

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Figure 1: Interstitial pulmonary inflammation in mice during acute MAS. A, representative histological sections of lung tissue from mice treated with PBS (control) or CpG to induce MAS. Section stained with H&E or with anti-CD3 monoclonal antibody where indicated. Top row 200x magnification; bottom row 400x magnification. B, number of CD3+, CD3+CD4+, and CD3+CD8+ lymphocytes in whole lung tissue from mice treated with PBS or CpG. Data expressed as cells/10,000 CD45+ cells. C, cytokine and chemokine levels from whole lung lysates of mice treated with PBS or CpG. Data are representative of three independent experiments (n=4). Error bars represent mean±SEM, *p<0.05, students t-test.
Figure 2: AMϕ demonstrate features of IFNg-mediated activation during acute MAS. A, relative proportion of cell types in BAL determined by flow cytometry. B, BAL fluid cytokine and chemokine levels. C-D, MHC class II and CD206 surface expression in lung macrophage populations as determined by flow cytometry. C, representative histograms. D, mean fluorescence index. E, cytokine and chemokine release by AMϕ from control mice or mice treated with CpG. F, AMϕ gene expression as determined by qRT-PCR. G, AMϕ microRNA expression. Data are representative of three independent experiments (n=4 or 8). Error bars represent mean±SEM, *p<0.05; **p<0.01; ***p<0.001, students t-test.
Figure 3: Distinct immune single-cell populations infiltrate the lung in MAS. A-B) UMAP projection of single-cell populations defined by the software ICGS2 following joint-analysis of (A) wild-type and (B) MAS lung. C) UMAP projection of gene expression for selected markers genes (grey = no expression, red = high). D) The percent of each cell population is shown, normalizing the number of cells in the MAS to wild-type (PBS) capillary endothelial (c24), which should have similar frequencies in wild-type and MAS captures. E) Heatmap of the top Monocyte and Macrophage cluster markers (top 60) from the MAS scRNA-Seq using the MarkerFinder algorithm. Top-enriched Gene Ontology terms and associated enrichment p-values (GO-Elite) are indicated on the left of the heatmap. The top-20 marker genes for each MAS-specific Macrophage cluster are shown to the right of the corresponding heatmap clusters.
Figure 4: Interferon signaling networks are broadly impacted in distinct MAS cell populations. A) Heatmap of MAS versus wild-type fold-changes (log2) for all differentially expressed genes comparing the same cell populations detected in wild-type (PBS) and MAS scRNA-Seq by the software cellHarmony. The heatmap is broken down according to broad and cell-type specific patterns of MAS induced gene changes, using the cellHarmony default display. Enriched PathwayCommons gene sets are displayed to the left of the heatmap with the associated GO-Elite enrichment p-values (blue) and example genes from each cellHarmony gene module are shown to the right of the heatmap. B) Filtered version of the heatmap from panel A, for Interferon signaling associated genes (ToppFun). C) The predicted gene regulatory network for MAS versus wild-type genes in the Classical Monocyte cell population (cellHarmony).
Figure 5: Persistent interstitial inflammation and AMϕ changes after systemic MAS resolution. A, representative histological sections of lung tissue from mice treated with PBS (control) or CpG to induce MAS, after three week recovery. Sections stained with H&E or with anti-CD3 monoclonal antibody where indicated. Top row 200x magnification; bottom row 400x magnification. B-C, BAL fluid relative cell proportions (B) and cytokine and chemokine levels (C) after systemic MAS resolution. D, MHC class II and CD206 surface expression in CD11c<sup>+</sup>CD11b<sup>var</sup> lung macrophages. E-F, AMϕ gene expression as determined by qRT-PCR (E) and microRNA expression (F) in mice with acute MAS (CpG) and MAS resolution (CpG resolution). G, chemokine release from cultured AMϕ from control and CpG-treated mice after systemic MAS resolution. Data are representative of three independent experiments (n=4 or 8). Error bars represent mean±SEM, *p<0.05; **p<0.01; ***p<0.001, student t-test (PBS vs CpG) or ANOVA with follow-up Dunnett’s multiple comparisons test.
Figure 6: Gene expression analysis of AMϕ in acute and resolving MAS. A, principle component analysis. B, hierarchical clustering of differentially expressed genes (DEG) between acute MAS and control macrophages (fold change >2, p<0.05). C, most significantly enriched gene ontology (GO) pathways of genes upregulated in acute MAS AMϕ vs control. D, Venn diagram of differentially regulated genes in acute and resolving MAS vs control AMϕ. E, hierarchical clustering of DEG between systemic MAS resolution and control. F, most significantly enriched GO pathways of genes upregulated in resolving MAS AMϕ vs control. G, most significantly enriched GO pathways of genes downregulated in resolving MAS AMϕ vs control.
Figure 7: Systemic features in mice with recurrent MAS. A-C, hemoglobin, platelet and absolute lymphocyte counts (A), spleen weights (B) and serum ferritin (C) in control mice, mice with acute MAS (PBS-CpG) or recurrent MAS (CpG-CpG). D, serum cytokine levels as determined by luminex assay. Data are representative of three independent experiments (n=4 or 8). Error bars represent mean±SEM, *p<0.05; **p<0.01; ***p<0.001, ANOVA with follow-up Dunnett’s multiple comparisons test.
Figure 8: Enhanced pulmonary inflammation and lung injury with repeated episodes of MAS. A, total BAL fluid cell counts in mice with one (PBS-CpG) or two (CpG-CpG) episodes of MAS. B, BAL fluid chemokine levels in control mice (PBS) or those with one (PBS-CpG) or two (CpG-CpG) episodes of MAS. C, in vitro CXCL9 release from AMΦ isolated from control mice (PBS) or those with one (PBS-CpG) or two (CpG-CpG) episodes of MAS. D, representative histological sections of lung tissue with one (left) or two (right) episodes of MAS. Sections were stained with H&E (top) or anti-CD3 (bottom). E-F, AMΦ gene expression as determined by qRT-PCR (E) and microRNA expression (F) in control mice (PBS) or those with one (PBS-CpG) or two (CpG-CpG) episodes of MAS. G, BAL IgM concentration as determined by ELISA. Data are representative of three independent experiments (n=4 or 8). Error bars represent mean±SEM, *p<0.05; **p<0.01; ***p<0.001, ANOVA with follow-up Dunnett’s multiple comparisons test.
Figure 9: Systemic and pulmonary inflammation in mice with MAS and IFNγ blockade. A-B, CpG + isotype control and CpG + anti-IFNγ treated mice demonstrate mild anemia (left), thrombocytopenia (center), and lymphopenia (right) (A) and splenomegaly (B) compared to mice treated with PBS alone. C, Cytokine levels as determined by luminex except for IL-18 which was determined by specific ELISA. Data are mean of 4 mice per condition, normalized for each analyte. D, BAL fluid CXCL9 levels. E, CXCL9 release by AMϕ. F, AMϕ MHC class II and CD206 surface expression as determined by flow cytometry. G-H, AMϕ gene (G) and microRNA (H) expression as determined by qRT-PCR. Data are representative of three independent experiments (n=4 or 8). Error bars represent mean±SEM, *p<0.05; **p<0.01; ***p<0.001, ANOVA with follow-up Dunnett’s multiple comparisons test.
Figure 10: Macrophage sensitivity to IFNγ is essential for full systemic cytokine storm and alveolar inflammation. A, Spleen weights in WT or MIIG mice treated with PBS or CpG. B, Hemoglobin, platelet, and lymphocyte counts in WT or MIIG mice treated with PBS or CpG. C, Serum ferritin level. D, Cytokine levels as determined by luminex, except for IL-18 which was determined by specific ELISA. Data are mean of 3 mice per condition, normalized for each analyte. E, Concentration of IL-18, CXCL9, and CXCL10 in BAL fluid. F, Concentration of CXCL9 secreted by ex vivo cultured AMΦ. G, AMΦ gene expression as determined by qRT-PCR. n=3 mice per condition. Error bars represent mean±SEM, *p<0.05; **p<0.01; ***p<0.001, ANOVA with follow-up Dunnett’s multiple comparisons test.