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We investigated mechanisms by which TLR9 signaling promoted the development of the protective response to Cryptococcus neoformans in mice with cryptococcal pneumonia. The afferent (week 1) and efferent (week 3) phase immune parameters were analyzed in the infected wild-type (TLR9+/+) and TLR-deficient (TLR9−/−) mice. TLR9 deletion diminished 1) accumulation and activation of CD11b+ dendritic cells (DCs), 2) the induction of IFN-γ and CCR2 chemokines CCL7, CCL12, but not CCL2, at week 1, and 3) pulmonary accumulation and activation of the major effector cells CD4+ and CD8+ T cells, CD11b+ lung DCs, and exudate macrophages at week 3. The significance of CCL7 induction downstream of TLR9 signaling was investigated by determining whether CCL7 reconstitution would improve immunological parameters in C. neoformans-infected TLR9−/− mice. Early reconstitution with CCL7 1) improved accumulation and activation of CD11b+ DCs at week 1, 2) restored early IFN-γ production in the lungs, and 3) restored the accumulation of major effector cell subsets. CCL7 administration ablated the difference in lung fungal burdens between TLR9+/+ and TLR9−/− mice at week 3; however, significant reduction of fungal burdens between PBS- and CCL7-treated mice has not been observed, suggesting that additional mechanism(s) apart from early CCL7 induction contribute to optimal fungal clearance in TLR9−/− mice. Collectively, we show that TLR9 signaling during the afferent phase contributes to the development of protective immunity by promoting the early induction of CCL7 and IFN-γ and the subsequent early recruitment and activation of DCs and additional effector cells in mice with cryptococcal pneumonia.

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Cryptococcus neoformans is a leading cause of fatal mycosis in HIV-positive individuals around the world (1) and is a common pathogen found in organ transplant recipients and patients with hematological malignancies (2) and other conditions characterized by impaired T cell function. Consistent with the clinical data, T cell-mediated immunity is required for clearance of C. neoformans in mouse models of cryptococcal infections (3–5). The protective anticycrococcal immune response in mice is marked by 1) the recruitment of the effector T cells (CD4+ and CD8+) into the lungs (3–6), 2) Th1-type immune polarization and robust IFN-γ production (7–11), and 3) the accumulation/activation of CD11b+ dendritic cells (DCs) and exudate macrophages (ExMs) in the lungs (12, 13). Thus, the interaction between the Th1-polarized effector T cells and the APCs in the lungs, primarily pulmonary DCs and macrophages, is needed for activation of the effector fungicidal mechanisms and clearance of C. neoformans (12, 14, 15).

Although the innate responses are insufficient to eliminate C. neoformans from the infected host, the innate immune system is the first line of defense against C. neoformans. Furthermore, the molecular signals elicited during the innate (afferent) phase of the immune response drive the development of the T cell-mediated responses (14). During the innate phase, fungal organisms are sensed via innate pattern recognition receptors, which results in a generation of “danger signals,” mainly cytokines and chemokines that trigger the development of the adaptive responses (14–16). TLRs represent the major class of pattern recognition receptors known to recognize components of viral, bacterial, and fungal pathogens and facilitate rapid innate responses to these organisms (18, 20–22). TLR2, TLR4, and TLR9 were demonstrated to recognize different components of C. neoformans (19, 23–26). However, only TLR9 signaling has been demonstrated to play a major role in generation of the protective immune response against cryptococcal lung infection in vivo (26, 27). Furthermore, the role of TLR9 signaling in response to various strains of C. neoformans has been reported (25–28).

Our previous study shows that TLR9 deletion had profound effects on several aspects of the adaptive immune response against C. neoformans (26). Furthermore, complementary in vitro studies show that TLR9 signaling is crucial for activation of DCs during their encounter with this fungal pathogen (25). Together, these findings suggest that TLR9 activation is likely to be important early during the priming and initial development of adaptive anticycrococcal immunity, which subsequently modulates the development of effector responses. However, the mechanism(s) by...
which TLR9 activation affects the development of protective anticytostoccal immunity in vivo are unknown. Experimental pulmonary infections with \textit{C. neoformans} in mice have demonstrated that the chemokine and cytokine response during the first week postinfection (wpi) directs the subsequent development and polarization of adaptive responses in \textit{C. neoformans}-infected lungs (17, 29, 30). The major “early” cytokines driving Th1 development are IFN-\(\gamma\), TNF-\(\alpha\), and IL-12 (31–34), and CCR2 ligands are critical for mononuclear leukocyte recruitment (12, 13, 15, 35, 36). This combination of an early cytokine/chemokine milieu is needed for optimal accumulation and maturation of DCs in the lungs, which in turn is required for the generation of protective Th1 immunity. Our previous studies demonstrated that CCR2 is required for the recruitment of CD11b+ lung DCs and ExMs (12, 13, 15) and Th1 polarization in \textit{C. neoformans}-infected lungs (37). CCR2 ligands, chiefly CCL2/MCP-1 and CCL7/MCP-3, are induced early during the \textit{C. neoformans} lung infection (13, 38). CCL2/MCP-1 was shown to contribute to the recruitment of CD4+ humanely euthanized by CO2 inhalation. All experiments were approved.

\section*{Materials and Methods}

\textbf{Mice}

Female wild-type BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME). TLR9+/+ and TLR9-/- mice on a BALB/c background were bred at the University of Michigan/Ann Arbor Veterans Administration Medical Center using microisolator cages covered with a filter top, with breeder at the University of Michigan/Ann Arbor Veterans Administration Institutional Animal Care and Use Committee. TLR9 +/+ and TLR9-/- mice were obtained from The Jackson Laboratory (Bar Harbor, ME)/2
to 3 weeks after challenge.

\textbf{Lung CFU assay}

For determination of microbial burden in the lungs, small aliquots of dispersed lungs were collected after the digest procedure. Series of 10-fold dilutions of the lung samples were plated on Sabouraud dextrose agar plates in duplicates of 10-\(\mu\)l aliquots and incubated at room temperature. \textit{C. neoformans} colonies were counted 2 d later, and the number of CFUs was calculated on a per-organ basis.

\textbf{Lung leukocyte isolation and culture}

The lungs from each mouse were excised, washed in RPMI 1640, minced with scissors, digested enzymatically at 37\(^\circ\)C for 30 min in 5 ml/mouse of digestion buffer (RPMI 1640, 5% FBS, penicillin and streptomycin (Invitrogen, Grand Island, NY); 1 mg/ml collagenase A (Roche Diagnostics, Indianapolis, IN); and 30 \(\mu\)g/ml DNase (Sigma, St. Louis, MO) and processed as previously described (29, 30). The cell suspension and tissue fragments were further dispersed by repeated aspiration through the bore of a 10-ml syringe and were centrifuged. Erythrocytes in the cell pellets were lysed by addition of 3 ml NH4Cl buffer (0.829% NH4Cl, 0.1% KHC03, and 0.0372% Na2EDTA, pH 7.4) for 3 min followed by a 10-fold excess of RPMI 1640. Cells were resuspended, and a second cycle of syringe dispersion and filtration through a sterile 100-\(\mu\)m nylon screen (Nitek, Kansas City, MO) was performed. The filtrate was centrifuged for 25 min at 1500 \(\times\) g in the presence of 20% Percoll (Sigma) to separate leukocytes from cell debris and epithelial cells. Leukocyte pellets were resuspended in 5 ml complete RPMI media and enumerated on a hemocytometer after dilution in trypsin blue (Sigma). Leukocytes were plated at 5 \(\times\) 10^6 cells/ml in 24-well plates, and supernatants were harvested at 24 h for cytokine analysis.

\textbf{Bronchoalveolar lavage}

Euthanized mice were lavaged after cannulation of the trachea with polyethylene tubing (PE50), which was attached to a 23-gauge needle on a tuberculin syringe. The lungs were lavaged twice with 1 ml PBS containing 5 \(\times\) 10^3 M 2-mercaptoethanol (Sigma) and protein inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN). The recovered fluid (1.8–1.9 ml total) was spun at 1500 rpm, and the supernatant was removed and analyzed for cytokines.

\textbf{Protein evaluation by ELISA}

Cytokine and chemokine protein concentrations were measured by DuoSet R&D Systems, Minneapolis, MN) and PromKine (PromoCells GmbH, Heidelberg, Germany) kits following the manufacturers’ specifications. All plates were read on a Versamaxplate reader (Molecular Devices, Sunnyvale, CA).

\textbf{Quantitative RT-PCR}

Total RNA was prepared using RNeasyPlus Mini Kit (Qiagen, Valencia, CA), and first-strand cDNA was synthesized using SuperScriptIII (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cytokine mRNA was quantified with SYBR Green-based detection using an MX 3000P system (Stratagene, La Jolla, CA) according to the manufacturer’s protocols. Forty cycles of PCR (94\(^\circ\)C for 15 s followed by 60\(^\circ\)C for 30 s and 72\(^\circ\)C for 30 s) were performed on a cDNA template. The mRNA levels were normalized to GAPDH mRNA levels, and relative expression was shown as percent of GAPDH.

\textbf{Abs and flow cytometric analysis}

For the flow cytometry experiment, the Abs were purchased from BioLegend (San Diego, CA), including rat anti-murine CD16/CD32 (Fc block), rat anti-murine CD45 conjugated to allophycocyanin, hamster anti-murine...
CD11c or CD8 conjugated to Pacific blue, rat anti-murine CD11b or CD4 conjugated to allophycocyanin–Cy7, rat anti-murine Ly6G conjugated to PE–Cy7, rat anti-murine CD3 conjugated to PerCP–Cy5.5, rat anti-murine CD19 conjugated to PerCP–Cy5.5, rat anti-murine MHC class II (IA), CD40, CD80, CD86, or CD69 conjugated to PE, and rat anti-murine Ly6C or CD45RB conjugated to FITC.

Staining was performed as previously described (15). Data were collected on a FACS LSRII flow cytometer using FACSDiva software (Becton Dickinson Immunocytometry Systems, Mountain View, CA). A minimum of 20,000 cells was evaluated from a predominantly leukocytic population identified by CD45+–stained cells per sample. The flow data were analyzed by FlowJo software (Tree Star, San Carlos, CA). Total numbers of each cell population were calculated by multiplying the frequency of the population by the total number of leukocytes (the percentage of CD45+ cells multiplied by the original hemocytometer count of total cells).

Calculations and statistics

Statistical significance was calculated using Student t test for individual paired comparisons or r test with Bonferroni adjustment whenever multiple groups were compared. Means with p values <0.05 were considered significantly different. All values are reported as mean ± SEM.

Results

Impaired pulmonary clearance of C. neoformans in TLR9-deficient mice is associated with the diminished accumulation and activation of the major effector leukocyte subsets during the efferent phase of the immune response in the lungs

Our first objective in this study was to determine if the impaired clearance in TLR9-deficient mice was associated with changes in the status of major effector cell subsets during the efferent phase of the adaptive immune response. The TLR9+/+ and TLR9−/− mice were infected with C. neoformans, and enzymatically dispersed lungs were evaluated for fungal burden and the number/activation status of T cells, DCs, and macrophages. Fungal clearance during the effector/efferent phase 3 wpi of the immune response was significantly diminished in TLR9−/− mice (Fig. 1A). This defect in clearance was associated with a diminished recruitment of specific effector cell subset populations evaluated by flow cytometric analysis. Specifically, both CD4+ and CD8+ cell numbers in the lungs were dramatically diminished, resulting in nearly 15 million fewer pulmonary CD3+ lymphocytes (T cells) in TLR9−/− mice compared with that in TLR9+/+ mice (Fig. 1B). The activation phenotype of CD4+ T cells in the lung was then determined. We observed that the percentage of CD45RB on CD4+ CD45RB low-density (CD45RB low−) cells and CD69 on total CD4+ T cells in TLR9−/− mice was moderately diminished compared with that in TLR9+/+ mice (Fig. 1C). No difference was observed in the numbers of CD4+ and CD8+ T cells in the lungs of uninfected TLR9+/+ and TLR9−/− mice (2.15 ± 0.71 × 10^6/lung versus 1.70 ± 0.27 × 10^6/lung and 1.31 ± 0.69 × 10^6/lung versus 0.73 ± 0.14 × 10^6/lung, respectively). Collectively, it is likely that the profound decrease in total T cell numbers in conjunction with their diminished activation contributes to the defect in cryptococcal clearance observed in TLR9-deficient mice.

Execution of the effector functions by T cells depends on their interaction with APCs (DCs and macrophages), which in turn results in activation of fungicidal mechanisms in these cells. We next sought to determine if TLR9 deletion affected the status of CD11b+ (conventional) DCs and major macrophage subsets in the lungs. DCs and macrophages were identified in the infected lungs using our established gating strategy (12). Briefly, autofluorescent macrophages were distinguished from nonautofluorescent DCs. Thereafter, relative expression of CD11b was used to separate CD11b+ alveolar macrophages (AMs) from CD11b− ExMs and to identify CD11b+ DCs. (Fig. 2A). Analysis of these three myeloid cell populations in the lungs at 3 wpi revealed a substantial defect in accumulation of CD11b+ DCs and ExMs in TLR9−/− mice, which contrasted with the robust recruitment of these subsets in TLR9+/+ mice at this time (Fig. 2A). Notably, the number of AMs was not affected by TLR9 deletion at 3 wpi (Fig. 2A). Next, we analyzed the expression of MHC class II and two costimulatory molecules (CD40 and CD80), as these myeloid cell activation markers are crucial for their interactions with anti-fungal T cells. The CD11b+ DCs and ExMs from TLR9-deficient mice expressed less MHC class II, CD40, and CD80 compared with that of these populations in the lungs of TLR9+/+ mice (Fig. 2B). In contrast, the AM population obtained from infected TLR9+/+ and TLR9−/− mice showed no significant differences in the expression of these activation markers (Fig. 2B), suggesting that TLR9 does not play a major role in activation of resident AMs and further supporting the notion that AMs are not an important effector cell during the efferent phase of pulmonary C. neoformans infection in mice (12, 40). No difference was observed in the numbers of AMs, ExMs, and CD11b+ DCs in the lungs of uninfected TLR9+/+ and TLR9−/− mice (56.2 ± 27.1 × 10^6/lung versus 56.3 ± 11.0 × 10^6/lung, 8.5 ± 3.6 × 10^6/lung versus 15.0 ± 3.4 × 10^6/lung, and 36.7 ± 11.5 × 10^6/lung versus 44.3 ± 30.2 × 10^6/lung, respectively). Collectively, the analyses of CFUs and pulmonary effector cell populations demonstrate that the impaired pulmonary clearance of C. neoformans in TLR9-deficient mice is linked to the impaired recruitment and activation of the major subsets of anticryptococcal effector cells.

FIGURE 1. Impaired clearance of C. neoformans in TLR9−/− mice during the effector phase of the immune response is associated with a diminished accumulation and activation of T cells. TLR9+/+ and TLR9−/− mice were inoculated intratracheally with 10^4 C. neoformans 32D. The lungs were enzymatically dispersed at 3 wpi for evaluation of fungal burdens (A) and pulmonary leukocyte analysis by flow cytometric analysis (B, C). Note that a 4-fold increase in fungal burden is accompanied by 50% decrease in total CD4+ and CD8+ T cell numbers (B) and diminished frequency of the activated effector CD4+ T cell markers (C). Data represent mean ± SEM pooled from two to three separate matched experiments; n = 6 and above for each of the analyzed parameters. *p < 0.05 (comparison between TLR9+/+ and TLR9−/− mice).
TLR9 deletion results in impaired early accumulation and activation of pulmonary DCs in C. neoformans-infected lungs

Having determined that TLR9 signaling is required for the recruitment and activation of the major groups of the effector cells in C. neoformans-infected lungs, we sought to identify possible upstream defects that could explain the defects in the effector response found in TLR9−/− mice. We focused on recruitment/activation of pulmonary DCs at 1 wpi, a crucial step in the development of the adaptive immune response (32, 36). Although an activation of pulmonary DCs at 1 wpi, a crucial step in the development of a protective cellular response against C. neoformans (32–34), Robust early expression of pulmonary inflammatory cytokines and chemokines is essential for the accumulation and activation of pulmonary DCs, which could explain the failure to develop a protective adaptive response against C. neoformans in TLR9−/− mice.

Defect in pulmonary DCs is associated with impaired early induction of CCR2 ligands and IFN-γ in C. neoformans-infected lungs of TLR9−/− mice

Robust early expression of pulmonary inflammatory cytokines and chemokines is essential for the accumulation and activation of DCs and their subsequent “immune priming” effect during the development of a protective cellular response to C. neoformans (35, 36). In particular, CCR2 ligands promote DC recruitment (13, 15, 41), and early induction of proinflammatory cytokines IL-12, TNF-α, and IFN-γ correlates with the development of a protective Th1 response in cryptococcal infections (32–34). To determine if TLR9 deletion resulted in diminished expression of CCR2 ligands, RNA was isolated from lung leukocytes of both TLR9+/+ and TLR9−/− mice infected with C. neoformans at 1 wpi and evaluated by quantitative RT-PCR. The relative expression data demonstrated that C. neoformans infection resulted in upregulation of all known murine CCR2 ligands (CCL2/MCP-1, CCL7/MCP-3, and CCL12/MCP-5) in TLR9+/+ mice. CCL7 exhibited the highest...
expression level, followed by CCL2 and CCL12 (Fig. 4A–C). By comparison, TLR9−/− mice showed a profound decrease in CCL7 and CCL12 induction, whereas there was no change in the relative expression of CCL2. The dramatic defect in CCL7 production by lung leukocytes from TLR9−/− mice at 1 wpi has been confirmed at the protein level (Fig. 4D). Whereas TLR9+/− mice demonstrate robust increase in CCL7 production at this time, TLR9−/− mice only show baseline (uninfected) level of CCL7 production. Additionally, the production of CCL2 protein was evaluated. Consistent with the outcomes of mRNA analysis, no difference between TLR9−/− and TLR9+/− mice was observed (data not shown). These findings suggested that the deficiency in CCL7 (and possibly CCL12), rather than CCL2, contributed to decreased early accumulation of DCs in TLR9−/− mice (see Fig. 3A).

We further explored if TLR9 deletion decreased the early induction of proinflammatory cytokines known to promote the development of a protective immune response. We observed a significant decrease in IFN-γ expression (but not TNF-α or IL-12) in TLR9−/− mice at 1 wpi (Fig. 5). Collectively, these data reveal a strong association between TLR9, the expression of the CCR2 ligands CCL7 and CCL12, and the accumulation and activation of pulmonary DCs in mice that develop a protective immune response against cryptococcal lung infection.

CCL7 reconstitution improves the accumulation and activation of pulmonary DCs and restores early IFN-γ production in TLR9-deficient mice during the afferent phase of the immune response to cryptococcal infection

TLR9 deletion resulted in a dramatic decrease in early CCL7 induction that occurred in concert with diminished early accumulation and activation of DCs and reduced IFN-γ induction in C. neofor-mans-infected lungs. Such defects are highly associated with the development of a nonprotective immune response against cryptococcal lung infection (32, 37, 42). To determine if decreased CCL7 was the mechanism leading to the immunological defects in TLR9−/− mice, we studied the effects of the reconstitution of CCL7 on the outcomes of these immune parameters in C. neofor-mans-infected TLR9−/− mice. After infection (day 0), we delivered CCL7 (100 ng/mouse in 20 μl sterile PBS via the intranasal route) or sterile PBS (negative control) to TLR9+/+ and TLR9−/− mice at days 3, 5, and 7. At 1 wpi, CCL7 reconstitution in TLR9−/− mice resulted in a significant improvement (compared with TLR9−/− mice treated with PBS) in the accumulation of pulmonary DCs to numbers comparable with those identified in TLR9+/+ mice treated with PBS (Fig. 6A). Note that the numbers of DCs in CCL7-treated TLR9−/− mice remained lower than those identified in CCL7-treated TLR9+/+ mice suggesting that CCL7 could promote some additional DC recruitment in wild-type animals. In addition to its effects on pulmonary DC number, CCL7 administration to

**FIGURE 4.** TLR9 deletion alters early induction of CCR2 ligands CCL7 and CCL12 during afferent phase of the immune response to C. neofor-mans in the infected lungs. RNA was extracted from isolated lung leukocytes from C. neofor-mans-infected TLR9+/+ and TLR9−/− mice, converted to cDNA, and evaluated by quantitative PCR for the relative expression of pulmonary chemokines. Chemokine protein levels were evaluated by ELISA in leukocyte culture supernatants. Baseline expression in uninfected mice 0 wpi and corresponding responses at 1 wpi are shown. Note a significant deficiency in the induction of CCR2 ligands CCL7/MCP-3 (B, D) and CCL12/MCP-5 (C) and equivalent expression of MCP-1/CCL2 (A) compared with matched responses in TLR9+/+ mice. Data are expressed as mean relative expression ± SEM pooled from two independent experiments; n = 6 and above for each of the analyzed parameters. *p < 0.05 (comparison with the matching TLR9+/+ mouse result).

**FIGURE 5.** TLR9 deletion alters early induction of Th1-type cytokine IFN-γ during afferent phase of the immune response to C. neofor-mans in the infected lungs. RNA was extracted from isolated lung leukocytes from C. neofor-mans-infected TLR9+/+ and TLR9−/− mice, converted to cDNA, and evaluated by quantitative PCR for the relative expression of pulmonary cytokines/chemokines. Baseline expression in uninfected mice 0 wpi and corresponding responses at 1 wpi are shown. Note a significant deficiency in the induction of IFN-γ compared with matched responses in TLR9+/+ mice and equivalent expression of TNF-α and IL-12p40. Data are expressed as mean relative expression ± SEM (% GAPDH) (n = 4 to 6). *p < 0.05 (comparison with the matching TLR9+/+ mouse result).
TLR9 deficient mice corrected the deficiencies in DC activation, as the expression of both MHC class II and CD40 in CCL7-treated TLR9−/− mice was no longer significantly different from that of their CCL7-treated TLR9+/+ counterparts (Fig. 6B). We also investigated whether CCL7 deficiency contributed to the diminished early induction of IFN-γ observed in TLR9−/− mice by assessing the level of IFN-γ in bronchoalveolar lavage fluid from CCL7- or PBS-treated TLR9+/+ and TLR9−/− mice at 1 wpi. ELISA analysis showed that CCL7 reconstitution restored pulmonary production of IFN-γ in CCL7-treated TLR9−/− mice compared with that in CCL7-treated TLR9+/+ mice (Fig. 6C). Thus, CCL7 reconstitution corrected the defect in early accumulation and activation of pulmonary DCs and restored the early IFN-γ production in the lungs of TLR9-deficient mice.

FIGURE 6. CCL7 reconstitution improves the accumulation and activation of CD11b+ DCs and restores IFN-γ induction in TLR9−/− mice at week 1. TLR9+/+ and TLR9−/− mice were inoculated intratracheally with 10^5 C. neoformans and treated with either CCL7 (100 ng/mouse) or sterile PBS in a volume of 20 µl via intranasal inhalation on days 3, 5, and 7 postinfection. Pulmonary leukocytes were isolated at 1 wpi and evaluated by flow cytometric analysis (A, B). The bronchoalveolar lavage fluid was analyzed by ELISA to assess IFN-γ expression (C). Note that CCL7 reconstitution significantly improved the accumulation of CD11b+ DCs in the lungs of TLR9−/− mice (A) and corrected the defects in DC activation (B) and pulmonary production of IFN-γ (C). Data (mean ± SEM) represent two separate, matched experiments; n = 6 and above for each of the analyzed parameters. *p < 0.05 (comparison between TLR9+/+ and TLR9−/− mice).

TLR9−/− mice corrected the deficiencies in DC activation, as the expression of both MHC class II and CD40 in CCL7-treated TLR9−/− mice was no longer significantly different from that of their CCL7-treated TLR9+/+ counterparts (Fig. 6B). We also investigated whether CCL7 deficiency contributed to the diminished early induction of IFN-γ observed in TLR9−/− mice by assessing the level of IFN-γ in bronchoalveolar lavage fluid from CCL7- or PBS-treated TLR9+/+ and TLR9−/− mice at 1 wpi. ELISA analysis showed that CCL7 reconstitution restored pulmonary production of IFN-γ in CCL7-treated TLR9−/− mice compared with that in CCL7-treated TLR9+/+ mice (Fig. 6C). Thus, CCL7 reconstitution corrected the defect in early accumulation and activation of pulmonary DCs and restored the early IFN-γ production in the lungs of TLR9-deficient mice.

Reconstitution of CCL7 in TLR9-deficient mice corrects deficiencies in the recruitment of CD4+ and CD8+ T cells, CD11b+ DCs, and ExMs during the efferent phase of the immune response to C. neoformans

Early CCL7 reconstitution corrected the major defects identified in TLR9−/− mice during the afferent phase of the immune response; we next evaluated the effect of early CCL7 reconstitution on the effector cell subsets during the efferent phase of the response (3 wpi). Using the protocol described earlier, we delivered CCL7 or sterile PBS (negative control) to TLR9+/+ and TLR9−/− mice at days 3, 5, and 7. Numbers of lung leukocyte subsets were then assessed by flow cytometric analysis at 3 wpi. As expected, we observed a significant defect in total lymphocyte numbers including the specific number of both CD4+ and CD8+ T cell subsets in PBS-treated TLR9−/− mice compared with that in PBS-treated TLR9+/+ mice (Fig. 7A). In contrast, results show that CCL7-treated TLR9−/− mice no longer displayed this impairment in T cell accumulation compared with that in CCL7-treated TLR9+/+ mice (Fig. 7B). Next, the effects of CCL7 treatment on myeloid cell accumulation were evaluated. As anticipated, PBS-treated TLR9−/− mice had reduced numbers of CD11b+ DCs and ExMs relative to PBS-treated TLR9+/+ mice (Fig. 8A). However, when TLR9−/− mice were reconstituted with CCL7, we observed...
equivalent accumulation of CD11b+ DCs and ExMs in the infected lungs in comparison with that in CCL7-treated TLR9+/+ mice (Fig. 8B). The AM population displayed neither deficiency in TLR9−/− mice nor the effect of CCL7 administration (A, B). Data (mean ± SEM) represent two separate, matched experiments; n = 5 and above for each of the analyzed parameters. *p < 0.05 (comparison between TLR9+/+ and TLR9−/− mice). NS indicates that there was no significant difference between TLR9+/+ and TLR9−/− groups.

CCL7 reconstitution in mice abolishes the difference in pulmonary clearance of C. neoformans between TLR9+/+ and TLR9−/− mice, but does not significantly improve fungal clearance

The final goal of this study was to determine if CCL7 reconstitution could abolish the effect of TLR9 deletion on C. neoformans clearance during the effector phase of the response (3 wpi). We analyzed the pulmonary fungal burden from infected TLR9+/+ and TLR9−/− mice that had received either CCL7 or sterile PBS, demonstrating that neither TLR9 signaling nor subsequent CCL7 production affected AM accumulation in the lungs at 3 wpi. Thus, CCL7 reconstitution during the first week of infection corrected the observed deficiency in the pulmonary accumulation of T cells, CD11b+ DCs, and ExMs during the effector phase of the immune response.

**Discussion**

The goal of this study was to evaluate the mechanism by which TLR9 promotes the development of protective immunity to C. neoformans. Our data demonstrate that C. neoformans infection results in a robust, early induction of CCR2 ligands CCL7 and CCL12, which is a crucial downstream signal that aids in the development of protective immunity against cryptococcal infection in the lungs. This conclusion is supported by the following novel findings. First, deletion of TLR9 resulted in 1) diminished induction of CCR2 ligands CCL7 and CCL12 and failure to induce INF-γ at the afferent phase of the immune response, 2) diminished early accumulation and activation of CD11b+ DCs, 3) impaired accumulation and activation of major effector leukocyte subsets such as T cells, CD11b+ DCs, and ExMs, and 4) diminished fungal clearance during the effector phase of the immune response in C. neoformans-infected lungs. Second, delivery of CCL7 into the lungs of TLR9−/− mice during the first week of infection 1) corrected early induction of IFN-γ and the accumulation/activation of pulmonary CD11b+ DCs during the afferent phase of the immune response, 2) restored accumulation of CD4+ and CD8+ T cells, CD11b+ DCs, and ExMs in the lungs of TLR9−/− mice during the effector phase of the response, and 3) alleviated the effect of TLR9 deletion on the fungal clearance. Collectively, these data reveal that early CCL7 induction downstream of TLR9 activation is a crucial step in the development of a protective immunity against cryptococcal lung infection, especially the recruitment of the essential effector cell subsets.
Our previous studies documented that TLR9 signaling was required for the development of the protective immune response in C. neoformans-infected lungs (26); however, the mechanism by which TLR9 contributed to the development of the immune protection in vivo has been unknown. The finding that TLR9 affected clearance of C. neoformans from 3 wpi onwards suggested that TLR9 signaling was particularly important for proper execution of the adaptive (effector) phase of the immune response. Consistent with this, we identified a group of major defects in the immunophenotype of TLR9−/− mice including diminished accumulation and activation of all major groups of cells engaged in execution of the effector phase of the immune response. We demonstrated that TLR9 signaling is required for pulmonary recruitment of CD4+ and CD8+ T cells and is required for optimal activation of CD4+ T cells (Fig. 1C). Furthermore, we found that TLR9 signaling contributes to the robust accumulation and activation of CD11b+ DCs and ExMs, which are critical contributors to the protective immune response against cryptococcal lung. The CD11b+ DCs have a major role in initiation/priming of the adaptive immune response; however, recent data also support their role in activation of the effector T cells coming into the lungs and thus maintaining a Th1 “cytokine environment” during the effector phase of anticytrococcal response in the infected lungs (13, 43). Our recently published studies also demonstrate that ExMs, and not AMs, are the dominant macrophage subset expressing fungicidal molecules and are efficient killers of C. neoformans in vitro (12). Collectively, our data show that TLR9 signaling contributes to the recruitment and activation of T cells, CD11b+ DCs, and ExMs, and the defect in accumulation and activation of these cells is associated with diminished adaptive clearance of C. neoformans (Fig. 1A).

Although the effects of TLR9 deletion were most profound during the effector phase of the immune response, TLR9 is abundantly expressed by cells of the innate immune system, and the importance of TLR9 signaling during the innate (afferent) phase of the immune response to C. neoformans has been demonstrated (25, 27). To understand the upstream mechanisms by which TLR9 signaling affected the development of the adaptive immunity, we analyzed the effects of TLR9 deletion on the afferent phase of the immune response. Consistent with our hypothesis, infected TLR9−/− mice displayed a profound defect in DC accumulation and activation (Fig. 3). Our data suggest that impaired DC accumulation may be attributable to diminished production of CCR2 ligands, notably CCL7 and CCL12, in TLR9−/− mice, a finding compatible with the previously described role of CCR2 in mediating the recruitment of these cells to the lungs (13, 15, 36). Furthermore, TLR9 deletion affected expression of early IFN-γ, known to be important for activation of DCs (32–34). Notably, TLR9 deletion was not associated with decreased induction of IL-12, TNF-α, or CCL2 (Fig. 5). Collectively, these data provided a mechanistic explanation for the decreased accumulation and maturation of DCs in the infected lungs, which in turn was a likely cause of the downstream impairments observed during the effector response in the lungs of TLR9−/− mice.

Because CCL7 is the most highly expressed chemokine in the infected lungs of the TLR9−/− mice at 1 wpi and is significantly abrogated in the infected TLR9−/− mice, we tested if CCL7 reconstitution in TLR9−/− mice would restore the immunological defects resultant from TLR9 deletion. CCL7 reconstitution to TLR9-deficient mice improved the accumulation and activation of CD11b+ DCs and restored early induction of IFN-γ in the infected lungs. This suggests that the robust CCL7 induction that occurs downstream from TLR9 activation has an important role in the cascade of molecular events leading to the development of the adaptive immunity to C. neoformans. Furthermore, the immunological defects downstream of TLR9 deletion occurred independently of CCL2 (Fig. 4A), which is consistent with a previous study that postulated the importance of the CCL2-independent CCR2 pathway in anticytrococcal host defenses (36). Our data reveal that early CCL7 has an important role in optimal accumulation and activation of CD11b+ DCs in the lungs and induction of Th1-priming IFN-γ during the afferent phase of the immune response.

Another interesting finding, underscoring the pivotal role of early TLR9-mediated CCL7 induction, is the profound effect that CCL7 reconstitution had on the effector cell subsets during the effector phase of the immune response. Although the intranasal instillation of CCL7 into the lungs does not perfectly mimic the indigenous production of this factor (which is most likely restricted to the infected areas of lungs and produced with continuous kinetics), CCL7 reconstitution in TLR9−/− mice during the first week of infection corrected the defect in accumulation of all major effector cell subsets present in the lungs at 3 wpi. Specifically, accumulation of CD4+ and CD8+ T cells, CD11b+ DCs, and ExMs in the lungs of C. neoformans-infected TLR9−/− mice was completely restored in CCL7-treated mice at 3 wpi (Figs. 7, 8). This result showed that the early TLR9-mediated CCL7 induction is required for proper accumulation of these effector cell subsets during cryptococcal lung infection.

Given that early CCL7 reconstitution corrected the defect of accumulation of the major effector cells at the effector phase, we expected that CCL7 reconstitution would restore the pulmonary clearance of C. neoformans-infected TLR9−/− mice. However, CCL7 reconstitution was not sufficient completely to correct the defect of pulmonary clearance in TLR9−/− mice. These results suggest that the presence of another TLR9-mediated mechanism(s), in addition to its role in mediating effector cell recruitment, contributes to the development of host defense against C. neoformans.

Apart from impaired CCL7 induction in TLR9−/− mice, we also observed a profound decrease in CCL12 induction at 1 wpi. This could be another potential mechanism that could contribute to the impaired clearance of C. neoformans. However, we believe that this hypothesis is rather unlikely. The relative expression of CCL12 is very low by comparison with other CCR2 ligands and is especially dwarfed by high expression of CCL7. Because both of these ligands are thought to act mainly via CCR2, it is hard to expect that CCL12 would be needed apart from CCL7 to restore the clearance of C. neoformans in TLR9−/− mice. Future studies will be needed to determine whether this or other additional mechanism(s) contribute to the full development of the protective immunity against cryptococcal lung infection downstream from TLR9 signaling.

In summary, our findings substantially enhance our understanding of how TLR9 signaling promotes the development of the adaptive immune response during cryptococcal infection in the lungs. The induction of CCL7, mediated by TLR9 signaling, is required for the recruitment and activation of CD11b+ DCs at the early time points postinfection and subsequent accumulation of CD11b+ DCs, ExMs, and CD4+ and CD8+ T cells during the effector phase of the immune response. These findings define a novel role for CCL7 in the cryptococcal infection model that appears to be unique and nonredundant. Future studies using CCL7 knockout mice and and/or neutralizing CCL7 will be needed to evaluate the full impact of CCL7 in cryptococcal infection. Providing that these effects will prove to be equally important in humans, TLR9 and the CCL7–CCR2 axis could become targets for the development of novel biologic therapeutics aimed at improving our ability to treat invasive fungal lung infections.
Disclosures
The authors have no financial conflicts of interest.

References


