Title

Cutting Edge: Neutrophils License the Maturation of Monocytes into Effective Antifungal Effectors.

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Authors

Espinosa, Vanessa
Dutta, Orchi
Wang, Keyi
et al.

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Neutrophils license the maturation of monocytes into effective antifungal effectors

Vanessa Espinosa[1], **Orchi Dutta**[1], **Lena J. Heung**[3], **Keyi Wang**[1], **Yun-Juan Chang**[2], **Patricia Soteropoulos**[2], **Tobias M. Hohl**[4], **Mark C. Siracusa**[1], **Amariliz Rivera**[1]

[1]Center for Immunity and Inflammation, New Jersey Medical School, Rutgers-The State University of New Jersey, Newark, New Jersey, USA.

[2]Genomics Research Program, New Jersey Medical School, Rutgers-The State University of New Jersey, Newark, New Jersey, USA.

[3]Women's Guild Lung Institute, Department of Medicine and Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, California, USA.

[4]Memorial Sloan Kettering Cancer Center, New York, NY

Abstract

Neutrophils are critical for the direct eradication of *Aspergillus fumigatus* conidia, but whether they mediate antifungal defense beyond their role as effectors is unclear. Here, we demonstrate that neutrophil depletion impairs the activation of protective antifungal CCR2^{+} inflammatory monocytes. In the absence of neutrophils, monocytes displayed limited differentiation into monocyte-derived-DCs (Mo-DC), reduced formation of reactive oxygen species (ROS), and diminished conidiacidal activity. Upstream regulator analysis of the transcriptional response in monocytes predicted a loss of STAT1-dependent signals as the potential basis for the dysfunction seen in neutrophil-depleted mice. We find that conditional removal of STAT1 on CCR2^{+} cells results in diminished antifungal monocyte responses while exogenous administration of IFN-γ to neutrophil-depleted mice restores Mo-DC maturation and ROS production. Altogether, our findings support a critical role for neutrophils in antifungal immunity not only as effectors but also as important contributors to antifungal monocyte activation, in part by regulating STAT1-dependent functions.

Introduction:

Neutrophils are well known for their essential role in defense against fungal pathogens and are rapidly recruited to the site of infection(1). Neutrophils can directly kill fungal pathogens via multiple mechanisms including phagocytosis, the release of proteases, neutrophil extracellular traps (NETs), and the generation of reactive oxygen species (ROS)(2). The enhanced susceptibility of neutropenic patients to invasive fungal infection (IFI) has thus been primarily understood as a failure of pathogen control due to the absence of these important effector cells. Emerging studies suggest that neutrophils can shape the response

Correspondence should be addressed to: Amariliz Rivera, PhD, Associate Professor, Rutgers University-NJMS, 205 So Orange Ave, Newark, NJ, 07103, riveraam@njms.rutgers.edu.

of other immune cell subsets, and can be relevant sources of cytokines and chemokines (3–7). Whether neutrophils have more nuanced contributions in antifungal protection that go beyond their traditional role as effectors remains to be determined.

In addition to neutrophils, $CCR2^+$ inflammatory monocytes ($CCR2^+$Mo) are innate cell precursors to macrophage and dendritic cell subsets that are crucial for defense against multiple pathogens including fungi (8–15). In previous studies, we established that $CCR2^+$Mo are essential for defense against invasive aspergillosis (IA) (14, 16). Upon infection with *Aspergillus fumigatus* (*Af*), $CCR2^+$Mo help activate antifungal neutrophils(16), they differentiate into $CCR2^+CD11b^+CD11c^+MHCII^+$ cells (Mo-DCs, same definition used throughout) (10, 14), that mediate fungal killing, produce cytokines, and help recruit pDCs; thus promoting optimal defense against IA (14, 17, 18).

Despite the important, antifungal functions of $CCR2^+$Mo and Mo-DCs, these cells are not able to protect against IA if neutrophils are depleted(14). Why Mo-DCs are unable to protect against IA in the absence of neutrophils is unclear. In this study, we set out to examine the impact of neutrophil depletion on the antifungal response of $CCR2^+$Mo.

Materials and Methods:

Mice, infection and histology

CCR2 reporter (CCR2-GFP), $CCR2^{Cre}$ and $STAT1^{fl/fl}$ were generated as previously described (19–21). Studies were performed in pathogen-specific free conditions according to Institutional Animal Care and Use Committee approved protocols. Fungal infections were done with, *Af*-DsRed (22), *Af*293, or FLARE (Fluorescent Aspergillus Reporter) conidia (23). FLARE were generated and analyzed as previously described and shown in supplementary fig 1 (23). Fungal culture, mouse infections tissue collection, and fungal growth assessments in infected lungs was done as previously described (14, 16).

Neutrophil Depletion and IFN administration

Mice were injected daily with 500 μg i.p and 250 μg i.t of 1A8 (anti-Ly6G) monoclonal antibody (BioXCell). Treatments were started at day −1. Mouse monoclonal IgG2a (2A3) was used as a control (BioXCell). For IFN treatment (Fig 3), neutrophil-depleted mice were treated with 1.0 ug i.p of murine IFN-γ (Peprotech) on the day of infection and D+1. For survival experiments (Fig 4 F), mice were injected with IFN-γ (1.0 ug i.p) on D0 and every other day.

Lung Cell Isolation and Flow Cytometry

Single cell suspensions from lung samples were done and analyzed as previously described (14, 16). BAL cells from infected mice were cultured with 1.0 μM of CM-H2DCFA (Life Technologies) for 60 minutes at 37°C for ROS detection. All flow cytometry was done in a BD Fortessa X-20 and analyzed with FlowJo software.

Quantitative RT-PCR and ELISA in Lung Tissue

RNA from lungs was extracted with Trizol (Invitrogen). Gene expression was calculated using ΔΔCT method relative to naïve sample using TaqMan probes (Applied Biosystems) and normalized to GAPDH. IFN-γ, IFN-λ, and TNF-α cytokines in lung homogenates were measured by ELISAs (ThermoFisher and R&D systems).

Cell Sorting, RNA Sequencing and Analysis

Live CCR2^{GFP+}CD45^{+} CD11b^{+} NK1.1^{-} Ly6C^{+} Ly6G^{-} (CCR2^{+} Mo) were isolated from the lung of *Af*-infected CCR2-GFP mice (48 hours) to >98.9% purity using a BD FACS ARIA II cell sorter. The transcriptional profile of CCR2^{+}Mo was examined by RNA-seq. The transcriptional profile of CCR2^{+}Mo was examined by RNA-seq. RNA processing for library generation and sequencing on an Illumina HiSeq instrument was done by the Genomic Research Core facility as described (14, 16). For each group, three different biological replicates were examined, and differentially regulated gene clusters were identified. RNA-seq data has been deposited under project PRJNA847069 BioProject's metadata is available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA847069. Neutrophils (CD45^{+}, Siglec F^{-}, CD11b^{+}, Ly6C^{int}, Ly6G^{+}), monocytes (CD45^{+}, Siglec F^{-}, CD11b^{+}, Ly6C^{hi}, Ly6G^{-}, CCR2^{+}, NK1–1^{-}), and AM (CD45^{+}, Siglec F^{+}, CD11c^{+}) were sorted from *Af*-infected (48 hrs after infection) and naïve lung; 1 × 10^5 cells were cultured overnight and examined for cytokine secretion in supernatants by ELISA.

Results:

Neutrophils are required to license antifungal monocytes

To examine the impact of neutrophils on global antifungal immunity, we examined the response of *Af*-infected mice after neutrophil depletion (α-Ly6G). Consistent with previous studies, neutrophil depletion resulted in increased mortality (Fig. 1A), increased fungal burden and susceptibility to IA (Fig. 1B–1C). We confirmed the efficient depletion of neutrophils in the lung at 36 hours after infection (Fig. 1D). The recruitment of CCR2^{+}Mo to the lung was increased in neutrophil-depleted mice (Fig. 1E). This observation is consistent with previous studies in neutropenic mice (24). Although the number of recruited cells was increased, Mo-DCs failed to upregulate MHC II expression (Fig. 1F), showed diminished killing of fungal conidia (Fig. 1G), and had reduced ROS (Fig. 1H) (a critical antifungal effector mechanism (25, 26). These observations suggest that in the absence of neutrophils, monocytes are impaired in their ability to become efficient antifungal effectors. These findings suggest that susceptibility to IA in neutropenic mice is likely a combination of lack of fungal conidia inactivation by neutrophils as well as a failure of monocytes to become competent antifungal effector cells.

Neutrophil depletion alters the antifungal transcriptional response of CCR2^{+} monocytes

To test if neutrophils shape the response of CCR2^{+} Mo, we performed a transcriptional analysis of CCR2^{+} Mo isolated from the lung of *Af*-infected mice in the presence or absence of neutrophils. Neutrophil depletion altered the response of CCR2^{+} Mo (Fig. 2A and Supplementary Table 1). Ingenuity Pathway Analysis (IPA) suggested that in the

context of neutropenia, CCR2^{+} Mo had impaired activation of genes in canonical pathways associated with DC maturation (Fig. 2B). Further, IPA analysis for upstream regulators of differentially expressed genes (threshold set at fold changes >2.5, Fig. 2C) predicted altered interferon activation as potential basis for dysfunctional responses of CCR2^{+}Mo in *Af*-infected, neutropenic mice (Fig. 2D).

In previous studies, we uncovered that CCR2^{+} Mo are required for the proper activation of interferon (IFN) responses that are necessary for the activation of antifungal neutrophils (16). The transcriptional analysis of CCR2^{+}Mo suggests that neutrophils might also be required to trigger the production of IFNs, and that these cytokines may act on monocytes to promote their maturation into antifungal effectors. We previously determined that all IFNs are produced in response to *Af* infection with distinct kinetics(16). Thus, we examined IFN expression at their respective peaks of expression in neutropenic mice. We observed that neutrophil depletion in *Af* infection resulted in reduced expression of all IFNs examined (Fig 3A–C). In contrast to IFNs, neutrophil depletion led to an increase in TNF production (Fig 3D), an observation consistent with previous studies (24, 27). In aggregate, our data suggest that neutrophils are required for optimal expression of IFNs during pulmonary aspergillosis, but are not linked to a global dysregulation of inflammatory responses.

To determine whether the impaired maturation of Mo-DCs in neutrophil-depleted mice was linked to defective IFN production, we tested if the antifungal response of CCR2^{+}Mo could be rescued by IFN administration. We chose to test the role of IFN-γ on this response based on the previously reported importance of this cytokine in the activation of Mo-DCs (28). We observed that neutrophil-depleted mice treated with IFN-γ had improved CCR2^{+}Mo responses that included increased ROS production (Fig. 3E), increased MHC II expression (Fig. 3F) and improved control of fungal burden (Fig 3G). Importantly, neutrophils sorted from the lung of *Af*-infected mice secreted significant amounts of IFN-γ as compared to neutrophils from naïve mice (Fig 3H). Mo and AMs sorted from the same mice also secreted some IFN-γ as compared to their naïve counterparts, but to a lesser extent than what was measured in neutrophils (Fig 3H). Altogether, these data support the idea that defective IFN production in neutrophil-depleted mice is linked to CCR2^{+}Mo dysfunction during *Af* infection.

A limitation of this approach is that exogenous IFN-γ may have improved antifungal CCR2^{+}Mo function via indirect effects. Thus, we employed a genetic loss-of-function model to test the impact of IFN-STAT1 signaling directly on CCR2^{+} Mo. STAT1 is critical in the signaling cascade of all interferons (29, 30). We thus generated mice with conditional knockout of the STAT1 gene in CCR2^{+} cells (CCR2^{cre}STAT1$^{fl/fl}$ mice)(20). CCR2^{+}Mo (Fig 4A) and neutrophil (Fig 4B) recruitment to the lung was not affected in CCR2^{cre}STAT1$^{fl/fl}$ mice. In contrast, control of fungal burden was diminished in *Af*-infected CCR2^{cre}STAT1$^{fl/fl}$ mice (Fig. 4E). Functionally, monocytes displayed diminished Mo-DC differentiation (Fig. 4C) and reduced production of ROS (Fig 4D). Importantly, CCR2^{cre}STAT1$^{fl/fl}$ succumbed to infection to *Af* (Fig. 4F), which was linked to IA (Fig. 4G–H). Administration of IFN-γ to CCR2^{cre}STAT1$^{fl/fl}$ was not able to rescue *Af*-infected mice (Fig 4F), thus supporting the importance of IFN-γ acting directly on CCR2^{+}Mo. In aggregate, these observations suggest that IFNs trigger a STAT1-dependent transcriptional program on CCR2^{+} Mo that is

necessary for the full activation of antifungal responses by CCR2^{+}Mo and their derivative effectors.

Discussion

Our observations support the idea that beyond their role as antifungal effectors, neutrophils are important for the regulation of antifungal CCR2^{+}Mo. Our findings suggest that one of the important ways in which neutrophils influence antifungal monocytes is by promoting the production of IFNs. The mechanisms of neutrophil-dependent IFN production could be as a direct cellular source and/or by regulating IFN production in other cells. Monocytes are known to be sensitive to the effects of both type I and II interferons, and previous studies have found an important role for IFNs in the local differentiation of monocytes into Mo-DCs (28, 31, 32). Furthermore, in a model of toxoplasmosis, neutrophils were found to be important, direct sources of IFN-γ (33, 34). Similarly, we observed that neutrophils sorted from the lung of *Af*-infected mice secreted IFN-γ to a significant extent. Thus, during *Af* infection, neutrophils contribute to IFN-γ levels, at least in part, via direct production. Additional contributions to global IFN levels during *Af* infection could be indirect via effects on other cells such as NK cells (35, 36). In turn, IFNs then help license CCR2^{+}Mo differentiation and acquisition of Mo-DC antifungal functions. The current study adds to our understanding of innate immune cell cross-talk during aspergillosis by placing neutrophils as both targets and regulators of antifungal monocytes. The observation that STAT1 is an important regulator of antifungal monocytes also suggest the expanded, therapeutic potential of IFNs in the treatment of fungal infections by activating essential antifungal effector responses in both neutrophils and monocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points:

- Monocytes have impaired antifungal effector function in neutropenic mice
- STAT1 expression on CCR2+ monocytes is required for defense against IA
- Neutrophils are a relevant direct source of IFN-g during Aspergillosis

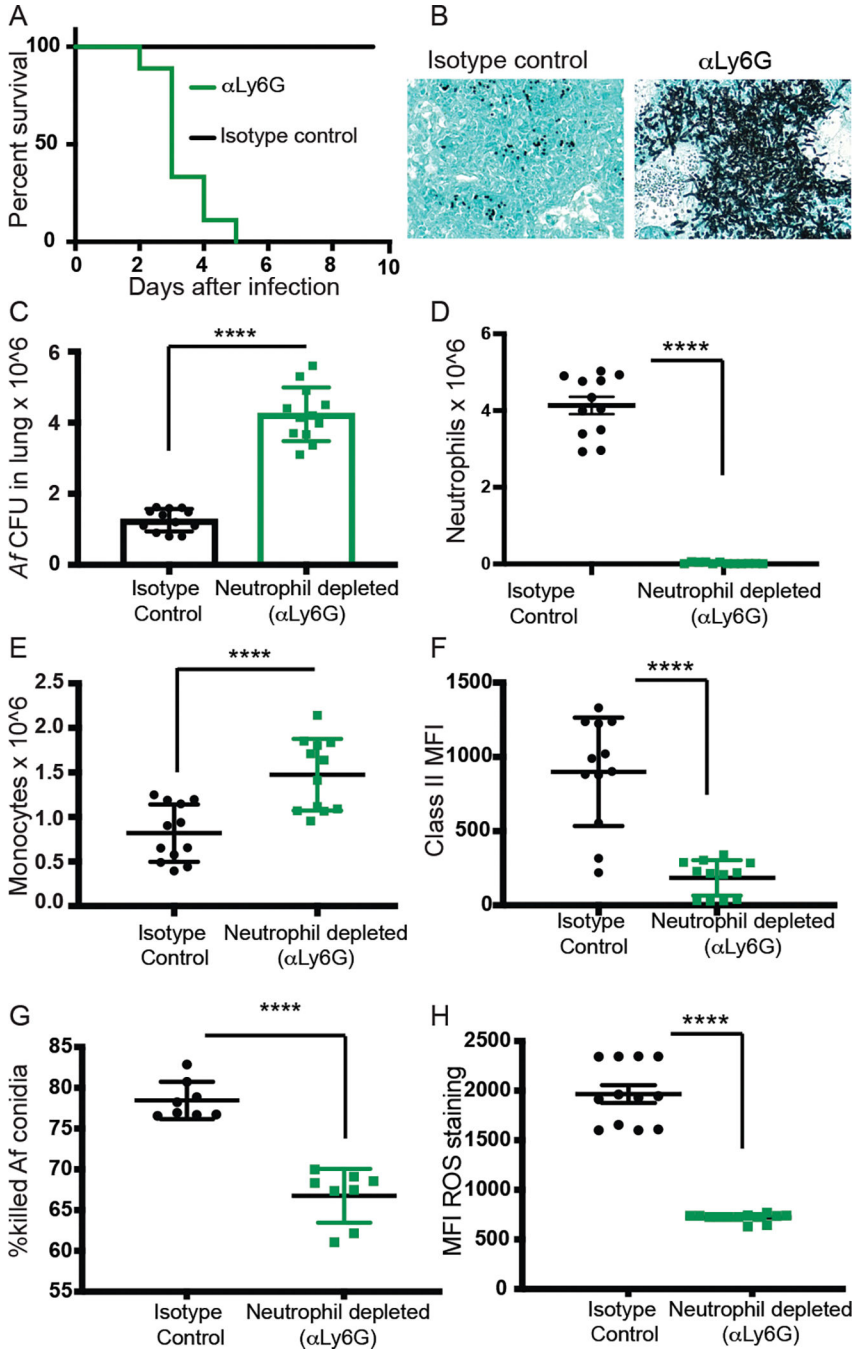

Figure 1:

A) Survival curve of neutrophil-depleted or control mice infected with 8×10^7 *Af* conidia. B) Representative photomicrographs of formalin-fixed GMS-stained lung sections at day 4 after infection. C) Fungal colony forming units in lung 36 hours after infection. D) Total number of neutrophils in isotype control mice and neutrophil-depleted mice. E) Total number of monocytes recruited to the lung. F) Mean fluorescence intensity for MHC Class II expression in Mo-DC gate. G) Frequency of killed FLARE conidia by Mo-DCs. H) ROS production by airway monocytes isolated from neutrophil-depleted or isotype control

treated mice at 36 hours after infection. Graphs show mean ± SEM of ROS MFI as assayed by FACS. In all graphs, each symbol represents one mouse. Data shown in this figure is cumulative of three independent experiments and show mean ± SEM. **** $p<0.0001$ as per Mann-Whitney test.

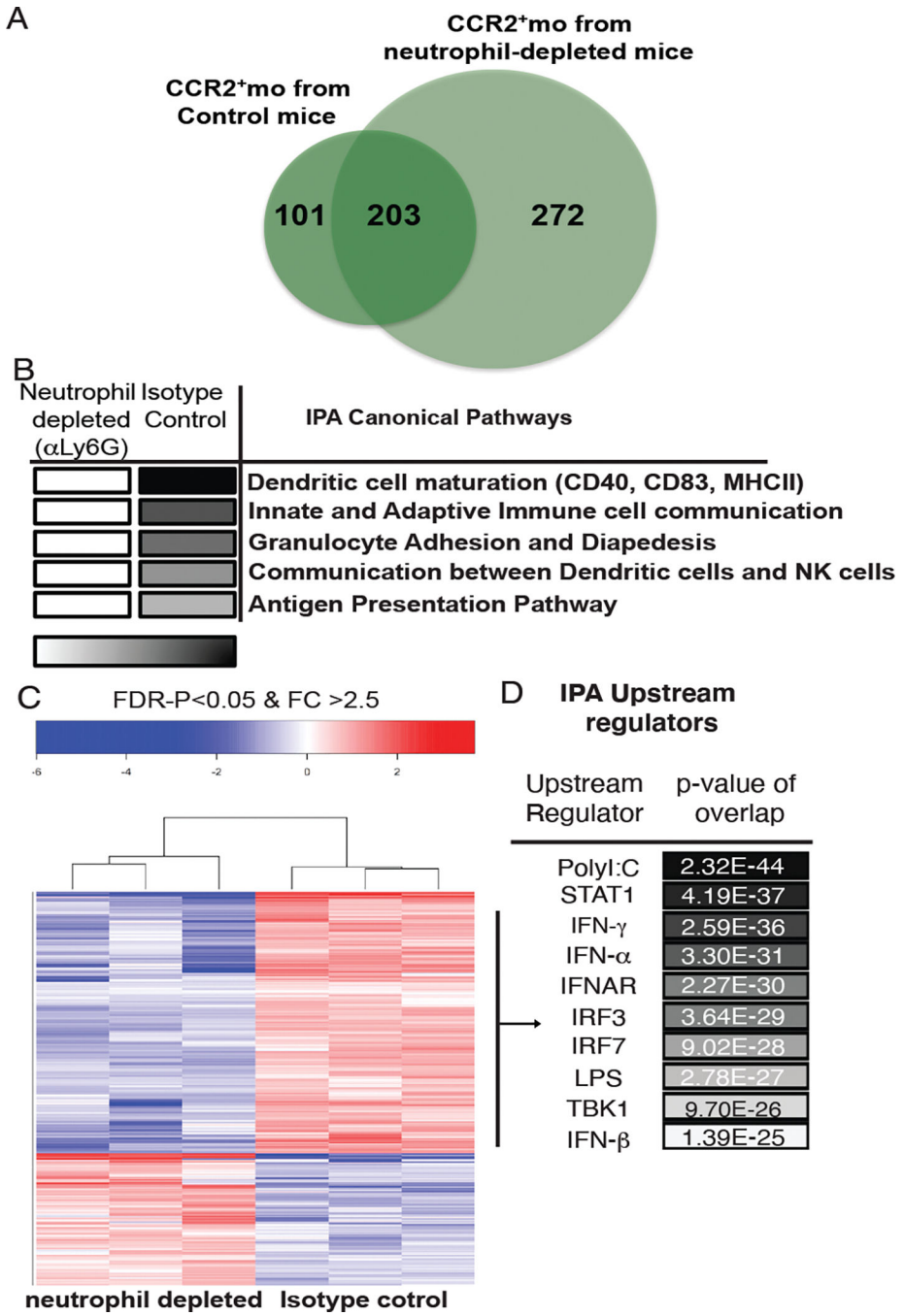

Figure 2:

Pulmonary CCR2^{+}Mo were isolated at 48 after *Af* infection from isotype-treated or neutrophil-depleted mice and analyzed by RNA-seq. A) Intersectional overview of common and differentially expressed transcripts in antifungal monocytes activated in the presence or absence of neutrophils. B) Canonical pathways differentially expressed in control CCR2^{+}Mo versus CCR2^{+}Mo from neutrophil-depleted mice as identified by Ingenuity Pathway Analysis. C) Heat map is for 531 genes differentially expressed at > 2.5 fold with a FDR-p <0.05. Each lane is for an indepenent biological replicate. D) Ingenuity Pathway

Analysis software prediction of upstream regulators of genes that were upregulated in monocytes isolated from isotype control mice but not in monocytes from neutrophil-depleted mice.

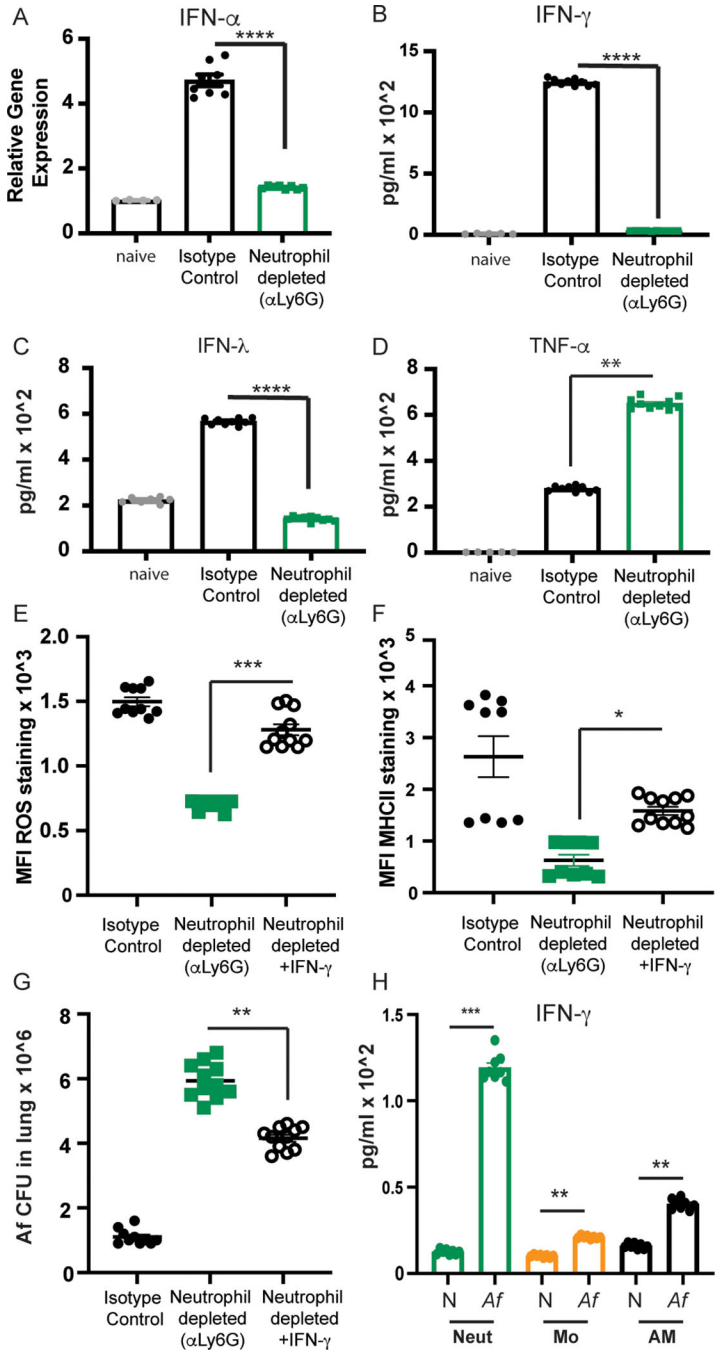

Figure 3:

A-D) Cytokine gene expression in naïve, *Af*-infected, isotype control treated or *Af*-infected, neutrophil depleted mice. A) Relative gene expression (ΔΔCT) of IFN-α at 6 hours. B-D) IFN-γ, IFN-λ, TNF-α in lung homogenates at 48 p.i. were measured by ELISA. E-G) Mice were treated with IFN-γ at day 0 and +1 of *Af* infection. At 48 hours post infection mice were examined for E) ROS production by BAL monocytes, F) MHCII MFI in pulmonary monocytes and G) Fungal burden. Data shown is cumulative of 2–3 independent experiments. Each symbol represents one mouse. Data shown is mean ± SEM.

H) Neutrophils, monocytes, and alveolar macrophages (AM) were sorted from the lung of mice that were naïve or infected with *Af* 48 hrs earlier. IFN-γ levels in supernatants was examined 24 hrs after culture by ELISA. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as per one-way ANOVA, multiple comparison analysis done with Prism software.

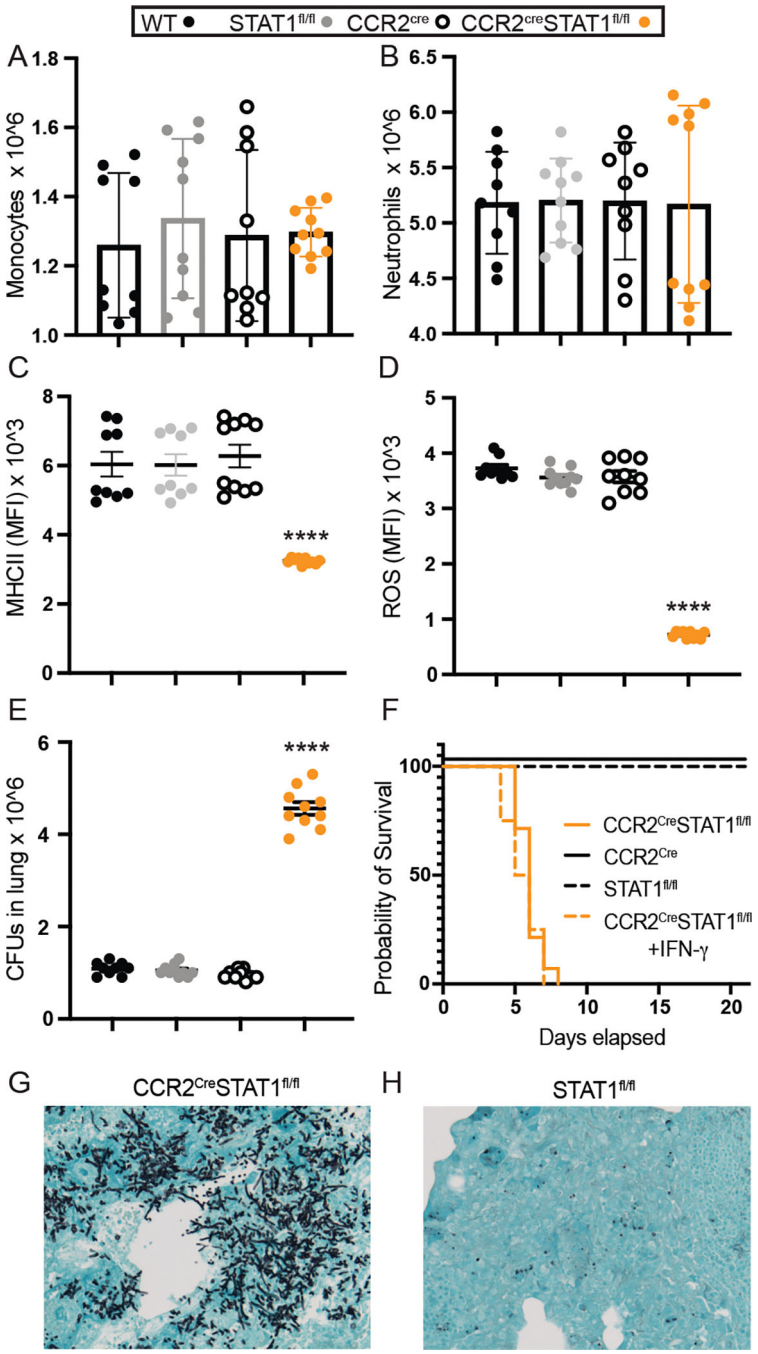

Figure 4:

CCR2^{cre}STAT1$^{fl/fl}$, CCR2^{cre}, STAT1$^{fl/fl}$ and control WT mice were infected with *Af* and examined at 48 hrs after infection. Recruited pulmonary monocytes (A) and neutrophils (B). C) MFI of MHCII in pulmonary monocytes. D) MFI of ROS staining in monocytes in BAL. E) Fungal burden. A-E) Data is cumulative of two independent experiments, each symbol represents one mouse. Data shown is mean ± SEM. WT mice (black symbols), STAT1$^{fl/fl}$ (gray circles), CCR2^{cre} (white circles), CCR2^{cre}STAT1$^{fl/fl}$ (orange circles). **** $p<0.0001$ as per one-way ANOVA, multiple comparison was done with Prism software.

F) Survival curve of *Af*-infected groups as indicated. A subset of CCR2^{cre}STAT1$^{fl/fl}$ were treated with IFN-γ the day of *Af*-infection and every other day thereafter. Data is cumulative of three independent experiments for a total of 10–15 mice per group. G) Representative GMS-stained lung sections at time of euthanasia.