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Type II Cytokines Impair Host Defense Against Intracellular Fungal Pathogen by Amplifying Macrophage Generation of IL-33

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Abstract

IL-4 subverts protective immunity to multiple intracellular pathogens including the fungus *Histoplasma capsulatum*. Previously, we reported that *H. capsulatum*-challenged CCR2$^{-/-}$ mice manifest elevated pulmonary fungal burden due to exaggerated IL-4. Paradoxical to our anticipation in IL-33 driving IL-4, we discovered the latter prompted IL-33 in mutant mice. In infected CCR2$^{-/-}$ animals, amplified IL-33 succeeded the heightened IL-4 response and inhibition of IL-4 signaling decreased IL-33. Moreover, macrophages, but not epithelial cells or dendritic cells from these mice expressed higher IL-33 in comparison to controls. Dissection of mechanisms that promulgated IL-33 revealed type-II cytokines and *H. capsulatum* synergistically elicited an IL-33 response in macrophages *via* STAT6/IRF-4 and Dectin-1 pathways respectively. Neutralizing IL-33 in CCR2$^{-/-}$ animals, but not controls, enhanced their resistance to histoplasmosis. Thus, we describe a previously unrecognized role for IL-4 in instigating IL-33 in macrophages. Furthermore, in presence of intracellular fungal pathogens, the type-II cytokine-driven IL-33 response impairs immunity.

Keywords

Lung; Immunity; Fungus; Cytokines; Macrophages

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Introduction

The prototypical T_H2 cytokine, IL-4, is vital for host immunity against helminth infections[1] and for wound healing and tissue repair[2]. Conversely, the cytokine has deleterious effects in the setting of intracellular infections[3,4] and allergic disorders like asthma and atopic dermatitis[5,6]. IL-4 polarizes macrophages to an alternatively activated phenotype, thereby providing a congenial environment for intracellular pathogens to proliferate freely[7]. Another key attribute of IL-4 is to differentiate naive $CD4^+$ T cells to the T_H2 lineage; however, undesired activation of T_H2 cells during certain microbial infections suppresses T_H1 immune responses[8]. Recently, considerable interest has been generated in the epithelial derived cytokines IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) that are important in initiating IL-4[9]. IL-33, in particular, acts on a variety of innate and adaptive immune cell populations including basophils, dendritic cells (DCs), macrophages and $CD4^+$ T cells via its transmembrane receptor ST2 to induce T_H2 immune responses[10].

H. capsulatum is a dimorphic intracellular fungal pathogen and the etiologic agent of histoplasmosis. It is found worldwide but there are regions of notably high incidence of infections, such as the Ohio and Mississippi River Valleys in the USA and regions of South America[11]. Each year, in the US alone, it is estimated that up to 25,000 people develop life threatening infections in endemic regions and 10-fold more individuals acquire mild or asymptomatic disease[12]. To establish extensive infection in humans, *H. capsulatum* transforms into its unicellular yeast phase and disseminates to other organs using macrophages as vehicles. Acute fungal infection in immunocompetent hosts is usually efficiently resolved; however, the more severe symptomatic infections occur in the setting of immunosuppression including patients with AIDS and individuals chronically receiving immunosuppressive drugs[13].

The successful clearance of *H. capsulatum* infection is largely based on the coordinated action of innate and adaptive arm of the immune system. The pathogen initially triggers a host response by activating innate immunity through pattern recognition receptors such as CR3 and Dectin-1 expressed on phagocytes[14,15]. This is followed by a robust T_H1 immune response characterized by proinflammatory cytokines IFN-γ, TNF-α, and GM-CSF and classical activation of macrophages[16]. In contrast, a defective immune response or an overproduction of T_H2 cytokines leads to alternative macrophage activation and ultimately increased susceptibility to *H. capsulatum*[3].

In previous work, we reported that in the absence of chemokine receptor 2 (CCR2), mice exhibit enhanced susceptibility to *H. capsulatum* in comparison to wild-type (WT) controls[3]. The major defect in immunity in these mutant mice is heightened production of IL-4 in the lungs that starts as early as day 3 of infection. In this manuscript, we report that $CCR2^{-/-}$ mice mounted an elevated IL-33 response after initiation of the IL-4 response. Paradoxical to the function of IL-33 in initiating a T_H2 immune response, we discovered that IL-4 induced it in the lungs of infected $CCR2^{-/-}$ mice. Moreover, macrophages were the central source of IL-33 in these mice. Dissection of the molecular mechanism revealed that IL-4 and *H. capsulatum* synergistically evoked IL-33 in macrophages and the signaling pathway was dependent on STAT6/IRF-4 and Dectin-1 respectively. Finally, IL-33 exhibited a

deleterious role in *H. capsulatum* infection by inducing alternatively activated phenotype in macrophages, and neutralizing its effects in the CCR2$^{-/-}$ mice resulted in decreased fungal burden in the lungs. Thus, we report IL-4 and *H. capsulatum* are inducers of IL-33 in macrophages, and the IL-4 driven IL-33 response during fungal infection orchestrates detrimental effects in the host that in turn leads to a collapse in immunity to the disease.

Results

Induction of IL-33 by IL-4 *in vivo*

H. capsulatum-infected CCR2$^{-/-}$ mice display exaggerated levels of IL-4 in the lungs in beginning as early as day 3[3]. Using these mutant mice as a model for dysregulated IL-4 in an intracellular infection, we explored the cytokines associated with regulation of IL-4. TSLP, IL-25 and IL-33 have been implicated in initiating a type II immune response[17]. To investigate their contribution in evoking the dominant IL-4 response in CCR2$^{-/-}$ mice, we measured concentrations in lung homogenates between days 0 and 3 of infection. This particular time frame was chosen because CCR2$^{-/-}$ mice exhibit elevated IL-4 beginning as early as day 3 of *H. capsulatum* infection. No differences were observed in protein concentrations of IL-25 (< 50 pg/ml) and TSLP (< 25 pg/ml) between days 0–3 in WT and CCR2$^{-/-}$ mice. Thus, it was unlikely that these cytokines instigated the exaggerated IL-4 response. We did observe slightly upregulated IL-33 transcripts in lungs of CCR2$^{-/-}$ mice at day 3 of infection (3.2 ±0.34 fold vs 1.9 ±0.18 fold in WT mice; $P<0.05$), but protein concentrations were similar between the two groups (Fig 1A).

To examine if IL-33 triggered IL-4 in CCR2$^{-/-}$ mice, we administered ST2 blocking antibody. Similar pulmonary IL-4 protein concentration following anti-ST2 or control IgG antibody treatment led us to conclude that IL-33 did not evoke the amplified IL-4 response in mutant mice (Fig 1B). Serendipitously, we discovered augmented IL-33 protein in the lungs of CCR2$^{-/-}$ mice at days 7 and 14 post-infection that succeeded, rather than preceded the exaggerated IL-4 response (Fig 1A). The unexpected kinetics of IL-4 and IL-33 prompted us to determine if IL-4 elicited an IL-33 response. We treated CCR2$^{-/-}$ mice with IL-4Rα blocking antibody prior to infection and analyzed pulmonary IL-33 concentration 7 days later. Although using anti-IL4-Rα antibody disrupts IL-4 and IL-13 signaling, IL-13 is not upregulated in *H.* capsulatum-infected CCR2$^{-/-}$ mice[3]. Inhibition of IL-4 engagement with its receptor decreased IL-33 in the lungs (Fig 1C).

IL-33 is generated by lung epithelial cells in response to helminth and fungal infections[18,19]. To investigate the cellular source of this cytokine in the lungs, its expression in pulmonary epithelial cells was analyzed by flow cytometry. WT and CCR2$^{-/-}$ mice displayed similar percentages and mean fluorescence intensity (MFI) of IL-33^{+} epithelial cells (CD45^{-} MHCII^{-} EpCAM^{+}) at 7 days of infection (Fig S1A). Although pulmonary epithelial cells are a source of IL-33, leukocytes synthesize it. IL-33 was upregulated in leukocytes at 7 and 14 days after infection in CCR2$^{-/-}$ mice (Fig 1D). Among leukocytes, macrophages and DCs are associated with IL-33 production in lungs[19,20]. Intracellular staining revealed that WT and CCR2$^{-/-}$ mice displayed similar percentages of IL-33^{+} macrophages but the latter expressed a higher IL-33 MFI (Supp Fig 1B and Fig 1E–G). To corroborate this finding, we sorted F4/80^{+} macrophages from the lungs of infected WT and mutant mice and measured

IL-33 expression. Macrophages isolated from CCR2$^{-/-}$ mice exhibited increased IL-33 mRNA (Fig 1H). No differences in the total percentage and MFI of IL-33^{+} DCs were observed (Fig 1F & G). These findings strongly suggested that IL-4 induces IL-33 in macrophages during *H. capsulatum* infection *in vivo*.

IL-4 and *H. capsulatum* instigate a strong synergistic IL-33 response in macrophages in vitro

To dissect the signaling pathway of how IL-4 drives IL-33, an *in vitro* system was established to study IL-33 expression in macrophages following IL-4 exposure. Stimulation of bone marrow-derived macrophages with IL-4 or infection with *H. capsulatum* yeasts resulted in a modest induction of IL-33 mRNA. IL-4-treated infected macrophages manifested a synergistic IL-33 mRNA response (Fig 2A). Similarly, peritoneal macrophages and alveolar macrophages synthesized IL-33 when exposed to IL-4 or *H. capsulatum* or both (Fig 2B and C). CCR2$^{-/-}$ macrophages generated an IL-33 response comparable to WT cells following incubation with IL-4 or *H. capsulatum* or both (Fig S2A). IL-33 in the lungs of WT and CCR2$^{-/-}$ mice was similar prior to infection (Fig 1B). These findings demonstrated that the exaggerated IL-33 response in CCR2$^{-/-}$ animals following infection was a result of heightened production of IL-4 rather than an intrinsic defect caused by the lack of CCR2. Thus, we utilized WT macrophages for the subsequent experiments. To examine if this response was restricted to *H. capsulatum*, macrophages were infected with a phylogenetically related fungus, *Blastomyces dermatitidis*. An analogous IL-33 expression profile was noted when macrophages were infected with *B. dermatitidis* alone or with IL-4 (Fig S2B).

Similar to other members in the IL-1 family, IL-33 lacks a classical secretory leader sequence that prevents its active release from the cells[10]. We did not detect IL-33 in culture supernatants. To ascertain if protein produced, we lysed cells with water and assessed the IL-33 concentrations. The intracellular protein concentration mirrored the mRNA response when macrophages were incubated with IL-4 or *H. capsulatum* or both (Fig 2D). There was no secretory defect in these infected macrophages since IL-1β and TNF-α were detected in culture supernatants. These cytokines were readily detected 24 h after infection (IL-1β- 75 ±4 pg/ml, and TNF-α- 174±44 pg/ml; n=5).

We investigated the kinetics of IL-33 transcription by IL-4. Macrophages required treatment with IL-4 for at least 48h to produce a maximal IL-33 response (Fig S2C). The magnitude of the response directly correlated with the quantity of IL-4 added to cultures (Fig S2D). Infecting macrophages with an increasing multiplicity of infection (MOI) of *H. capsulatum* yeasts augmented IL-33 mRNA (Fig S2E). Thus, IL-4 or *H. capsulatum* alone elicits modest upregulation of IL-33 in macrophages; however when both are present, macrophages mount a strong synergistic IL-33 response.

IL-4-induced IL-33 response in macrophages is dependent on STAT6 and IRF-4

IL-4 mediates its downstream effects through the transcription factor STAT6[21]. To determine if this factor was necessary for elicitation of IL-33, STAT6$^{-/-}$ macrophages were subjected to IL-4 or IL-4 plus *H. capsulatum*. The IL-33 response was abolished in these

macrophages when stimulated with IL-4 (Fig 3A). The synergistic IL-33 response in STAT6$^{-/-}$ cells was lost when they were exposed to a combination of IL-4 and *H. capsulatum*. Treatment of cells with IL-13, another cytokine that activates STAT6, produced effects analogous to IL-4 (Fig 3B).

IRF-4 is a STAT6-inducible transcription factor that is rapidly transcribed when macrophages are stimulated with IL-4[22]. To determine if IRF-4 was requisite for the enhanced IL-33 response, IRF-4$^{-/-}$ macrophages were exposed to IL-4 or *H. capsulatum* or both. IL-33 expression in these cells was comparable to STAT6$^{-/-}$ macrophages (Fig 3A). Hence, the induction of IL-33 is dependent on an IL-4/STAT6/IRF-4 signaling cascade in macrophages.

Synergistic induction of IL-33 is not a result of increased expression of IL-4Rα or autocrine IL-4 and IL-13 generated by macrophages during infection

The intracellular pathogen *Mycobacterium tuberculosis* induces the expression of IL4-Rα in macrophages[23]. A possible explanation for the synergistic elicitation of IL-33 was that *H. capsulatum* upregulated the expression of IL4-Rα on the macrophage surface, which in turn potentiated reactivity to IL-4. To test this, expression of IL-4Rα was analyzed in macrophages infected with *H. capsulatum*. Exposure to the fungus did not significantly upregulate IL-4Rα mRNA compared to controls (Fig 4A). Another possibility was that infected macrophages secreted IL-4 and/or IL-13 that acted in an autocrine manner to drive IL-33. We assessed IL-4 and IL-13 in culture supernatants of infected macrophages, but did not detect either (IL-4 and IL-13 < 2 pg/ml). Western blot analysis of infected cell lysates indicated that STAT6 was not phosphorylated during the course of infection (Fig 4B). We exposed IL-4$^{-/-}$ macrophages to IL-4 or *H. capsulatum* or both and observed an analogous IL-33 expression profile as WT cells (Fig 4C). To demonstrate that the synergistic evocation of IL-33 was not a result of autocrine IL-4 or IL-13, IL4-Rα$^{-/-}$ macrophages were utilized. These did not mount an IL-33 response when stimulated with IL-4 alone but had a similar induction of IL-33 as the WT macrophages upon infection with *H. capsulatum* (Fig 4D). The synergistic elicitation of IL-33 was not a result of increased expression of IL-4Rα or autocrine IL-4/IL-13 produced by infected macrophages.

H. capsulatum-induced IL-33 response in macrophages is dependent on Dectin-1 signaling pathway

Dectin-1 exerts an auxiliary role in triggering innate responses when macrophages interact with *H. capsulatum* yeasts *in vitro*[15]. The significance of Dectin-1 signaling pathway in the inception of IL-33 response was examined following *H. capsulatum* exposure. We infected Dectin-1$^{-/-}$ macrophages treated with IL-4 or vehicle for 24 h and analyzed the expression of IL-33. The synergistic IL-33 response in Dectin-1$^{-/-}$ macrophages was greatly diminished compared to WT cells (Fig 5A). A potential caveat with this experiment was an attenuation in binding of *H. capsulatum* yeasts to Dectin-1$^{-/-}$ macrophages and hence a subsequent decrease in phagocytosis. We quantified the association index of green fluorescent protein (GFP)-expressing *H. capsulatum* to Dectin-1$^{-/-}$ macrophages. No differences were observed in binding of *H. capsulatum* yeasts to either WT or mutant macrophages at 5 or 10 MOI (Fig 5B). To substantiate the importance of Dectin-1 in IL-33

generation, macrophages were stimulated with the Dectin-1 agonist, curdlan. Treatment of WT cells with curdlan resulted in modest induction of IL-33 (4 hours post treatment) and a vigorous synergistic IL-33 response in the presence of IL-4 (24 hours post treatment) (Fig 5C).

To demonstrate the involvement of Dectin-1 signaling in the *H. capsulatum*-induced IL-33 response, we treated macrophages with the Syk-inhibitor, piceatannol and incubated them with *H. capsulatum* alone or IL-4 and *H. capsulatum*. A marked decrease in evocation of IL-33 was observed when cells were incubated with *H. capsulatum* or IL-4 plus *H. capsulatum* (Fig 5D). Treatment with this agent did not influence IL-4-induced IL-33 transcription. The Syk inhibitor at a concentration greater than 20μM retards phagocytosis of *H. capsulatum* yeast particles[15]. We used the inhibitor at a concentration that did not interfere with uptake of yeasts by macrophages (data not shown). Thus, these findings strongly suggest that Dectin-1 signaling is crucial for the *H. capsulatum* induced IL-33 response in macrophages.

Blocking IL-33 signaling decreases fungal burden *in vivo*

To determine the impact of IL-33 during *H. capsulatum* infection in CCR2$^{-/-}$ mice, we administered αST2 antibody to one group of mice, while the other received isotype control antibody. A significant lowering of fungal burden was noted in CCR2$^{-/-}$ mice that received ST2 blocking antibody at day 7 post infection (Fig 6). Infected WT mice do not mount an exaggerated IL-33 response and treating them with αST2 antibody did not lower their pulmonary fungal burden. We investigated the mechanism by which IL-33 exacerbated infection in the host. IL-33 has been shown to induce genes associated with alternative activation in macrophages[24]. We observed a similar upregulation in *Arg-1*, *Chil-3* and *Retnla* in macrophages stimulated with IL-33 for 24 hrs (Fig 6B). Moreover, infected-CCR2$^{-/-}$ mice treated with αST2 antibody exhibited reduced mRNA expression of alternative activation markers, in comparison to isotype control-treated mutant animals (Fig 6C). The IL-33 primed macrophages were found to be more permissive to intracellular fungal growth, in comparison to resting macrophages (Fig 6D). Thus, our data indicate the detrimental attribute of IL-33 in driving an alternatively activated phenotype in phagocytes that in turn, enhances susceptibility to *H. capsulatum* in CCR2$^{-/-}$ mice.

Discussion

H. capsulatum-infected CCR2$^{-/-}$ mice mount an exaggerated and sustained IL-4 response that is localized to the lungs. The elevated IL-4 is associated with impaired host resistance and results in the death of mice from a progressive infection[3]. In this study, we discovered a new facet of IL-4 biology using CCR2$^{-/-}$ mice infected with *H. capsulatum*. In a search for an inducer of IL-4 in these mutant mice, we found that IL-4 was not a target of IL-33 but rather than the converse. The latter is firmly established as a potent initiator of IL-4 synthesis in various cell populations such as basophils, NKT cells and CD4^{+} T cells[10]. Examination of the molecular mechanism revealed that an exaggerated IL-33 response was kindled only when macrophages were exposed to IL-4 and an intracellular pathogen. The

generation of IL-33 exerted a deleterious role in pulmonary mycosis as evidenced by decreased fungal burden in CCR2$^{-/-}$ mice treated with ST2 blocking antibody.

Given our previous findings in CCR2$^{-/-}$ mice and the knowledge concerning IL-33 initiating T_H2 immunity, we initially sought to determine if IL-33 instigated the exaggerated IL-4 response in CCR2$^{-/-}$ mice. To study the *in vivo* function of IL-33, we blocked interaction of this cytokine with its receptor ST2. Although antibodies may act as agonists or antagonists, this monoclonal antibody has been used to block ST2 engagment in diverse experimental models[19,25–27]. In each of these studies, the antibody does not trigger an agonistic effect on the receptor ST2. Much to our surprise, generation of IL-33 in the lungs of these mice was actually observed during the onset of the adaptive phase of the immune response to *H. capsulatum* rather than the innate immune phase. Even more surprising was the fact that it was macrophages that differentially produced this cytokine and not pulmonary epithelial cells. Equally peculiar, there was at least a 4 day hiatus between the time when IL-4 was upregulated in the lungs of CCR2$^{-/-}$ mice (day 3) and the detection of enhanced IL-33 (day 7). This finding implies that the exaggerated IL-33 response by macrophages must be shaped by a cascade of interactions rather than a constitutive process. This delay was mimicked partially *in vitro* in which we did not observe peak IL-33 transcription until 48 hours following exposure to IL-4.

The unexpected inverse relationship between IL-4 and IL-33 prompted us to investigate the possibility that the former triggered an IL-33 response. Indeed, interruption of IL-4 signaling using anti-IL-4Rα antibody diminished pulmonary IL-33 protein content in the lungs of infected CCR2$^{-/-}$ mice. Although antibody to this receptor blocks both IL-4 and IL-13, the latter is not altered in the lungs of infected CCR2$^{-/-}$ mice[3]. Thus, the impact of receptor blockade is strictly on IL-4. Disrupting the same signaling pathway in WT controls did not affect IL-33 since these mice inherently mount a weaker IL-4 response in infection as compared to the mutant mice. This finding suggests that there exists a threshold for elicitation of IL-33 by IL-4. IL-4 has been reported to evoke IL-33 in murine splenocytes and peritoneal exudate cells *in vitro*, but the precise mechanism was not identified[28]. In concordance, we did detect a modest IL-33 response to IL-4 in uninfected macrophages *in vitro* that was magnified upon infection. Yet, to the best of our knowledge, IL-4-driven IL-33 *in vivo* has not been described.

Although epithelial cells and DCs represent potential sources of IL-33 in lungs, the quantity of cytokine in these cell populations was comparable between WT and CCR2$^{-/-}$ mice. On the other hand, macrophages were the prominent producers of IL-33 in the lungs of mutant animals. An explanation for upregulation of IL-33 in the macrophage population is that alternatively activated macrophages exhibit enhanced expression and function of Dectin-1[29]. Activation of both the Dectin-1 and IL-4 signaling pathway were critical for triggering the synergistic IL-33 response. We detected intracellular IL-33 (but no extracellular release) protein that mirrored its transcription when cells were exposed to IL-4 or *H. capsulatum* or both. Failure to detect IL-33 in the culture supernatants was not a result of defective secretory machinery as evidenced by detecting TNF-α and IL-1β in culture supernatants, but most likely a result of the lack of leader peptide sequence required for secretion of IL-33 from cells. Others have reported release of this cytokine from cells in response to external

stimuli including ATP or lysophosphotidylcholine (LPC)[18,30]. We did not detect release of IL-33 from macrophages following similar treatments (data not shown). A probable consideration for the contradictory findings is that the prior studies used cell lines.

IRF-4 is a transcription factor that is engaged following activation of STAT6 and controls the expression of genes linked with alternative activation in macrophages[22]. Activation of STAT6 and IRF-4 were crucial for the IL-4-prompted IL-33 response. A recent report has indicated that IRF-4 directly binds to the IL-33 locus to activate the gene[31]. A similar mechanism might be in effect in macrophages stimulated with IL-4. However, an indirect role for IRF-4 in regulating IL-33 expression cannot be excluded.

Apart from its function in triggering cytokine responses to *H. capsulatum* yeast cells, Dectin-1, has been linked with recognition and innate immune responses to other fungal pathogens such as *Candida*, *Coccidioides* and *Aspergillus*[32–34]. Macrophages deficient in Dectin-1 had a marked reduction in IL-33 when infected with *H. capsulatum* yeasts. This finding is congruent with a recent report implicating Dectin-1 in regulating IL-33 in an allergic model of chronic exposure to the fungus *Aspergillus fumigatus*[35]. This study did not address the cellular source of IL-33 during the allergic phase, but its results suggest that engagement of Dectin-1 by β-glucan on the *A. fumigatus* surface is critical for evoking IL-33 in the lungs. β-glucan is a vital component of *H. capsulatum* cell wall in several of the North American isolates, and engagement of this constituent by Dectin-1 activates the receptor to transduce signals that lead to IL-33 in macrophages.

H. capsulatum-prompted IL-33 is reliant on Dectin-1/Syk signaling pathway. In human neutrophils and peripheral blood mononuclear cells, engagement of IL-4 with its receptor IL4-Rα activates Syk kinase that ultimately augments cell adhesion and delays apoptosis[36]. Thus, there is a possibility that IL-4 initiated IL-33 transcription partially depends on Syk kinase. This consideration is unlikely since IL-4 stimulation of macrophages that had been previously treated with the Syk inhibitor yielded an intact IL-33 response. The synergistic elicitation of IL-33 was significantly ablated when either the IL-4 or Dectin-1 signaling pathway was disrupted. These findings strongly support that both the signaling pathways act autonomously to evoke IL-33 in a robust manner.

We demonstrated the deleterious aspect of IL-33 during intracellular fungal infection and provide proof that it contributes to host susceptibility. Others have reported adverse effects of this cytokine but they have largely been a consequence of inducing T_H2 cytokines. In experimental cryptococcal infection or in allergic sensitization with *Aspergillus* or *Alternaria*, IL-33 is detrimental to the host by skewing the immune response to a T_H2 phenotype[35,37,38]. However, IL-33 did not contribute to the instigation of IL-4 response in *H. capsulatum* infection. Rather, the latter evoked IL-33 in the lungs to aggravate *H. capsulatum* infection in $CCR2^{-/-}$ mice.

One mechanism by which IL-33 predisposed these mutant mice to histoplasmosis was by triggering an alternatively activated phenotype in macrophages. Alternative activation was demonstrated previously in macrophages from infected $CCR2^{-/-}$ mice at 7 days p.i. coincident with elevated IL-33[3]. Exposure of macrophages to IL-4 induces arginase-1, an

enzyme that diminishes nitric oxide required for fungicidal activity[39]. Moreover, IL-4 blocks the activating properties of GM-CSF and enhances intracellular zinc that supports the fungal growth[40,41]. In addition to serving as an extracellular cytokine, IL-33 might function as a nuclear factor to downregulate proinflammatory genes and in turn, augment susceptibility to intracellular infections[10].

Given previous data regarding the influence of IL-4 in causing the demise of CCR2$^{-/-}$ mice, a reexamination of that work indicates that the likely cause of the collapse of immunity is attributable to IL-33. The reason why neutralization of IL-4 restored immunity was a consequence of reducing IL-33, rather than exerting an IL-33-independent effect. This finding represents a novel paradigm in T_H2 immunity since IL-33 is principally an initiator of IL-4 and other T_H2 cytokines. The work establishes the existence of a novel positive feedback loop between two key type II immunity inducing cytokines. The knowledge from this study can be extrapolated to other intracellular infections in which IL-4 dampens protective immunity. Indeed, an IL-4/IL-33 axis could exist that exacerbates infection, and IL-33 may be target for interdiction in progressive intracellular infections. Our findings are important for non-infectious T_H2 diseases including allergic asthma and eczema where a similar IL4/IL33 coupling might be in effect. Our work highlights the detrimental effect of IL-33 in host defenses to an intracellular pathogen. Comprehensive understanding of how exaggerated amounts of IL-33 condition the host to be more susceptible to *H. capsulatum* or other intracellular pathogens is a necessary pursuit.

Methods

Mice

Male C57BL/6 and breeding pairs of CCR2$^{-/-}$ (C57BL/6 background) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Bone marrow cells from IL-4$^{-/-}$, IL4-Rα$^{-/-}$ and STAT6$^{-/-}$ mice (C57BL/6 background) were generously provided by Senad Divanovic (Cincinnati Childrens' Hospital Medical Center). Bone marrow cells from Dectin-1$^{-/-}$ and IRF-4$^{-/-}$ mice were a gift from Stuart Levitz at University of Massachusetts, Worcester, MA and Lu Runqing at University of Nebraska, Omaha, NE respectively. Animals were housed in isolator cages and maintained by the Department of Laboratory Animal Medicine, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All animal experiments were performed in accordance with the Animal Welfare Act guidelines of the National Institutes of Health, and all protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Preparation of H. capsulatum and infection of mice

H. capsulatum yeast strain G217B and GFP$^+$ yeasts were grown for 72h at 37°C as described[42]. To infect mice, 6–8 week old animals were inoculated intranasally with 2×10^6 yeasts in ~ 30μl of HBSS (HyClone, Logan, UT).

Organ culture for H. capsulatum

Organs were homogenized in sterile HBSS and serially diluted and plated onto mycosel-agar plates containing 5% sheep blood and 5% glucose. Plates were incubated at 30°C. The limit of detection was 10^2 CFU.

In vivo blocking of IL4-Rα and T1/ST2

For blocking IL-4Rα, mice were injected intraperitoneally with 500 μg of rat anti-mouse IL4-Rα (AMGEN, Thousand Oaks, CA) or control antibody (Bio X Cell, Lebanon, NH) on day 0. For disrupting IL-33 signaling, mice were injected with 300μg of rat anti-mouse T1/ST2 antibody (AMGEN) on day 0 and day 3 of infection.

Isolation of lung leukocytes

Lungs were homogenized with the gentleMACS™ dissociator (Miltenyi Biotec, Auburn, CA) in 5ml of HBSS with 2mg/ml of collagenase D (Roche, Mannheim, Germany) and 40U/ml of DNase I (Roche) for 30min at 37° C. The homogenate was percolated through a 60μm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA) and washed 3 times with HBSS. Leukocytes were isolated by separation on Lympholyte M (Cedarlane Laboratories, Burlington, ON).

Flow cytometry and cell sorting

The phenotype of cells from mouse lungs was determined by incubating lung leukocytes with the indicated antibodies and CD16/32 to limit nonspecific binding. Leukocytes were stained at 4°C for 15 min in PBS containing 1%BSA and 0.01% sodium azide. Cells were stained with combinations of the following antibodies: FITC-conjugated I-A^b; PE-conjugated CD11c; PerCP-conjugated CD45; and APC-conjugated F4/80 and EpCAM from BD Biosciences. For intracellular IL-33 staining, cells were incubated with Cytofix/ Cytoperm (BD Biosciences, San Diego, CA), washed in Permeabilization Buffer (BD Biosciences), and stained for 30 min with PE-conjugated IL-33 (R&D systems, Minneapolis, MN). Cells were washed and resuspended in 1% paraformaldehyde. Isotype controls were used. Data was acquired using BD Accuri™ C6 cytometer and analyzed using FCS Express 4.0 Software. For cell sorting experiments, F4/80^+ leukocytes from the lungs of WT and CCR$2^{-/-}$ mice were isolated at day-7 p.i. using 5-laser FACS Aria II (BD Biosciences).

Generation of bone marrow derived, peritoneal, and alveolar macrophages

Bone marrow was isolated from the tibia and femurs of 6–10 week old mice by flushing with HBSS. Cells were dispensed into tissue culture flasks at a density of 2×10^5 cells/ml of RPMI-1640 supplemented with 10% fetal bovine serum, 0.1% gentamicin sulfate, 5×10^{-5}M 2-mercaptoethanol, and 10ng/ml of mouse granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ). Flasks were incubated at 37°C in 5% CO_2. Macrophages were harvested at day 7. Non-adherent cells were removed and trypsin-EDTA was added for 10min at 37° C. Cells were collected, washed with HBSS and dispensed into culture dishes. Resident peritoneal macrophages from mice were isolated and adhered overnight. The following day, non-adherent cells were removed. Alveolar macrophages

were isolated by lavaging the lungs of uninfected WT mice with PBS. Non adherent cells were washed off and adherent macrophages were used.

Binding of H. capsulatum yeast to macrophages

Bone marrow derived macrophages were seeded in 12-well plates and cultured overnight. The following day, plates were cooled on ice for 20min before the addition of non-opsonized GFP^+ *H. capsulatum* to the wells at final yeast: macrophage ratio of 5:1 or 10:1. The plates were placed on a shaker at a rotation speed of 150rpm for 2min and left on ice for another 60min. This process allowed GFP-labeled yeasts to settle on and contact with macrophages before phagocytosis took place. The plates were then placed in a CO_2 incubator to allow phagocytosis to have a synchronous start. After 60min of incubation at 37°C, cells were washed twice in warm HBSS. Macrophages were then detached by treatment with cell dissociation buffer (Invitrogen, Carlsbad, CA) and fixed in 1% paraformaldehyde. Finally, to determine the Association Index (AI), percentages of GFP^+ cells were quantified by flow cytometry.

In vitro culture conditions

For most experiments, bone marrow derived, peritoneal or alveolar macrophages (except the control or *H. capsulatum* only group) were primed with 10ng/ml of IL-4 (Peprotech) for 24h. Subsequently, fresh media was replenished and macrophages were subjected to IL-4 or *H. capsulatum* or both for 24h. For inhibition studies, Piceatannol (TOCRIS, Bristol, UK) was added to macrophages 90min before infection.

RNA Isolation, cDNA synthesis, and quantitative real time reverse transcription PCR (qRT-PCR)

Total RNA from whole lungs of mice was isolated using TRIzol (Invitrogen) and from *in vitro* macrophage cultures using RNAeasy Kit (Qiagen, Valencia, CA). Oligo(dT)-primed cDNA was prepared by using the reverse transcriptase system (Promega, Madison, WI). qRT-PCR analysis was performed using TaqMan master mixture and primers obtained from Applied Biosystems (Foster City, CA). Samples were analyzed with ABI Prism 7500. The hypoxanthine phosphoribosyl transferase (HPRT) housekeeping gene was used as an internal control. The conditions for amplification were 50°C for 2min and 95°C for 10min, followed by 40 cycles of 95°C for 15s and 60°C for 1min.

Growth inhibition assays

100, 000 bone marrow derived macrophages were plated on a 96-well in culture media with or without IL-33 (10ng/ml) for 24h. The following day, cells were infected with 0.1 MOI of yeasts. Fungal growth was assayed 24h after infection. Macrophages were lysed and yeasts plated. Colonies were counted 10 days later.

Measurement of IL-33 by ELISA

IL-33 protein was quantified in lung homogenates and macrophage-cell lysates (lysed using DI water) by using an ELISA kit (R&D Systems).

Statistics

Analysis of variance (ANOVA) with Dunn's test was used to compare multiple groups, while Student's *t* test was used to compare two groups. *P* value of < 0.05 was considered significant. For all graphs, * P=0.01–0.05, ** P=0.005–0.01, and *** P<0.005.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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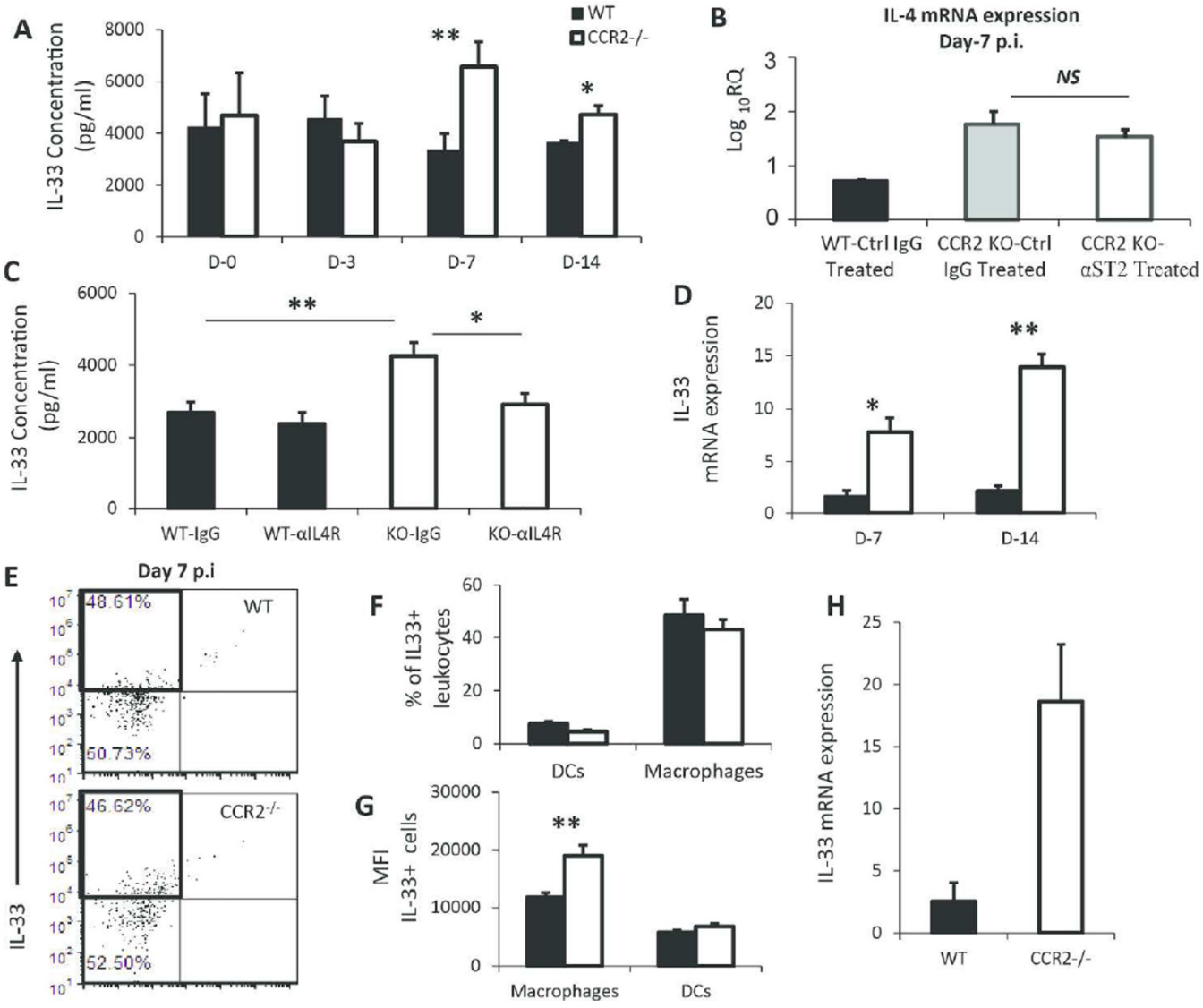

Figure 1. IL-4 prompts an IL-33 response in macrophages *in vivo*

(A) IL-4 mRNA expression in whole lung homogenates of mice treated with anti-ST2 or isotype control antibody. Transcription is expressed as $\log_{10}$ relative quantification (RQ) normalized to uninfected WT lung. (B) IL-33 protein concentration measured by ELISA in lung homogenates of WT and CCR2$^{-/-}$ mice at indicated time points. (C) IL-33 protein concentration measured at day-7 p.i in lung homogenates of mice treated with anti-IL-4Rα or isotype control antibody. (D) IL-33 mRNA expression in isolated lung leukocytes from WT and CCR2$^{-/-}$ mice at indicated time points. Data normalized to lung leukocytes from uninfected WT animals. (E) Intracellular staining of IL-33 in pulmonary macrophages from WT and CCR2$^{-/-}$ mice at 7 days p.i (gating strategy depicted in Fig S1B); x-axis depicts empty channel. Representative plot of one of eight mice from 2 independent experiments. (F & G) Analysis of IL-33 expression and mean fluorescence intensity by flow cytometry in macrophages and DCs at day-7 p.i. (H) IL-33 mRNA expression in sorted F4/80^{+} macrophages from lungs of WT and CCR2$^{-/-}$ mice at 7 days p.i. Data normalized to

uninfected WT macrophages. Pooled values of 8–12 mice from 1 of 2 similar experiments. For all other *in vivo* experiments, data are represented as mean ± SEM, n=6–8 from 2 independent experiments.

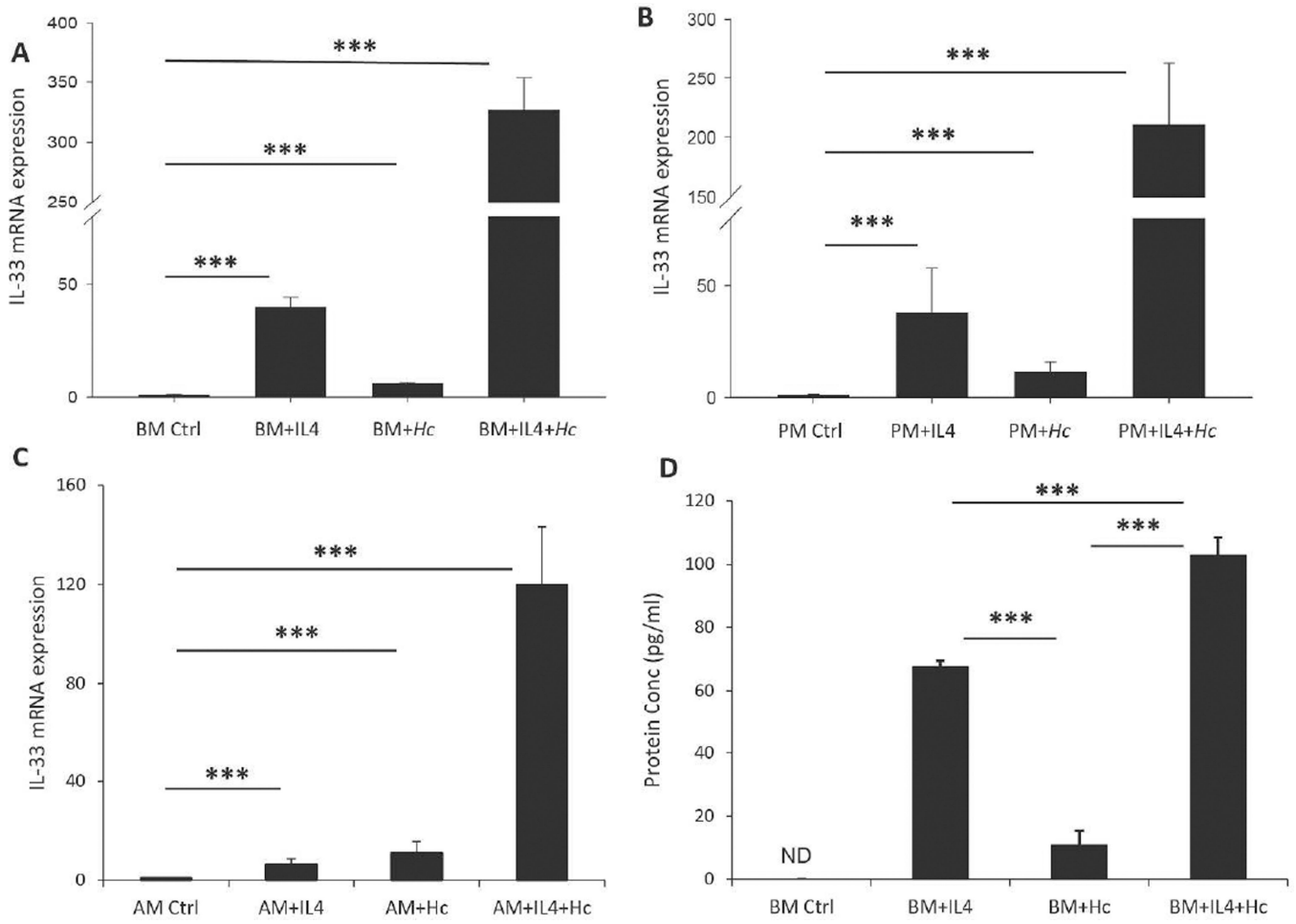

Figure 2. Synergistic induction of IL-33 by IL-4 and *H. capsulatum (Hc) in vitro*

(A) IL-33 mRNA expression measured by qRT-PCR in WT bone marrow derived macrophages (BM) that were exposed to IL-4 (10 ng/ml), or *H. capsulatum* (5 MOI), or both for 24 hrs. All macrophages exposed to IL-4 or IL-4+*H. capsulatum* were initially primed with IL-4 (10 ng/ml) for 24 hrs. Values normalized to untreated macrophages. All treatment groups are significantly different ($P<0.05$) from each other. (B and C) IL-33 mRNA expression in resident macrophages (PM) and alveolar macrophages (AM) isolated from WT mice that were exposed to IL-4 (10 ng/ml), or *H. capsulatum* (5 MOI), or both *in vitro* for 24 hrs. All treatment groups are significantly different ($P<0.05$) from each other. (D) Intracellular IL-33 protein content in whole cell lysates (cells lysed with deionized water) measured by ELISA after 24 hrs of exposure to the stimulus. All treatment groups are significantly different ($P<0.05$) from each other. Values depicted in all the above experiments are mean ± SEM from at least 5 independent experiments.

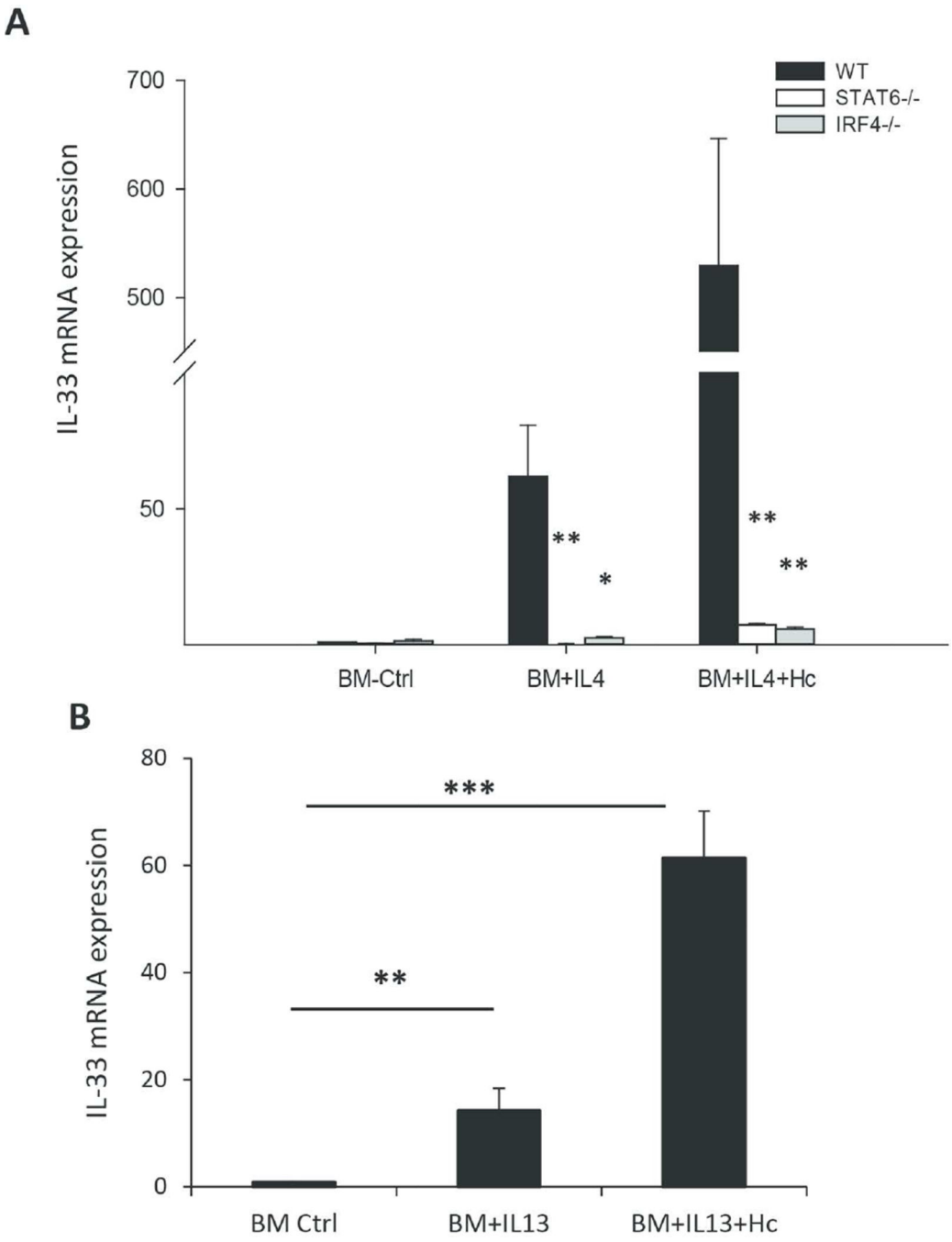

Figure 3. IL-4-induced IL-33 response is dependent on STAT6 and IRF-4

(A) IL-33 mRNA expression in bone marrow derived macrophages from WT, STAT6$^{-/-}$ and IRF-4$^{-/-}$ animals that were subjected to IL-4 treatment or *H. capsulatum* infection or both *in vitro* for 24 hrs. Values normalized to untreated WT macrophages. Data are mean ± SEM from at least 3 independent experiments. (B) IL-33 transcription measured in WT macrophages exposed to IL-13 (10 ng/ml) or IL-13 plus *H. capsulatum* (5 MOI) for 24 hrs. Data are mean ± SEM from 5 independent experiments.

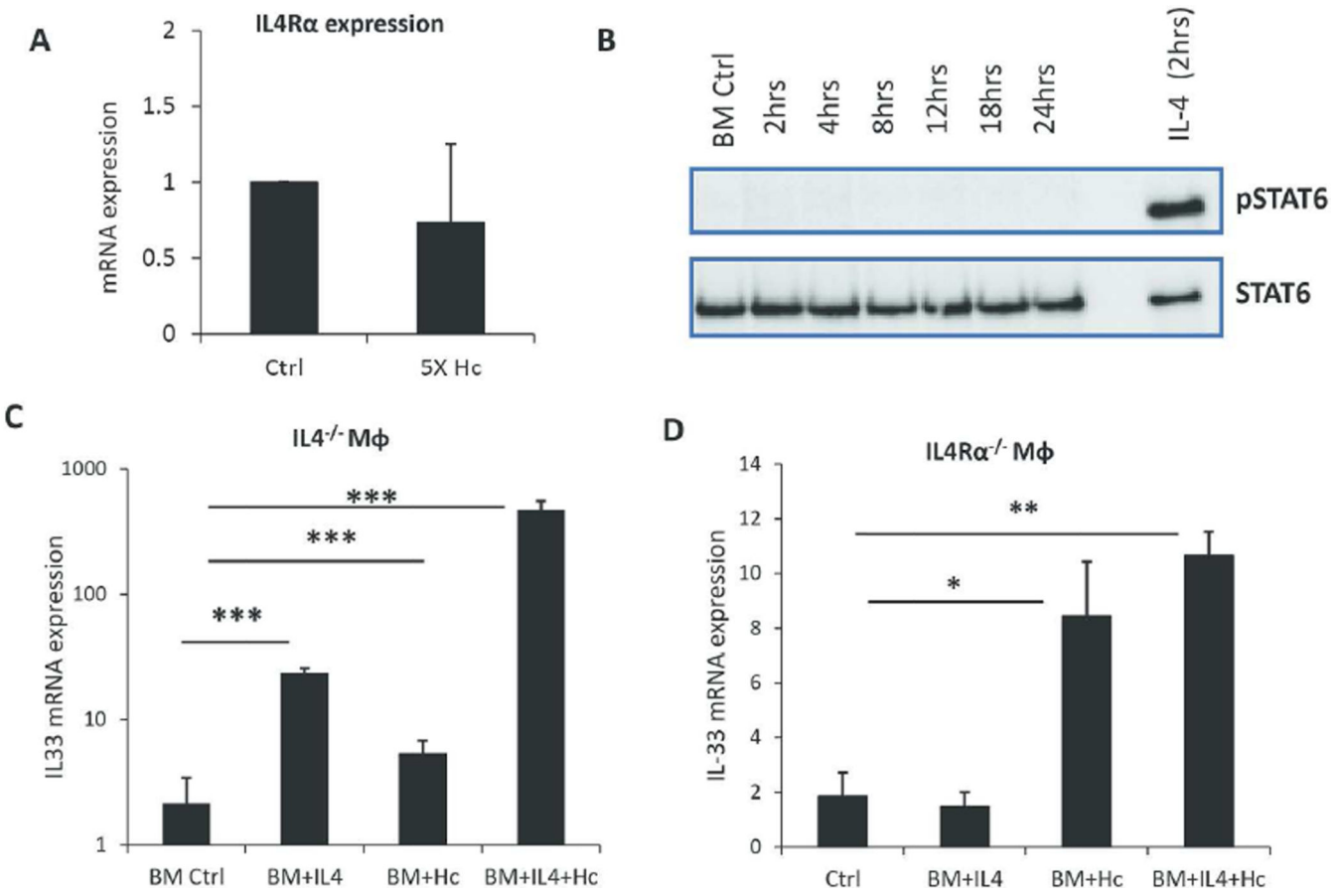

Figure 4. Synergistic induction of IL-33 is not a result of increased expression of IL-4Rα or autocrine IL-4/ IL-13

(A) IL-4Rα mRNA expression in macrophages quantified by qRT-PCR after 24 hrs of *H. capsulatum* infection (5 MOI). Data are mean ± SEM from 3 independent experiments. (B) Immunoblot of phospho-STAT6 and total STAT6 from whole cell lysates of macrophages infected with *H. capsulatum* for the indicated time. Macrophages stimulated with IL-4 were used as positive control. Representative blot of 3 similar experiments. (C & D) IL-33 mRNA expression in IL-$4^{-/-}$ and IL-4R$\alpha^{-/-}$ macrophages exposed to IL-4 or *H. capsulatum* or both for 24 hrs. Values normalized to unstimulated WT macrophages. Data are mean ± SEM from 3 independent experiments. All treatment groups of IL-$4^{-/-}$ macrophages are significantly different ($P<0.05$) from each other.

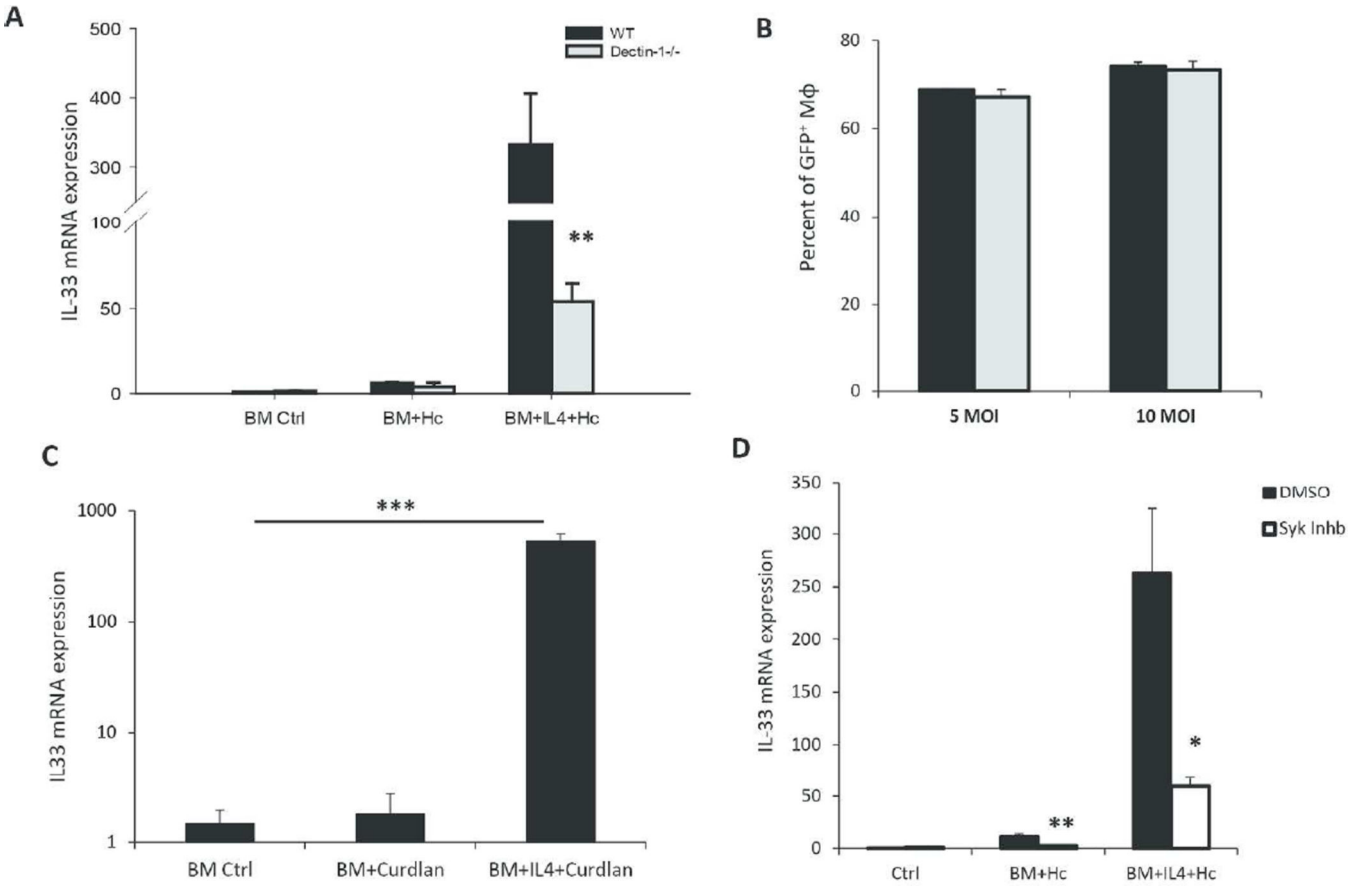

Figure 5. *H. capsulatum*-induced IL-33 response is dependent on Dectin-1 signaling

Effect of *H. capsulatum* (5 MOI) or IL-4 plus *H. capsulatum* on IL-33 transcription in Dectin-1$^{-/-}$ macrophages after 24 hrs of challenge. Data normalized to uninfected WT macrophages. (B) FACS analysis quantifying the uptake of GFP^{+} yeasts (5 or 10 MOI) by WT and Dectin-1$^{-/-}$ macrophages 1 hr after infection. (C) IL-33 mRNA expression in WT macrophages stimulated with curdlan (100 μg/ml) or IL-4 plus curdlan for 24 hrs. (D) IL-33 mRNA expression in macrophages pre-treated with DMSO or Syk inhibitor (100 nM) for 90 minutes before exposure to *H. capsulatum* or IL-4 plus *H. capsulatum*. Values depicted in all the above experiments are mean ± SEM from 3–5 independent experiments.

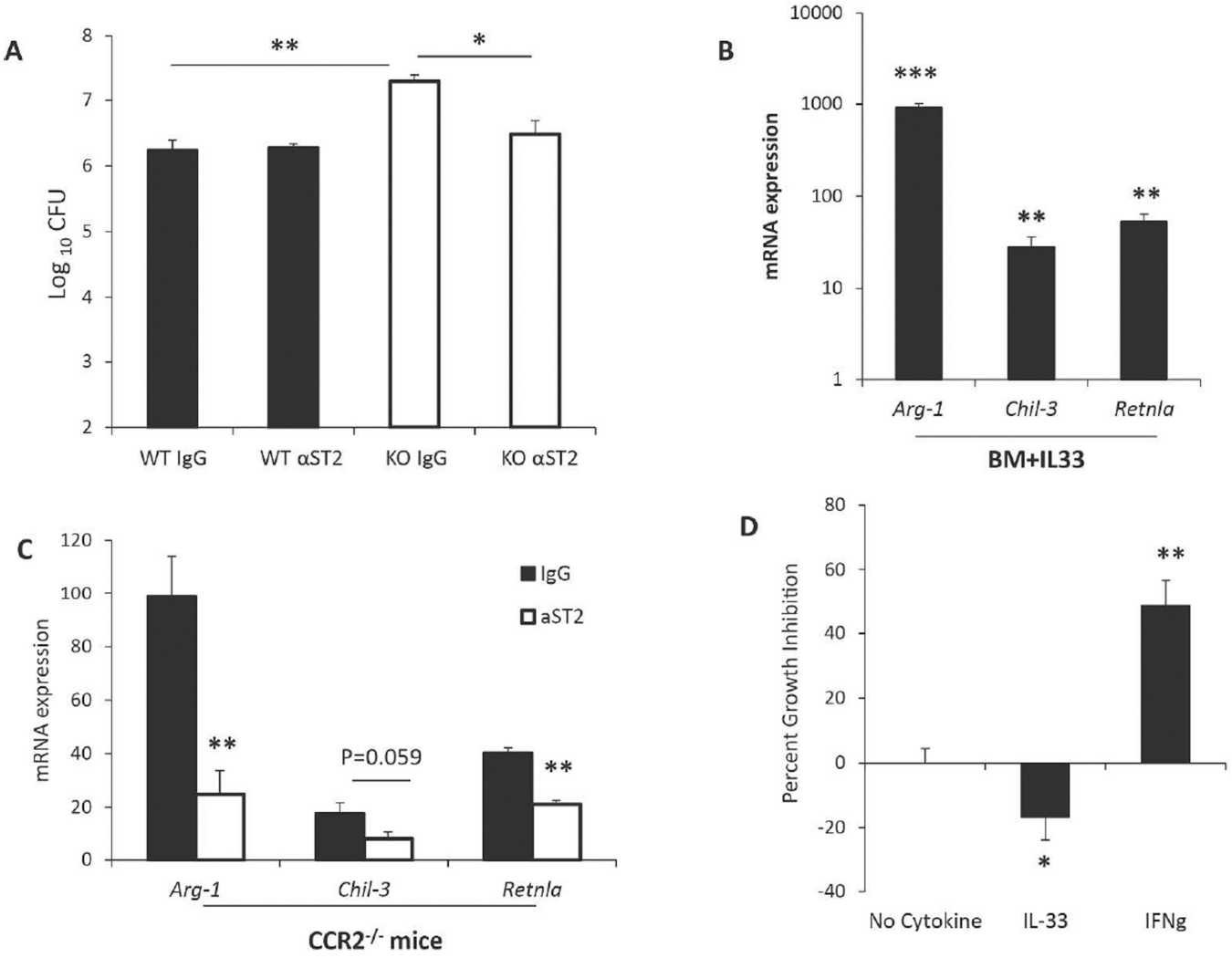

Figure 6. Detrimental effect of IL-33 during *H. capsulatum* infection

(A) Infected WT and CCR2$^{-/-}$ mice were i.p treated with ST2 blocking or isotype control antibody. Animals were euthanized on day 7 of infection and pulmonary fungal burden was quantified. Values are represented as $\log_{10}$ CFU from 7–8 mice/group from two independent experiments. (B) mRNA expression of genes associated with alternatively activated phenotype in bone marrow derived macrophages stimulated with IL-33 (10 ng/ml) for 24 hrs. Data are mean ± SEM from 3 independent experiments; normalized to unstimulated macrophages. (C) qRT-PCR analysis of genes associated with alternative activation. Analysis done on mRNA from whole lung homogenates of control IgG or αST2-treated CCR2$^{-/-}$ mice after 7 days of infection, N=7–8 from two independent experiments. (D) Percent of fungal growth inhibition in unstimulated or IL-33-stimulated macrophages 24 hrs after *H. capsulatum* challenge (0.1 MOI). Data normalized to fungal growth in unstimulated macrophages. IFN-γ-primed macrophages used as positive control for the growth inhibition study. Data compiled from values of 3 independent experiments.