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Axl Receptor Blockade Protects from Invasive Pulmonary Aspergillosis in Mice

Takehiko Shibata,^{*,1} David M. Habel,[†] Ana Lucia Coelho,[†] and Cory M. Hogaboam^{*}

Aspergillus fumigatus is a sporulating fungus found ubiquitously in the environment, which is quickly contained in the immunocompetent host but can cause lethal invasive aspergillosis in the immunocompromised host. We have recently demonstrated that Axl (one member of the Tyro3, Axl, MerTK receptor family) is a key regulator of antiviral immune responses in the lung. In this study, we investigated the role of Axl in antifungal immunity in a model of invasive pulmonary aspergillosis (IPA). In this model, *Aspergillus fumigatus* conidia were administered into the lungs of neutrophil-depleted mice, and the mice were monitored for survival, lung inflammatory response, and fungal clearance. The lethal effect of IPA was significantly reduced in anti-Axl mAb-treated mice compared with IgG control-treated mice. Targeting Axl significantly inhibited pulmonary inflammation, including the expression of IL-1 β , IL-6, TNF- α , and chitinase-like proteins in whole lung. Further, anti-Axl mAb treatment significantly increased M1 macrophages that highly expressed inducible NO synthase and decreased M2 macrophages that expressed Arginase 1 and were found in inflammatory zone protein (Fizz1). More importantly, anti-Axl mAb treatment significantly increased the number of IFN- γ -producing T cells and NK cells compared with the IgG control group during IPA. Together, our results demonstrate that the Axl mAb treatment is protective during invasive aspergillosis in neutropenic mice. Collectively, these data suggest a potential deleterious role for Axl during primary immune responses directed against *A. fumigatus* and novel therapeutic strategy for IPA. *The Journal of Immunology*, 2014, 193: 3559–3565.

A *Aspergillus fumigatus* is a ubiquitous sporulating fungus, and the spores or conidia from this fungus are generally rapidly cleared from an immunocompetent individual (1–3). In contrast, *A. fumigatus* infections primarily occur through inhalation of airborne conidia with subsequent germination and hyphal growth in the lungs, resulting in invasive aspergillosis. Individuals suffering from chronic lung disease, such as cystic fibrosis, or those who are immunocompromised, especially, transplant recipients, cancer patients undergoing chemotherapy, and patients receiving corticosteroids, have an increased risk of contracting aspergillosis with high morbidity and mortality rates (1, 4). Further, a developing body of evidence suggests that *A. fumigatus* conidia exacerbate allergic and asthmatic diseases (3, 5, 6). Currently available antifungal therapies have many side effects, and many are difficult to administer for prophylaxis to prevent invasive pulmonary aspergillosis. Nevertheless, experimental and clinical data demonstrate that the pulmonary immune response to *A. fumigatus* conidia in the immunocompromised and

the allergic host is skewed toward a Th2-type immune response rather than a protective Th1-type immune response (7). Therefore, therapeutic redirection of the immune response toward a protective Th1-type immunity is an attractive option for the containment of invasive fungal growth in the immunodeficient host and *A. fumigatus*-induced allergic disease.

Investigations of the innate immune receptors and soluble factors that inhibit or enhance Th1-type protective immune activation during IPA led us to examine the role of Axl receptor activation in this disease. Previously, we showed that anti-Axl mAb treatment increased IFN- γ production from T cells and NK cells, thereby enhancing viral clearance during respiratory syncytial virus infection in mice (8). Axl is one member of the Tyro3, Axl, and MerTK (TAM) receptor family. TAMs are distinct receptor protein-tyrosine kinases expressed by various immune and nonimmune cells, which are activated by vitamin K-dependent protein growth arrest-specific protein 6 (Gas6) and protein S (9). First described as critical receptors for the clearance of apoptotic cells and as proproliferative mediators on normal and neoplastic cells, it is now known that TAM receptors have potent inhibitory functions on TLR- and cytokine-driven immune responses (10–13). Thus, the current study focused on the role of Axl in the pulmonary immune response directed against *A. fumigatus* during experimental IPA.

Materials and Methods

Mice

Female C57BL/6 mice (6–8 wk of age) were purchased from Taconic Farms (Germantown, NY). Prior approval for mouse use in the protocols described below was obtained from the University Committee on Use and Care of Animals at the University of Michigan Medical School.

Invasive aspergillosis model

A well-described model of invasive aspergillosis was used in the current study (14). To induce neutropenia, mice were injected with anti-Ly-6G Ab (100 μ g in PBS; BioXcell, West Lebanon, NH) 24 h prior to intratracheal challenge. Briefly, mice were anesthetized with a mixture of ketamine and xylazine, and 5.0×10^6 *A. fumigatus* (*A. fumigatus* Fresenius, anamorph;

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Abbreviations used in this article: DC, dendritic cell; GMS, Grocott methenamine silver; IM, interstitial macrophage; IPA, invasive pulmonary aspergillosis; TAM, Tyro3, Axl, and MerTK.

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American Type Culture Collection, Manassas, VA) conidia suspended in 30 μ l PBS with 0.1% Tween 80 were administered by intratracheal injection. For survival studies, mice ($n = 10$ per group) were monitored every 12–24 h after the *A. fumigatus* challenge and euthanized if they appeared moribund. In other groups of mice ($n = 5$ –10 per group), whole-lung samples were processed for quantitative PCR, histological, or flow cytometric analysis (described below) at either day 1 or day 2 after conidia injection. In our previous studies, the extent of neutropenia was ~90%, and rebound of neutrophil numbers began to occur 24–48 h after injection of anti-Ly-6G Ab (15). Full reconstitution of neutrophil numbers typically occurred by days 3–5 after anti-Ly-6G Ab injection. In experiments involving Ab treatment, groups of mice were treated via i.p. injection with human IgG1 (i.e., control group; 5 μ g per dose) or anti-Axl mAb (monoclonal anti-human Axl mAb YW327.6S2; 5 μ g/dose; Genentech). Note that the anti-Axl mAb recognizes both mouse and human Axl with high affinity (16).

Quantitative PCR analysis

Total RNA was isolated from whole lung or cultured cells using TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA). Purified RNA was treated with DNase, and 0.2 μ g RNA was reverse transcribed into cDNA using TaqMan reverse transcription reagents (Foster City, CA). Quantitative gene expression assays were used to quantify *Il1b*, *Il6*, *Tnf*, *Chia*, *Chi3l3*, *Chi3l1*, *Inos*, *Arg1*, *Fizz1*, *Ifng*, *Cxcl9*, *Ccl17*, *Ccl22*, and *Ccl2* according to the manufacturer's directions (Applied Biosystems, Carlsbad, CA). The fold changes in transcript expression were calculated by comparing the gene expression in whole-lung or cell culture samples with that of transcript levels in naive whole lung or control cell conditions, and the levels in the latter two types of samples were assigned a value of 1.

Whole-lung immunohistochemical and histological analysis

Whole lungs were fully inflated with 10% formalin, dissected, and placed in fresh 10% formalin for 24 h. Routine histological techniques were used to paraffin embed the entire lung, and 5- μ m sections of whole lung were stained with H&E and Grocott methenamine silver (GMS) stain. Microscopy analysis was performed using a microscope (OLYMPUS BX43, Tokyo), camera (OLYMPUS DP73), and software (cellSens Dimension 1.7, OLYMPUS).

Flow cytometry analysis

Whole-lung samples were incubated with collagenase (1 mg/ml; Roche, Mannheim, Germany) at 37°C for 45 min. Whole-lung cell suspensions were then incubated with anti-CD16/32 (2.4G2) and further labeled with fluorescent dye-conjugated mAb (CD45, CD11b, F4/80, CD11c, Ly6G, CD3, CD4, CD8, NK1.1, Nkp46; eBioscience, San Diego, CA) for 30 min. Tissue culture-generated cells were incubated with the same Abs for the same amount of time. For intracellular staining of cytokines, lung cells (1.0×10^6 cells per well) that were stained with anti-CD3, anti-CD4, anti-NK1.1, or anti-NKp46 Abs were resuspended in a fixation/permeabilization solution (BD Cytfix/Cytoperm Kit; BD Biosciences Pharmingen) and stained with anti-IFN- γ (PBL Interferon Source, Piscataway, NJ) for 30 min. Data were acquired on an LSR II machine using

FACSDiva software (BD Biosciences), and all data were analyzed using a FlowJo software package (TreeStar, Ashland, OR).

Statistical analysis

All results are expressed as the mean $\pm$ SEM. A Student *t* test or an ANOVA and a Student–Newman–Keuls multiple comparison test were used to determine statistical significance between groups. A *p* value ≤ 0.05 was considered statistically significant.

Results

Anti-Axl mAb protects from experimental IPA

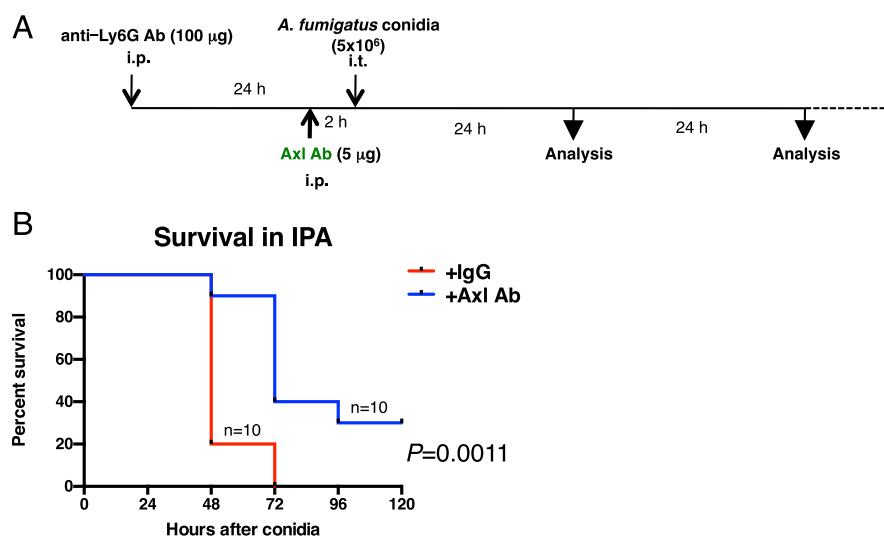
First, we modeled IPA by depleting neutrophils with 100 μ g of anti-Ly6G Ab injection 24 h before *A. fumigatus* conidia challenge of C57BL/6 mice (Supplemental Fig. 1A). Control groups of C57BL/6 mice received 100 μ g of nonspecific IgG at the same time prior to conidia challenge. Both neutrophil infiltration in the bronchoalveolar space and fungal containment were observed in the IgG control group at 24 h after conidia challenge, as determined by analysis of bronchoalveolar lavage (BAL) samples for leukocytes and GMS-stained fungus, respectively (Supplemental Fig. 1B, 1D, left panels; Fig. 1C). In contrast, neutrophil numbers were significantly decreased and fungal staining markedly increased at 24 h after conidia challenge in anti-Ly6G-treated neutropenic mice (Supplemental Fig. 1B, right panels; Fig. 1C), and the germination of *Aspergillus* conidia was observed in these mice (Supplemental Fig. 1B, 1D, right panels).

To investigate the role of Axl during the model of IPA, we targeted this TAM receptor using a mAb (16). Accordingly, neutropenic mice were i.p. injected with one dose of 5 μ g of control IgG or anti-Axl mAb at 2 h prior to conidia instillation (Fig. 1A), and inflammation, fungal clearance, and survival were monitored up to 48 h (monitoring cell infiltration and fungal clearance) or 120 h (monitoring survival) after conidia administration. In the IgG1-treated control group, mice began to die 24 h after conidia infection, and all mice in this group were dead by 72 h post infection (Fig. 1B). Conversely, treatment of neutropenic mice with anti-Axl mAb significantly reduced the lethal effects of conidia, and ~40% of these Ab-treated mice were alive at 96 h post infection (Fig. 1B).

Anti-Axl mAb suppresses the inflammatory response and fungal growth during experimental IPA

As shown in Fig. 2A, a severe pulmonary inflammatory response was evident in H&E-stained whole-lung samples at 24 and 48 h after conidia infection in IgG-treated neutropenic mice. Con-

FIGURE 1. Anti-Axl mAb increases survival during IPA. Mice were treated with IgG1 (+ IgG1 Ab) or anti-Axl mAb (+ Axl mAb) prior to IPA (A). Kaplan–Meier survival plot of IgG1-treated and Axl mAb-treated mice during IPA (B). Results are expressed as the percent of survival of a starting cohort of 10 mice per group.



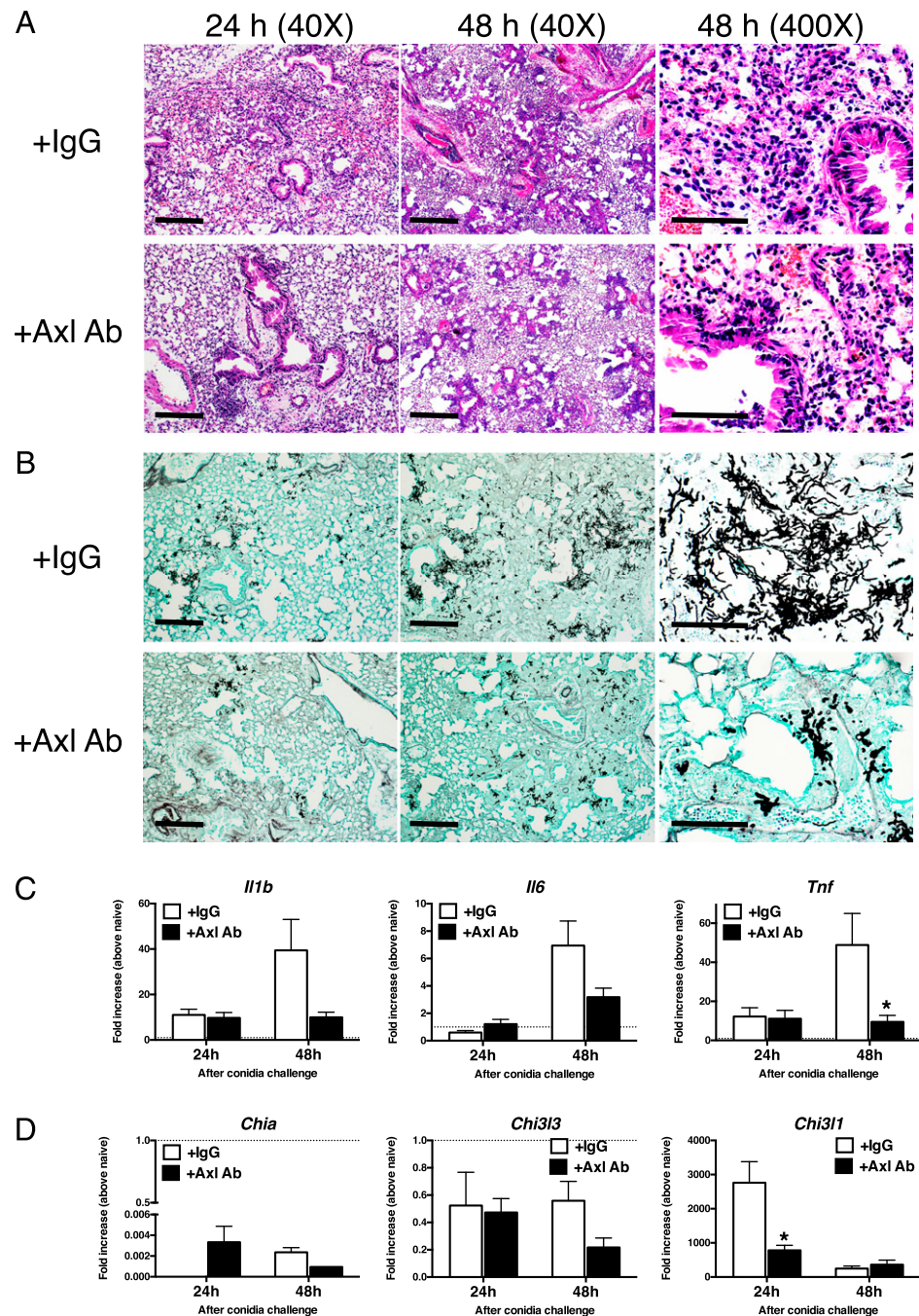


FIGURE 2. Anti-Axl mAb suppresses both the inflammatory response and fungal growth during IPA. Representative H&E-stained lung tissue sections from IgG1- and Axl mAb-treated mice at 24 and 48 h after conidia challenge. Original magnification $\times 40$ or $\times 400$ (**A**). Scale bar, 200 μm ($\times 40$), 100 μm ($\times 400$). Results are expressed as the mean $\pm$ SEM for $n = 5$ per group. Representative GMS-stained lung tissue sections from IgG1- and Axl mAb-treated mice at 24 and 48 h after conidia challenge. Original magnification $\times 40$ or $\times 400$ (**B**). Scale bar, 200 μm ($\times 40$), 100 μm ($\times 400$). Quantitative PCR analysis of *Il1b*, *Il6*, *Tnf* (**C**), *Chia*, *Chi3l3*, and *Chi3l1* (**D**) transcript levels in whole-lung samples at 24 and 48 h after conidia challenge. Results are expressed as the mean $\pm$ SEM of $n = 5$ per group. * $p < 0.05$ versus the IgG control group.

versely, anti-Axl mAb treatment markedly inhibited this pulmonary inflammatory response at both 24 and 48 h after conidia challenge. This reduced pulmonary inflammatory response coincided with dramatically reduced GMS-stained fungal material at both time points in the anti-Axl mAb treatment group (Fig. 2B). We then investigated the mechanism through which Axl mAb treatment suppressed the inflammatory response and conidia growth in the lung. First, the expression of proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α was examined. The expression of these proinflammatory cytokines was significantly increased after conidia challenge in the IgG control group, but not the anti-Axl mAb group, at 48 h (Fig. 2C), results consistent with our findings of reduced inflammatory cell infiltration in the latter group. We further quantified the effect of anti-Axl mAb treatment on other host markers of fungal infection, including *Chia*, which encodes acidic chitinase (AMCase); chitinase-related genes chitinase 3-like 3 (*Chi3l3*); and

chitinase-related genes chitinase 3-like 1 (*Chi3l1*). The pulmonary transcript expression of *Chia* was strongly suppressed by conidia challenge in neutropenic mice regardless of Ab treatment, compared with naive lung (Fig. 2D), presumably reflecting the fact that Ly6G $^{+}$ cells are the primary source of this acidic chitinase. Similarly, the transcript levels of *Chi3l3* (also known as Ym-1) were lower in both the IgG and anti-Axl mAb treatment groups compared with a naive group. However, *Chi3l1* (also known as YKL-40) was significantly induced in IgG-treated neutropenic mice at 24 h after conidia infection, but anti-Axl mAb treatment significantly suppressed its expression at 24 h after conidia challenge, compared with the IgG control group.

Anti-Axl mAb increased the numbers of M1 macrophages and dendritic cells during IPA

To further elucidate the mechanism whereby targeting Axl promoted antifungal immunity in the neutropenic mouse, we next

examined the role of this TAM receptor in the numbers and/or activation status of myeloid cell populations, including macrophages and dendritic cells (DCs), in the lung (17). A representative flow cytometric analysis of F4/80 and CD11c populations in lung samples at 24 h after conidia challenge of IgG or anti-Axl mAb-treated neutropenic mice is shown in Fig. 3A. This analysis revealed that targeting Axl in neutropenic mice significantly increased the numbers of alveolar macrophages (CD11c⁺F4/80⁺CD11b⁻) and DCs (CD11c⁺F4/80⁻CD11b⁺) at 24 h after conidia challenge (Fig. 3A). Conversely, the number of interstitial macrophages (IMs) was significantly decreased in the anti-Axl mAb-treated group compared with the IgG one. Cell number differences were less apparent at 48 h after conidia challenge (Fig. 3A), but we noted significant differences in the transcript expression of key macrophage activation markers between the IgG and anti-Axl mAb treatment groups at the later time point (Fig. 3B). Specifically, whole-lung transcript levels of *Inos* (an M1 activation marker) were significantly increased, whereas *Arg1* and *Fizz1* (M2 activation markers) were absent or markedly lower compared with the IgG control group at both times after conidia challenge (Fig. 3B). Thus, the targeting of Axl in neutropenic mice affected the numbers and activation status of key myeloid cells, including macrophages and DCs, after conidia challenge.

Anti-Axl mAb increases IFN- γ production by T cells and NK cells during IPA

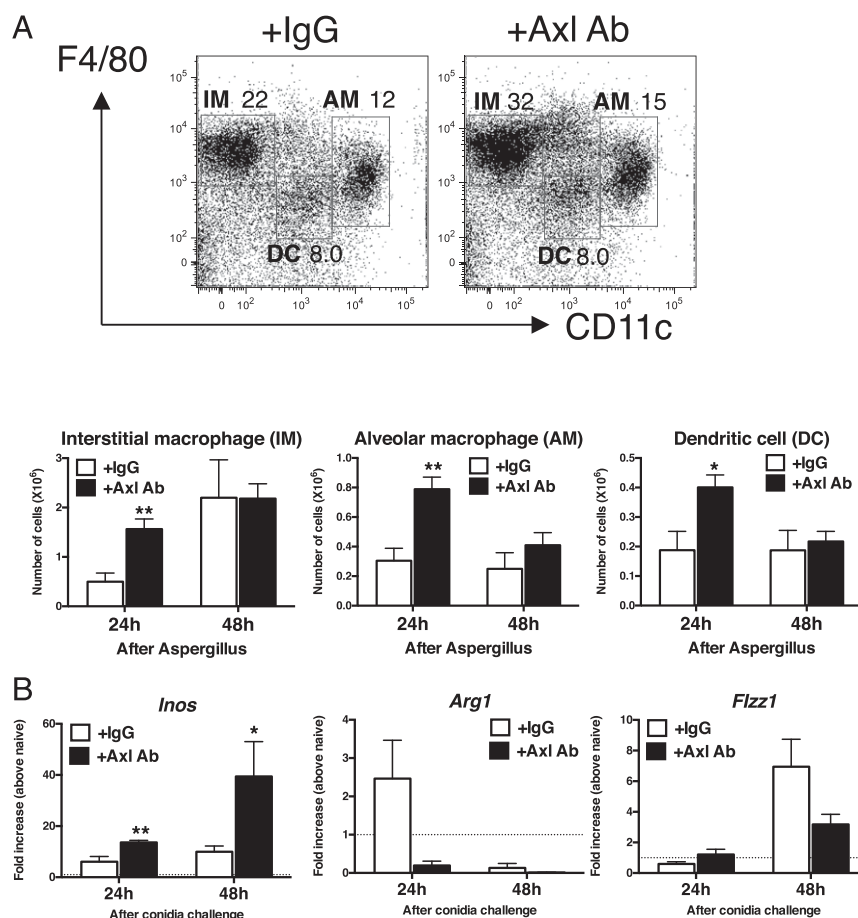
M1 macrophages are generally induced in the context of the IFN- γ -induced Th1-type environment (18). Consequently, we examined the impact of targeting Axl on IFN- γ expression in experimental IPA. Whole-lung IFN- γ transcript levels in the IgG group were lower than naive lung at both 24 and 48 h after conidia

challenge, whereas compared with this control group, anti-Axl mAb treatment significantly increased whole-transcript IFN- γ expression (Fig. 4A). To investigate the cellular source of IFN- γ in anti-Axl mAb-treated mice, we examined its intracellular staining in various immune cell types. We observed that CD4⁺ T cells (Fig. 4B) and NK cells (Fig. 4C) expressed the highest levels of IFN- γ , and the numbers of these IFN- γ -producing cells were significantly increased in the anti-Axl mAb group versus the control group. Further analysis of whole-lung levels of Th1-type and Th2-type chemokines further confirmed that the targeting of Axl promoted a Th1-type immune response. Specifically, the level of *Cxcl9* was significantly increased in the anti-Axl mAb group versus the IgG control group, whereas Th2-type chemokines such as *Ccl17* and *Ccl22* were suppressed in the anti-Axl mAb treatment group compared with the control group (Fig. 4D). Finally, transcript levels of *Ccl2*, which is a potent chemotactic signal for NK cells, were increased in the anti-Axl mAb treatment group compared with the IgG treatment group at 24 h after conidia challenge (Fig. 4E). Together these findings highlight the potent immunomodulatory effect of Axl on the IFN-mediated antifungal response in the neutropenic host.

Discussion

Unequivocally, neutrophils are the most important innate immune cell in the containment of *A. fumigatus* growth in the lung (2, 19–21). Without these critical antifungal immune effectors, immunocompromised patients are very susceptible to infection, and antifungal agents, such as voriconazole and amphotericin B, provide first-line defense against the development of IPA (22). However, antifungal therapeutics have limitations owing to the risk of severe kidney and liver damage, thus necessitating

FIGURE 3. Anti-Axl mAb treatment increases the number of M1-activated macrophages and DCs during IPA. Representative flow cytometry analysis of IMs, alveolar macrophages (AM), and DCs in IgG1- and Axl mAb-treated lung at 24 h after conidia infection and the quantification of IMs, AMs, and DCs in IgG1- and anti-Axl mAb-treated lung at 24 and 48 h after conidia challenge (A). Quantitative PCR analysis of *Inos*, *Arg1*, and *Fizz1* (B) transcript levels in whole-lung samples at 24 and 48 h after conidia challenge. Results are expressed as the mean $\pm$ SEM of $n = 5$ per group. * $p < 0.05$, ** $p < 0.01$ versus the IgG control group.



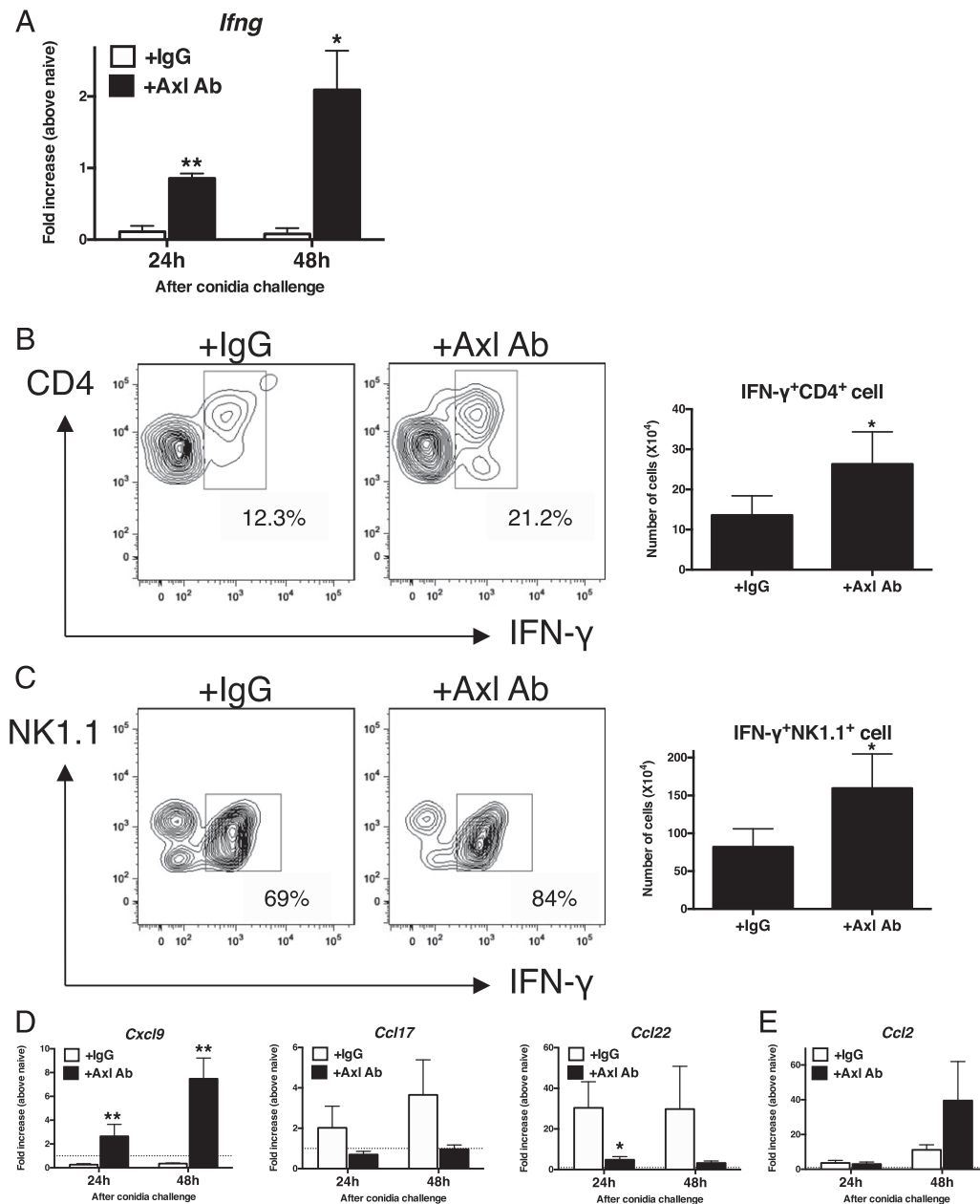


FIGURE 4. Anti-Axl mAb increases the quantity of IFN- γ -producing T cells and NK cells during experimental IPA. Quantitative PCR analysis of *Ifng* transcript levels in whole-lung samples at 24 and 48 h after conidia challenge (**A**). * $p < 0.05$, ** $p < 0.01$ versus the IgG control group. Results are expressed as the mean $\pm$ SEM of $n = 5$ per group. Representative flow cytometry data indicating IFN- γ -expressing CD4⁺ cells (**B**) and NK cells (**C**) in IgG1- and Axl mAb-treated lung at 24 h after conidia challenge. The number of IFN- γ -producing CD4⁺ cells and IFN- γ -producing NK cells (**B** and **C**, right panels) in IgG1- and Axl mAb-treated lung. * $p < 0.05$, compared with IgG1-treated mice. Results are expressed as the mean $\pm$ SEM for $n = 5$ per group. Quantitative PCR analysis of *Cxcl9*, *Ccl17*, *Ccl22* (**D**), and *Ccl2* (**E**) transcript levels in whole-lung samples at 24 and 48 h after conidia challenge (**A**). Results are expressed as the mean $\pm$ SEM of $n = 5$ per group. * $p < 0.05$, ** $p < 0.01$ versus the IgG control group.

the identification of novel antifungal mechanisms that can be used in the immunocompromised host (23). With our background findings indicating that targeting Axl via a mAb approach increased IFN- γ production and viral clearance during respiratory syncytial virus infection in mice (8), we undertook the current study to ascertain whether this mechanism might be activated during antifungal responses in neutropenic mice. Results from the current study demonstrate that anti-Axl mAb treatment significantly enhanced both the numbers and activation of IFN- γ -producing T cells and NK cells, and M1 macrophages during IPA. As a result, anti-Axl mAb-treated neutropenic mice were protected from the lethal effects of invasive fungal growth and a hyper-inflammatory response driven by IL-1 β , IL-6, and TNF- α . Thus,

targeting Axl via a mAb-mediated process enhanced the antifungal response mediated by IFN- γ -producing T cells and NK cells during experimental IPA.

During antifungal responses by the innate immune system in the lung, activated alveolar macrophages initially orchestrate antifungal immune cell (i.e., neutrophils and monocytes) migration and release mediators that facilitate the activation of IMs and DCs (24). In the current study, we observed that the numbers of IMs and DCs were increased by anti-Axl mAb treatment of neutropenic mice challenged with *A. fumigatus* conidia. Although the mechanism or mechanisms via which anti-Axl mAb treatment increased the number of these cells remain unclear, increasing the number of these myeloid cells might be indirectly involved in the

promotion of clearance of *Aspergillus*. Indeed, IFN- γ -stimulated, M1-activated macrophages have a distinct role in antifungal immune responses (25). It is also interesting that key antifungal inflammatory mediators, including IL-1 β , IL-6, and TNF- α (26), were decreased in anti-Axl mAb-treated mice, highlighting the fine balance that must be achieved between effective antifungal immunity and collateral lung tissue damage. Thus, the targeting of Axl altered both the quantity and activation status of key antifungal myeloid cell populations during experimental IPA.

Axl mAb treatment increased the numbers of IFN- γ -producing T and NK cells in the lung, which coincided with the effective clearance of *Aspergillus* from neutropenic mice. It is likely that IFN- γ directly and indirectly affects the growth of *Aspergillus* in the immunocompromised lung. Of interest, IFN- γ appears to have a direct antifungal effect (27). Further, IFN- γ indirectly affects the fungal growth via its activation of inducible NO synthase-expressing M1 macrophages (28) and monocyte-derived DCs (24), both of which have potent antifungal properties. Within the lung, NK and T cells appear to be major sources of IFN- γ during antifungal responses directed against *Aspergillus* (29). In the current study, we also noted that targeting Axl appeared to have an inhibitory effect on the *Aspergillus*-induced Th2 immune response, which is much less effective in fungal clearance and can markedly heighten the risk of allergic airway inflammation (6, 30). Therefore, increased IFN- γ production by T cells and NK cells via the targeting of Axl enhances an appropriate and effective antifungal response in experimental IPA.

Although chitinase also has antifungal activity, the transcriptional levels of *Chia* and *Chi3l3* were suppressed in neutropenic mice and anti-Axl mAb did not reverse this suppression. Nevertheless, *Chi3l1* was significantly increased in neutropenic lung, and the anti-Axl mAb treatment significantly reduced levels of this transcript in whole-lung samples. It is of note that YKL-40 lacks chitinase activity owing to mutations within the active site of this molecule (31), and although the exact physiological role of YKL-40 is at present unknown, it has been implicated in the development of lung allergic inflammatory diseases such as asthma (32).

We recently reported on the role of Gas6/TAM signaling in a well-established fungal allergic model (8, 33). Gas6/Axl signaling in this model was found to regulate adaptive Th2-mediated immune responses, including Th2 cytokine production, airway hyperresponsiveness with airway inflammation, goblet cell metaplasia, and increased IgE production (8, 33). Similar to the fungal allergic airway model, Axl mAb treatment in mice with IPA was protective via demonstrable effects on cells of myeloid origin. However, the Axl targeted cell in the allergic airway model appeared to be the DC, whereas the lung macrophage appeared to be a major target of this Ab treatment during IPA. Accordingly, levels of inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , were suppressed by Axl mAb treatment in the IPA model, whereas this mAb treatment did not alter these cytokines in the fungal allergic airway model. Thus, Gas6/Axl activation has prominent immunomodulatory effects in both acute infectious and chronic allergic processes in the lung elicited by *Aspergillus fumigatus*.

In summary, we have demonstrated that the TAM receptor Axl has a key immunoregulatory role during invasive pulmonary aspergillosis. The blockade of this receptor via a biologic approach revealed that Axl regulates key innate immune responses in the lung that facilitate both invasive aspergillosis and immunopathology. Taken together, these data suggest that targeting the Axl receptor might provide an effective clinical therapy for invasive aspergillosis. Given these findings, further study is warranted to determine the manner in which Axl signaling regulates the innate immune response against key lung pathogens.

Disclosures

The authors have no financial conflicts of interest.

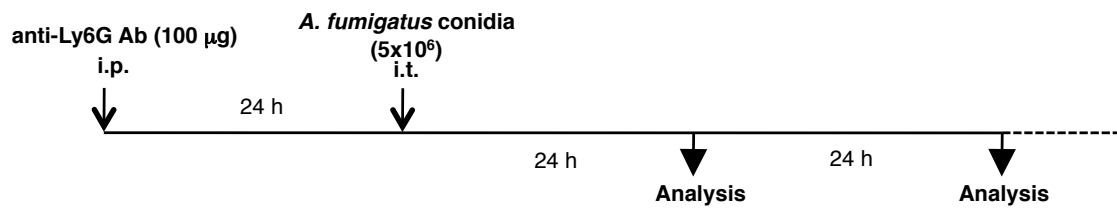
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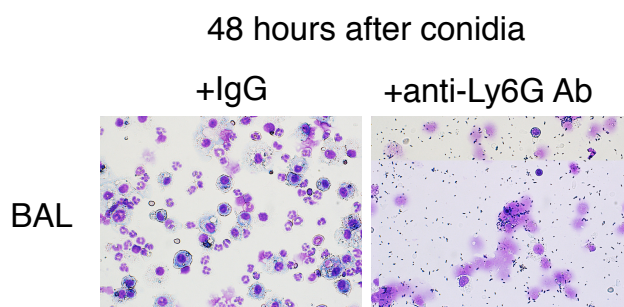
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Supplementary Figure 1

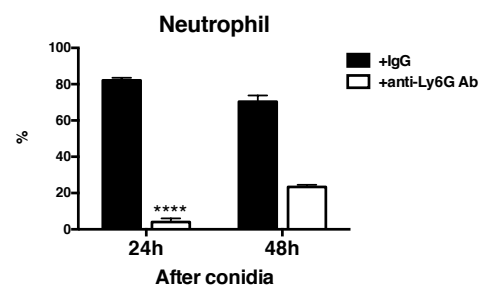
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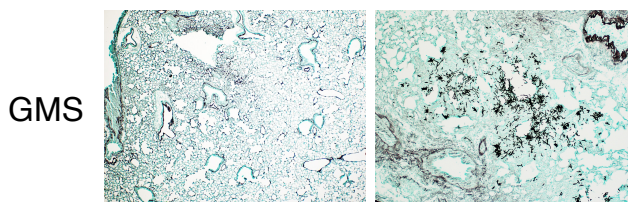
B



C



D



Supplementary FIGURE1. A model of invasive pulmonary Aspergillosis (IPA). To induce neutropenia, mice were injected with anti-Ly-6G Ab (100 µg in PBS) 24 h prior to i.t. *A. fumigatus* conidia challenge (5.0×10^6 *A. fumigatus* conidia by i.t. injection) (A). BAL samples harvested from IgG1- and anti-Ly6G Ab-treated mice were added to microscope slides via a cytopsin (B) and the percentage of neutrophils in BAL (C) are shown. Original magnification, $\times 400$. GMS of IgG1- and anti-Ly6G Ab-treated lung are shown (D). Original magnification, $\times 40$.