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Role of dendritic cell–pathogen interactions in the immune response to pulmonary cryptococcal infection

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Abstract

This review discusses the unique contributions of dendritic cells (DCs) to T-cell priming and the generation of effective host defenses against *Cryptococcus neoformans* (*C.neo*) infection. We highlight DC subsets involved in the early and later stages of anticryptococcal immune responses, interactions between *C.neo* pathogen-associated molecular patterns and pattern recognition receptors expressed by DC, and the influence of DC on adaptive immunity. We emphasize recent studies in mouse models of cryptococcosis that illustrate the importance of DC-derived cytokines and costimulatory molecules and the potential role of DC epigenetic modifications that support maintenance of these signals throughout the immune response to *C.neo*. Lastly, we stipulate where these advances can be developed into new, immune-based therapeutics for treatment of this global pathogen.

Keywords

cryptococcal virulence factors; *Cryptococcus neoformans*; dendritic cells; epigenetics; mouse model; TLR9; TNF- α

Introduction: why dendritic cells?

The central role of dendritic cells (DCs) in the generation of antifungal immunity is increasingly appreciated in fungal immunology, and cryptococcosis is no exception. The interplay between innate immunity that initially detects the pathogen and adaptive immunity that is essential to combat the infection hinges on DCs [1–7]. The function of DCs as antigen presenting cells is to gather antigens; discriminate between danger and nondanger; process

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the antigen for presentation, and to prime T-cell responses that are specific to the genre of the original danger signal (i.e., virus, fungi, tissue damage, etc.) (reviewed in [8]). In addition to initiation of adaptive immunity, there is increasing evidence of a role for DCs in the effector responses in the infected tissues, by both effector T-cell restimulation and the ability of DCs to phagocytose and kill fungal pathogens [9–11].

In this review, we first provide a brief overview of *Cryptococcus neoformans*, the related species *Cryptococcus gattii* and the generation of protective immunity to cryptococcosis. We then systematically identify critical evidence demonstrating that DCs are the architects of anticryptococcal immunity, from their initial testing and surveying of the local micro-environment, to their capacity to initiate naive T cell responses within regional lymph nodes, and lastly, to the ability of newly recruited DC to orchestrate effective adaptive immunity directly within infected tissues. In parallel, we will highlight cryptococcal virulence factors and alterations in the host cytokine milieu that can modulate DC responses and impair host defenses.

Pathogens & epidemiology

Two related cryptococcal species, *Cryptococcus neoformans* (*C.neo*) and *Cryptococcus gattii* (*C. gattii*), are etiological agents of cryptococcosis, an invasive mycosis capable of disseminating to the CNS with oftentimes-lethal effects. The global burden of cryptococcal infections is substantial; one million new cases are diagnosed each year associated with a staggering mortality rate of over 60% despite antifungal therapy [12]. Inhalation of desiccated yeast cells or spores from various environmental sources is thought to be the major gateway for cryptococcal infections [13,14]. The fungi propagate within the host as encapsulated budding yeasts [15,16]. Protective immunity against these highly adaptable opportunistic pathogens requires the successful interplay between intact innate and adaptive immune responses. Thus, patients with impaired T-cell defenses such as those with AIDS [17] and transplant recipients [18] are especially susceptible.

Although most infected patients have a clearly identifiable immunocompromising condition, in the USA, up to 25% of cryptococcosis cases occur in patients without any previously identified immunodeficiency [19]. Some of these infections are caused by *C. gattii* [20–22], whereas others result from a previously unrecognized cause of immunosuppression, as illustrated by recent studies identifying the presence of high titers of anti-GM-CSF autoantibodies in the serum and CNS in a subset of infected patients [23–25] (reviewed in [26]). The rapidly expanding development and use of newer immune modulating agents may also increase susceptibility to cryptococcal infections, as best illustrated by the increased incidence of infection in patients treated with anti-TNF- α antibody therapy [27–31]. The identification of these new risk factors for primary infection, increasing concerns about recurrent or latent disease [32–35], and the limited efficacy of current antifungal therapies, motivates new studies focused on protective immunity to cryptococci and mechanisms of cryptococcal persistence in the host. A major goal of these investigations is to translate these findings into therapeutic strategies that augment host immunity while minimizing damage associated with nonprotective immune responses.

The first key: an overview of host defenses against *C. neoformans*

The immune response to cryptococci may be divided into two phases: the afferent phase where the innate responses are present and the adaptive responses are in the developing stage, and the efferent phase in which the effector immune responses are orchestrated by the developed adaptive arm.

During the afferent phase, the innate immune system recognizes the pathogen, generates signals that propel the development of adaptive immunity, and activates mechanisms of limited fungal control. The major players during the afferent phase are resident macrophages and DCs, which initiate anticryptococcal immune responses by recognizing and ingesting the yeasts [36]. However, the ingested fungus is able to escape phagocytosis and lysosomal killing by inducing phagolysosome damage within macrophages [37]. The DCs are thought to show greater ability to both phagocytose and kill *C.neo* [10–11,38]. However, innate defenses are insufficient to eliminate the pathogen and their major role is to orchestrate the development of adaptive responses.

Upon the uptake of *C.neo* in the lungs, DCs process cryptococcal antigens after its initial elaboration within endosomal/lysosomal pathway and present it in the context of the major histocompatibility complexes (MHC I and MHC II) [10,38–39]. This is concurrent with DC maturation, defined by enhanced surface expression of a number of functional surface molecules. Activated DCs upregulate chemokine receptor CCR7 (responsible for homing of DC to the lung-draining lymph nodes), MHC II (responsible for presenting cryptococcal antigen to the naive T cells) and costimulatory molecules, including CD40, CD80 and/or CD86 (necessary for effective presentation of *C.neo* antigen). Resultant activation of naive antigen-specific T-helper cells in the regional nodes promotes their expansion and initiates T-cell polarization [40]. While the innate defenses are insufficient to control fungal growth, perturbations to the afferent phase responses mediated by macrophages and DCs may result in death of the host due to acute inflammation that damages the lung architecture [41].

The efferent phase follows the afferent phase and is orchestrated by antigen-sensitized T cells and is characterized by recruitment of nonresident leukocytes, which collectively execute adaptive immunity. In this phase, the lung-resident and recruited macrophages that initially phagocytosed and sequestered – but did not eradicate the invading fungus – become activated by the signals from antigen-sensitized T cells emigrating from regional lymph nodes [38,42]. The T-cell-derived activation signals for the phagocytes are critically dependent upon interactions between the newly arrived T cells and nonresident monocyte-derived CD11b⁺ DC in the lung environment [43,44]. Antigen-specific restimulation of the effector T cells in the infected lung provides the final signal required for production of the effector cytokines, which in turn regulate the behavior of the innate effector cells to support active clearance of the infection or, in some situations, other less-favorable outcomes. Robust Th1 and Th17 responses promote gradual but progressive clearance of cryptococci after it reached its peak growth at the end of afferent phase [9,45]. By contrast, nonprotective responses such as Th2 immunity, dysregulated immunity (mixed cytokine response), or responses that develop in the absence of T cells, result in persistently elevated fungal burdens that can be developed into a persistent steady state infection or progressive fungal

expansion with dissemination to the spleen and CNS [3,46–49]. The most severe impairments in CD4⁺ and CD8⁺ T-cell effluent responses result in uncontrolled pulmonary growth and dissemination to the CNS leading to fatal meningoencephalitis in humans [17,35,50–51] and mouse models [52–56]. DCs, as the bridge between innate and adaptive immunity (reviewed in [57]), are the lynchpin linking these afferent and efferent responses. The factors influencing the outcome of DC:T-cell interactions will be further detailed in the following sections.

The second key: DCs in the context of murine models & human disease

Much of the information provided in the preceding overview of host defense was obtained using murine models of cryptococcal infection. In the more detailed information about DC phenotype and function to follow, it will be helpful to understand that different mouse models of cryptococcosis can be employed to mimic distinct clinical patterns observed in human patients. These can be modeled by selecting the appropriate strain of *Cryptococcus*, the magnitude of inoculum or the route of infection. These strategies can also be combined with selection of genetic background of mice and/or manipulations of host molecular or cellular components. For example, the highly pathogenic strain H99 is almost uniformly lethal in all mouse strains (reviewed in [58]); thus, manipulation of its virulence factors sheds light on the contributions of various microbial factors on host immunity (see below). Conversely, infection with less virulent strains of *Cryptococcus* (JEC21, 52D) into resistant (CBA, BALB/c) or susceptible (C57BL/6) strains of mice facilitates investigations into the cellular and molecular mechanisms determining whether infections are cleared, are contained but persist or progress with uncontrolled growth and lethal CNS dissemination.

Observations in human patients have directed murine modeling of cryptococcosis, which has identified T cells, and their associated cytokine responses, as important ‘downstream’ determinants of adaptive immune responses against the organism. Both CD4⁺ and CD8⁺ T cells contribute to adaptive immunity, as studies in mice lacking helper and/or cytotoxic T cells show significant impairment in anticryptococcal host defenses [52,56,59–62]. Mice with both T cell subsets, but deficient in components of the IFN- γ or TLR9 signaling pathways, succumb to infection with a milder strain of *C.neo* earlier and/or at greater numbers than their wild-type counterparts [63–67]. These observations indicate that T cells alone are necessary but not sufficient to mount a protective immune response. Hallmarks of protective T cell-mediated immunity to *C.neo* include: robust production of Th1 and Th17 cytokines, particularly, IFN- γ , TNF- α , IL-12 and IL-17A; the presence of classically activated (M1) macrophages and DCs (DC1); and progressive clearance of infection and limited dissemination of the microbe [3,45,47–48,63,65,68–74]. Conversely, nonprotective immunity to *C.neo* infection in mice is associated with: increased Th2 cytokine production, particularly IL-10, IL-4, IL-5 and IL-13; the presence of alternatively activated macrophages (M2) with resultant YM-1 crystal deposition; eosinophilia and high serum IgE; and progressive growth of the microbe in the lungs followed by multiorgan dissemination [3,32,75–79]. Nonprotective immune responses may result in either a progressive infection and lethal CNS dissemination [17–18,51], or a persistent lung infection where the microbe is contained but not cleared by the immune system and may exacerbate upon loss of immune function [32–35]. Thus, the importance of murine models in identifying these unique T cell-

mediated immunophenotypes led to many of the additional investigations discussed throughout this review that have dissected and delineated the role of DCs in orchestrating these responses.

Lastly, it is important to integrate data related to DC function from both human and murine studies. Similar to mice, human T-cell responses to *C. neo* may be mixed and determined by the net balance between several potentially competing cytokine profiles. For example, a number of studies have shown that low levels of Th1 cytokines (IFN- γ and TNF- α) are associated with a poor outcome in patients with cryptococcosis [80,81], consistent with findings using murine models. In addition, limited evidence suggests that infected patients displaying features of a strong Th2 bias also do poorly [82,83]. Whether DCs are involved in skewing these T cell profiles in human cryptococcosis patients is uncertain and not well studied. However, data obtained from relatively few human studies paired with observations in mouse models strongly suggest that DCs and their activation play a key role in anticytotoxic host defenses. For example, a particularly virulent strain of *C. gattii* R265, which infects immunocompetent individuals, was found to suppress the host DC's TNF- α production [84], an observation that may be paired with the increased risk of cryptococcosis in patients receiving TNF- α -blocking monoclonal antibody therapy for treatment of autoimmune disorders [27–31] and studies in mice modeling this phenomenon [45,85]. In addition, recent reports have identified autoantibodies against granulocyte macrophage colony stimulating factor (GM-CSF), an important DC growth and differentiation factor, in a subset of patients with cryptococcosis who had not previously been identified as immunodeficient [23,24], consistent with the role of GM-CSF in *C. neo*-infected mice [25]. Collectively, we believe murine models recapitulate many features of human host defenses against *C. neo* and provide insights into immunological mechanisms that cannot be thoroughly studied in humans. This review will proceed to illustrate the integral role of DCs in the immune responses against the organism based on available data from human studies and a larger body of groundwork conducted in mouse models.

The master key: subsets & nomenclature of dendritic cells

During infection, the composition of the host DC population changes dramatically as resident DCs migrate to peripheral lymphoid organs and additional DCs, derived from recruited DC precursors, accumulate at the site of infection (see Table 1 and Figure 1). We refer to conventional DCs (cDCs) simply as DC, and the findings discussed do not represent plasmacytoid DC unless explicitly stated otherwise. Two separate subsets of DC residing in uninfected mouse lungs are identified and distinguished based on surface expression of CD103 and CD11b molecules: CD103⁺/CD11b⁻ DC and CD103⁻/CD11b⁺ DC (for all subsets described here, see Table 1 for the equivalent human DC subset markers). Both subsets express CD11c, MHCII, and feature low levels of autofluorescence by flow cytometry [86–88]. These DC are distinct from resident alveolar macrophages, which are large, autofluorescent, CD11c⁺/CD11b⁻, MHCII^{low} cells residing in alveolar spaces [43,54,87,89–91]. Cryptococcal infection triggers the rapid and substantial recruitment of Ly-6C^{high} ‘inflammatory’ monocytes that differentiate into CD11b⁺ monocyte-derived DC and exudate macrophages. The latter two populations are CD11c⁺, CD11b⁺, and MHCII^{pos} but are ExM are distinguished by their larger size and higher autofluorescence along with

lower MHCII expression [43,87,89,92]. The differentiation of these CD11b⁺ DC from Ly-6C^{high} monocytes and their CCR2 dependent accumulation suggests these DC are related to and likely encompass a subset of TNF- α -iNOS producing DCs (TIP-DCs) that have been shown to be important effector microbicidal cells in *Listeria monocytogenes* [93] and *Histoplasma capsulatum* infections [43–44,89,94–97]. Additional subsets of DCs found in the lymph nodes express distinct combinations of CD4, CD8 and Dec205; the CD4⁺8⁺DEC-205⁺, the CD4⁺8⁺DEC-205⁺ and the CD4⁺8⁺DEC-205⁺ subsets are node resident DCs, and these can be distinguished from other DCs that have trafficked to the lymph node by expressing lower amounts of costimulatory molecules, lacking CD11b expression and lacking CCR7 expression [98,99]. Within the lymph node, Langerhans DCs may play a role, and are defined as DEC205^{low}/FSC^{high}/CD8 α ^{low}/CD11b^{low} [40,88].

Plasmacytoid dendritic cells in cryptococcal infection

Although this review focuses primarily on the phenotype and function of conventional DCs, the potential role of plasmacytoid DCs (pDCs) in host defenses against *C.neo* deserves some attention. Plasmacytoid DCs (CD11b⁺/CD11c⁺/B220⁺/Gr-1⁺) are IFN- α / β -producing cells best known for their antiviral capabilities. Our understanding regarding the potential role of pDCs in anticryptococcal defense is limited. Early *in vitro* studies demonstrated that murine bone marrow derived pDCs do not upregulate MHCII and CD86 in response to *C.neo* strain 1841 similar to bone marrow derived macrophages but in sharp contrast to bone marrow-derived conventional DCs [100]. However, pDCs stimulated *in vitro* with a combination of cryptococcal mannoprotein and TLR9 ligand CpG DNA will secrete large amounts of IL-12 and IFN- α [101]. Furthermore, pDCs *in vitro* are capable of phagocytosing *C.neo* and displaying fungistatic properties [102].

Ongoing *in vivo* research points toward pDCs as possible effector cells of protective immunity in the lungs. In mice infected with an IFN- γ -producing strain of *C.neo* [103], pDCs are recruited to the lungs within 7 days and display potent anticryptococcal activity [102]. CXCL9, CXCL10 and CXCL11 appear to be responsible for recruitment of pDCs to the lungs of mice via IFN- γ and STAT1 signaling [102]. However, one study suggested that pDC might have detrimental role as they accumulated in the regional lymph nodes following lung exposure with a nonprotective cryptococcal antigen. This was in contrast with preferential accumulation of myeloid (conventional) DCs and Langerhans DCs when mice were immunized with a protective cryptococcal antigen [40]. Thus, the role of pDCs in cryptococcal lung infection warrants further investigation.

DCs as gatekeepers: pathogen recognition & dendritic cell activation

All fundamental DC functions during microbial infections, including cryptococcosis, are initiated with recognition of pathogens (Figure 2). This occurs via a system of receptors defined as pattern recognition receptors (PRRs). PRRs are intra- and extracellular molecules designed to detect a matching set of microbial pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns released by injured tissues. The PRRs trigger signaling cascades that result in DC activation and subsequent interactions that orchestrate behavior of other cells of the immune system. Specifically, PRR signaling is

crucial for: antigen uptake; discrimination between relevant and nonrelevant antigens; processing of antigens for presentation; activation/maturation; and generation of signals for priming and execution of the adaptive immune response by DCs. Major types of PRRs of particular or emerging importance to cryptococcal infection are the Toll-like receptors (TLRs), Nod-like receptors (NLRs), scavenger receptors, C-type Lectin receptors and the functionally-related complement receptors (discussed in [104]). While these receptors are intimately tied to DC activation, microbes frequently employ counterstrategies to evade detection by these receptors or co-opt them to manipulate host responses.

TLRs in cryptococcal infection

TLRs are PRRs expressed on the cell surface and in endosomes or phagosomes that recognize various types of PAMPs. TLR2 and TLR4 are active during cryptococcal infection and bind cryptococcal polysaccharides with the help of CD14 and CD11/18 [105,106]. LPS, the canonical TLR4 ligand, can be found in the polysaccharide capsule of *C.neo*, likely due to the ubiquity of LPS and the stickiness of the capsule (reviewed in [107]), and can also activate TLR4 during cryptococcal infection. Despite its involvement in the initial immune response, studies in TLR4-deficient mice show that it is ultimately dispensable to anticytotoxic immunity [108,109]. TLR2 appears to be more important in the immune response to cryptococcal infection. Yauch *et al.* showed that the presence of TLR2 conferred a slight but statistically significant survival advantage and reduction in pulmonary fungal burden during intranasal infection with a relatively high dose of serotype A strain 145A *C.neo* [108], while Biondo *et al.* showed a more robust role for TLR2 in survival using serotype A strain H99 [110]. In contrast, Nakamura *et al.* 2006 showed no significant observable differences in the immune responses between WT and TLR2^{-/-} mice infected with serotype A strain YC-13 [109]. Differences in cryptococcal strain (and thus capsule thickness), inoculum dose, and route of infection may all account for the variable effects of TLR2 signaling during cryptococcal infection. Thus, TLR2 signaling may play a differential role in different sets of circumstances.

Numerous studies emphasize the importance of TLR9 in anticytotoxic immunity. Early work showed that administration of TLR9 agonist cytosine-phosphate-guanosine (CpG) DNA oligonucleotides during cryptococcal infection promotes protective responses, including: increasing early IL-12 secretion by myeloid cells; promoting expression of protective Th1 and decreasing nonprotective Th2 immune hallmarks; and ultimately, improving fungal clearance [111]. TLR9 in DC infected with *C.neo* was shown to co-localize with *C.neo*-containing lysosomes and in its absence, TLR9^{-/-} DC show impaired cytokine production [67]. Consequently, TLR9^{-/-} mice show impaired control of cryptococcal infections resulting from the development of altered adaptive immune response [64,66–67,112]. MyD88, as the adaptor molecule for canonical signaling through TLRs 2, 4 and 9 [113–115] and also IL1R, is required for mounting effective immune responses against cryptococcal infection; its deficiency during cryptococcal infection induces equivalent defects as the TLR9 deficiency alone [64,66,108,110,112]. This response is characterized by diminished Th1 elements, elevated Th2 elements and reduced numbers of CD4⁺ and CD8⁺ T cells in lungs, nodes and spleen, and alternative activation of macrophages [66]. The kinetics of this impaired fungal clearance becomes apparent

primarily after 2 weeks of infection, while impaired DC activation develops within first week of infection in TLR9^{-/-} mice [64,66,108,110,112], supporting a role for TLR9-deficient DCs steering the development of the impaired immunity as a downstream effect.

Defective immunity in response to *C.neo* in the absence of TLR9 can be attributed to failed DC activation combined with the impaired myeloid cell recruitment from the bone marrow [64]. Cryptococcal DNA is a critically important TLR9 ligand, as demonstrated by Nakamura *et al.* and Tanaka *et al.* [67,116]. CpG DNA, the canonical TLR9 ligand, is taken up by DCs and directed to the lysosome where it interacts with TLR9 [117]. In side-by-side *in vitro* experiments, fluorescently-labeled CpG or cryptococcal DNA from acapsular mutants displayed similar trafficking patterns in BMDCs via fluorescence microscopy, and elicited similar production of proinflammatory cytokines and surface expression of costimulatory molecules from BMDC [67]. Later work showed that DNA of the URE5 gene (important for uracil production) is particularly effective at stimulating DC1 polarization in BMDC through TLR9 [116]. This and other experiments investigating properties of oligonucleotides that activate BMDCs suggest that active and inactive DNA, rather than specific base sequences, interact differently with TLR9. This may explain why virulence genes activated and used by *C.neo* during infection elicit a response from BMDCs. Nucleotide sequence, activity and differential trafficking of oligonucleotides may each account for the effects of CpG DNA on BMDCs during cryptococcal infection.

Regarding its role in activation of anticytotoxic immunity in *C.neo* infection, additional evidence points toward TLR9 signaling being particularly important within DC, as opposed to other cell subsets. Both hypercapsular and acapsular mutant strains of *C.neo* can overcome TLR9-dependent CpG DNA priming of macrophages specifically [118], but these effects are not seen in DCs [67]. Studies in *M. tuberculosis* may shed light on the mechanism behind this. In *M.tb* infection, IL-12p70 secretion is lighter in BMM than in BMDC, similar to that in *C.neo* infection, and this is due to BMM relying on TLR2 for IL-12p70 production and secretion while BMDC rely on TLR9 for IL-12p70 production and secretion [119]. The ability of DCs to utilize TLR9 as an exclusive PRR-induced danger signal induced by *C.neo* could explain the greater fungicidal activity of unstimulated DCs compared with macrophages in which TLR9 signaling can be effectively suppressed by capsular factors [10,100,120–121]. Thus, TLR9 signaling within DCs appears to be quintessential for the development of protective immunity to *C.neo*.

NLRs in cryptococcal infection

Recent studies have identified NLRs as intracellular PRRs that critically contribute to antifungal immunity (reviewed in [122]). Stimulation of NLRs induces IL-1 β and IL-18 production in many cell types, including DCs. Little is published about NLRs during cryptococcal infection. Recent work from Cordero *et al.* shows that acapsular mutants of *C.neo* activate the NLRP3 inflammasome in peritoneal and bone-marrow derived macrophages, and that macrophage activation is inhibited by the <1 kd fraction purified from conditioned media taken out of capsular *C.neo* cultures [123]. NLRP3 activation resulted in decreased cryptococcal replication and escape from macrophages *in vitro* [123]. Studies in other fungal infection models suggest at least a potential for NLR to contribute to

some aspects of anticryptococcal immune responses. For example, NLRP3 is instrumental in controlling IL-1 β /IL-18 production in *C. albicans* and *A. fumigatus* infections [124,125]. While the precise PAMP activating NLRP3 remains to be determined, both *C. albicans* and *A. fumigatus* must be in a filamentous form to activate NLRP3 [125,126]. This is in contrast with *C. neo*, in which the invasive form is an encapsulated yeast particle. Regardless of this major difference between *C. neo*, *Candida* and *Aspergillus* biology, the role of inflammasome activation in cryptococcal infection is implicated by studies showing that inflammasome-activated cytokines IL-1 β and IL-18 are linked to protective immunity against *C. neo* [127,128]. However, more recent studies suggest that inflammasome activation leading to IL-18 activation is more important than generation of active IL-1 as deletion of the IL-18 receptor, but not the IL-1-receptor, improves survival of H99-infected mice [112]. Further investigations with other *C. neo* strains in mice, and more corresponding data from human studies, are needed to clarify the role of NLR and the downstream inflammasome activation in the generation of protective responses to *C. neo*.

Scavenger & mannose receptors & immune modulation in cryptococcosis

Numerous scavenger receptors aid macrophages and DCs in priming protective immune responses to *C. neo*. Using an *in vitro* shRNA screen of mouse macrophages during cryptococcal stimulation, Means *et al.* demonstrated that the scavenger receptors CD36, SCARF1 and SCARB2 promote IL-1 β generation and are protective during cryptococcal immune responses [129]. Conversely, studies using Scavenger Receptor A (SRA)-deficient mice demonstrated that SRA signaling contributed to nonprotective immunity via decreased DC and CD4⁺ T-cell accumulation, decreased DC1 polarization and an increase in the hallmarks of nonprotective immune responses including eosinophilia and IL-10 production [130]. This suggests that *C. neo* exploits SRA to modulate the host response, promoting responses that favor survival and persistence of the microbe. The roles of other SR family members in the development of protective immunity to cryptococcal infection remain to be elucidated.

The mannose receptor (CD206) represents a double-edged sword for anticryptococcal immunity: it is necessary for the generation of protective immunity to *C. neo* [131], but its overexpression is also a hallmark of alternative activation in macrophages and DCs (reviewed in [132]), which contribute to nonprotective immunity to *C. neo* [78]. CD206 on the surface of macrophages and DCs binds cryptococcal MP on the cell wall of acapsular *C. neo* [10,133]; the addition of zymosan or glucan particles to macrophages in culture can bind competitively to CD206 and prevent uptake of *C. neo* [133]. BMDCs derived from CD206-deficient mice did not stimulate robust T-cell expansion or activation (relative to WT mouse-derived BMDCs) [131]. However, uptake of *C. neo* was unhindered in CD206-deficient BMDCs relative to WT BMDCs, suggesting that another phagocytic receptor may be compensating for the loss of CD206 in *C. neo* uptake [131]. Stimulation of macrophages and BMDCs through CD206 induced TNF- α mRNA and protein production [10,133], but in other models, it inhibits IL-12 production and promotes IL-10 production [134,135]. Thus the effects of CD206 signaling appear complex and it is possible that high CD206 expression, linked with alternative activation of phagocytes, promotes the intracellular survival, and not killing, of *C. neo* [136]. Together, these studies show that CD206 may

contribute to both protective and nonprotective responses, depending on other signals that occur during DC-*C.neo* interactions. More work to clarify the role of CD206 in T-cell activation and CD206 signaling pathways is necessary to more fully understand its complex role in protective immune responses to *C.neo*.

Dectin-1 & -2 in cryptococcal infection

Dectin-1, required for β -glucan recognition and immunity against *C. albicans* and *A. fumigatus*, is not required for anticryptococcal immune responses [137]. Consistent with this finding, a recent study shows that Dectin-2 deficient mice have similar fungal burden as infected WT mice [138]. However, infected Dectin-2-deficient mice display numerous Th2 hallmarks: increased IL-4, IL-5 and IL-13, increased mucin, lower TNF- α and IL-12 production, and decreased costimulatory molecule expression [138]. This phenotype is most often associated with impairments in cryptococcal clearance, yet none were observed in this study. Thus, while dectin-2 signaling has clearly some effects on the phenotype of the immune response, its net effect on fungal clearance appears to be neutral. This is further supported by the equivalent induction of nitric oxide in myeloid cells in the presence and absence of dectin-2 signaling and the mildly protective effect of mucin production, which in turn could counterbalance some negative effects associated with IL-4 induction down stream of dectin-2 signaling [138]. While IL-4 is generally considered to be nonprotective in cryptococcal infections, recent study showed that IL-4 receptor signaling can temporarily promote *C.neo* control during the early, but not late, phase of cryptococcal infection in mice [138,139]. Together these findings highlight the need for further investigations into the mechanisms by which PRRs alter DC phenotype and function to influence the outcomes of cryptococcal infections.

The fungus strikes back: cryptococcal virulence factors influence dendritic cells

Several cryptococcal virulence factors interfere with the development of protective immune responses in the infected host. The majority of these effects are linked to their interference with antigen presenting cells (APCs) of myeloid lineage including DC, macrophages and their monocyte precursors (Figure 3). These cells share many functional and metabolic pathways, and thus the virulence-associated genes not surprisingly affect these mononuclear APCs in a similar fashion. Functional pathways commonly affected by fungal virulence factors include those associated with pathogen uptake and degradation, APC cell activation/maturation, classical versus alternative polarization, cytokine production and crosstalk of APC with the antigen specific T cells. As a consequence of cryptococcal interference with these pathways, *C.neo* not only directly alters innate functions of DCs and other APCs, but also induces profound downstream effects on the adaptive/efferent arm of the immune response. For example, cryptococcal laccase [140–142], VAD1 [143,144], cryptococcal Hsp70 homologue Ssa1 [145], and PIK1, RUB1 and ENA1 [146] were shown to interfere with the innate APC pathways upstream of their effects on the development of adaptive immunity. While future studies are needed to dissect specific interactions of these factors with DC, some cryptococcal components have been already shown to modulate maturation

and polarization of DC and their monocyte precursors. Among these factors are capsular polysaccharides, mannoproteins and cryptococcal urease.

Polysaccharide capsule & GXM

The *C.neo* polysaccharide capsule is a major virulence factor that inhibits phagocytosis, as illustrated by acapsular mutant strains of *C.neo* being avirulent and readily ingested by phagocytes in mice [147–150]. The addition of capsular material co-cultures of myeloid cells with acapsular *C.neo* prevents phagocytosis of the acapsular *C.neo* [151]. The polysaccharide capsule, comprised primarily (80%) of glucuronoxylomannan (GXM) [152–159], exerts effects on dendritic cell activation in both human and murine monocytic cells. *In vitro* exposure of murine CD11c⁺ splenic DC to *C.neo* polysaccharide capsule decreases their secretion of TNF- α and chemokine ligands (CCL3, CXCL10, CCL4), and impairs CCR7 expression [160].

Monocytes encountering acapsular *C.neo in vitro* upregulate MHC II and costimulatory molecules similar to those induced by treatment with LPS [43,161]; in contrast, these effects are not observed when monocytes are exposed to encapsulated organisms [162,163]. In work performed with human tissue, the addition of purified capsule component GXM to human monocytes treated with acapsular *C.neo* modulated their function as evidenced by their: decreased TNF- α and IL-1 β production [164]; increased IL-10 secretion [162]; decreased surface expression of MHCI and MHCII; and impaired phagocytosis of acapsular *C.neo* [163]. However, when human monocytes were incubated with encapsulated *C.neo* treated with anti-GXM, the human monocytes were nonetheless unable to upregulate MHCII or costimulatory molecules [163], suggesting that another, as of yet unidentified component(s) of the capsule, can suppress MHCII expression. The cryptococcal polysaccharide capsule is thus an extremely important and incompletely understood virulence trait with important effects on DC activation.

Mannoprotein

Cryptococcal mannoprotein (MP) promotes CD4⁺ T-cell stimulation [165,166], although it was initially unknown which phagocyte was primarily responsible for presenting it to T cells. Early studies showed that MP induced replication in human PBMCs [167] and enhanced IL-12 secretion from human monocytes [168]. Later work showed that multiple lectin receptors on DCs were responsible for MP uptake via MMR [169], and that uptake induced IL-2 production from CD11c⁺ splenic DCs [170] (reviewed in [171]). However, the effects of MP on DC maturation differ with stimulant duration, model system and the presence or absence of other immunostimulatory molecules. MP1 and MP2 were shown to induce DC maturation over 48 h at concentrations between 5 and 20 μ g/ml via upregulation of costimulatory molecules, MHCI and MHCII, and downregulation of phagocytic receptors comparable to LPS in human PBMCs differentiated to DCs using IL-4 and GM-CSF [169]. In another study, a mixed population of MP1 and MP2 at a concentration of 50 μ g/ml for 24 h maintained DC immaturity in murine BMDCs differentiated with GM-CSF and IL-4 when compared with LPS stimulation [85]. Finally, the addition of TLR receptor ligands CpG DNA, dsRNA and others (discussed above) to MP preparations *in vitro* resulted in greatly

enhanced DC activation [101]. Thus, the context in which DCs encounter MP may be a deciding factor in whether its effects are immunostimulatory or immunosuppressive.

Urease

The cryptococcal virulence factor urease, an extracellular enzyme that hydrolyzes urea, promotes CNS dissemination and facilitates microvascular sequestration of *C.neo* in the brain [172]. Mice infected intratracheally with a urease-deficient strain of *C.neo* have fewer hallmarks of nonprotective Th2 immunity: lower pulmonary fungal burden, less extrapulmonary dissemination, dramatically less eosinophil recruitment and less YM-1 crystal deposition [49]. The urease-deficient strain accumulated fewer immature DC in the LALN compared with wild-type *C.neo* [43], which was consistent with the hypothesis that urease promoted development of nonprotective immunity by interfering with maturation of DC in the *C.neo* infected lungs. These findings were also consistent with other mouse studies showing a strong association between immature DC phenotype and the development of nonprotective Th2 immune responses to *C.neo* [47,85]. As we will discuss in the next section, the critical role of DC in anticryptococcal defenses is being strengthened by the accumulating evidence linking impaired DC numbers and activation with higher fungal burdens in the lung and increased risk of lethal CNS dissemination.

Host factors that influence dendritic cells

GM-CSF in human cryptococcosis patients and mouse models of fungal infection

GM-CSF is a cytokine instrumental in generating DCs (reviewed in [173]). As noted above, GM-CSF is increasingly appreciated as a necessary factor in anticryptococcal immunity as evidenced by anti-GM-CSF autoantibodies, such as those found in patients who either have or go on to develop pulmonary alveolar proteinosis correlating to higher rates of cryptococcosis with either *C.neo* or *C.gattii* [23]. Furthermore, the presence of anti-GM-CSF autoantibodies results in higher rates of dissemination of *C.gattii* to the CNS in human patients [24]. The data generated in mice and rats are consistent with the requirement of GM-CSF for generation of protective response; Chen *et al.* showed that both mice and rats deficient in GM-CSF have a significantly higher pulmonary fungal burden at the efferent phase of the immune response and in association with some impairment in T cell numbers and cytokine responses [25,174]. Of note, TNF- α production in infected GM-CSF-deficient mice was significantly reduced relative to wild-type mice [25]. No dissemination to the CNS was reported in the manuscript in either genotype of mouse, but the strain of *C.neo* used (52D) exhibits little neurotropism [175]. Much remains to be learned about the role of GM-CSF in cryptococcal infection through murine modeling, but lessons from *Histoplasma capsulatum* suggest that GM-CSF is required for generation of adaptive immunity, likely due to its role in spurring DC development and priming of adaptive immunity. Mice treated with anti-GM-CSF antibodies during primary exposure to *H.capsulatum* had higher fungal burdens and survival defects, while mice treated with anti-GM-CSF antibodies during secondary exposure to *H.capsulatum* did not exhibit these defects [176]. Studies investigating the effects of GM-CSF depletion on DCs and modeling the mechanism behind *C.gattii* CNS invasion in GM-CSF autoantibody-producing patients will be necessary to address this emerging phenomenon.

Importance of TNF- α in human patients & in mouse models of cryptococcal infection

Several different lines of evidence point to TNF- α as a crucial cytokine in the host response to *C.neo*. Along with IFN- γ , TNF- α is the major protective biomarker in *C.neo* infected patients [80,81]. Furthermore, human patients that are receiving TNF- α blocking antibody therapy (Infliximab, Etanercept, Adalimumab) and who are otherwise immunocompetent, are at increased risk of *C.neo* infection [30,177]. The resistance of CBA/J mice to strain 52D can be overcome by TNF- α depletion [45,72,85,178]. Conversely, C57BL/6 mice (susceptible) given an adenoviral vector expressing TNF- α prior to intratracheal cryptococcal infection increased their number of accumulated monocytes, decreased the numbers of accumulated eosinophils, displayed progressive clearance of the infection similar to CBA/J mice and increased the hallmarks of Th1 response relative to mice that were infected with *Cryptococcus* that received a control adenoviral vector [179]. In addition to host-specific effects on TNF- α -production, different strains of *C.neo* also vary in the amount of TNF- α elicited from immune cells derived from the same strain of mice. Strains of *C.neo* that have been induced to express high melanin via culture in asparagine salts agar induce baseline levels of TNF- α production from cultured alveolar macrophages *in vitro*, while *in vivo*, naturally occurring high-melanin-producing strains of *C.neo* yield levels of TNF- α comparable to uninfected mice in BAL fluids [180,181]. Similar effects were observed with *C. gattii* strains that cause invasive disease in patients with no known immunodeficiencies. Infection resulted in downregulation of the host TNF- α response and skewed the adaptive immune response away from Th1/Th17 [84,182]. Thus, the removal or addition of TNF- α is sufficient to fundamentally alter whether *C.neo* is or is not cleared.

TNF- α & dendritic cells during *C. neoformans* infection

Studies using murine models consistently identify the first 7 days of infection while the afferent immune response develops as the period in which TNF- α signaling has the most profound effect on the generation of protective immunity against *C.neo*. [45]. Transient depletion of TNF- α before day 7 post infection by injection of TNF- α blocking antibodies induces profound, long-term effects on anticryptococcal immunity, while short-term depletions of TNF- α after day 7 of infection do not prevent development of protective immunity [45]. In other studies, administration of exogenous human TNF- α to mice during days 1–3 post infection was sufficient to extend survival times by over 30% [72]. The temporal relationship between TNF- α production (or blockade) and the afferent immune response provides important evidence that TNF- α is critically associated with priming of the immune response and that DCs, as primers of the immune response, may represent the cell subset most significantly affected by TNF- α signaling. While early studies did not distinguish between macrophages, DCs and monocytes [45], they noted a dramatic decrease in mononuclear phagocyte accumulation during TNF- α depletion, which resolved upon recovery of TNF- α levels [45,85,178]. Further experiments showed a correlation between the phenotype observed when mice were depleted of TNF- α and the phenotype observed when infected mice had received adoptively transferred immature dendritic cells pulsed with cryptococcal mannoprotein prior to infection. Mice receiving adoptively transferred immature DC developed nonprotective Th2 immunity in subsequently infected recipient mice relative to infected mice that received LPS- and MP-pulsed mature DC [85]. Thus, DC

maturation and/or polarization are of the utmost importance in generation of protective immunity to *C.neo*.

Future direction: ‘training’ of dendritic cells against *C. neoformans*

A series of novel paradigm-shifting studies demonstrated that epigenetic modifications substantially influence patterns of DC and macrophage activation and gene expression (reviewed in [183]). Epigenetic modifications are biochemical modifications (methylation, acetylation, phosphorylation, among others) added to DNA or histones that stably regulate gene expression by altering a gene’s accessibility to the cell’s transcriptional machinery (reviewed in [184]). These modifications can be either activating or suppressive. Histone acetylation is typically activating, while DNA hypermethylation in promoter-region CG islands is transcriptionally repressive. Histone methylation can be either activating or repressive depending on the residue of the histone tail that receives the methyl group(s). Histone methylation marks are deposited by histone methyltransferases (reviewed in [185]) and removed by histone demethylases. DNA methyltransferases add methyl groups to DNA, while demethylation is induced by cytidine deaminases or Tet proteins, (reviewed in [186]). Many of the signaling pathways, protein complexes and enzymes that affect DC and macrophage polarization through epigenetic modification are beginning to be elucidated. The studies enumerated below provide evidence that these pathways can modulate DC responses to cryptococcal infection.

TNF- α signals through MAPK and NF κ B pathways (reviewed in [187]), and epigenetic modification of key immune genes is known to occur through both the MAPK [188,189] and NF- κ B [190] signaling pathways. Exciting new work from Diermeier *et al.* has shown that TNF- α signaling directly changes chromatin structure around NF- κ B-associated gene regions [191]. MAPK signaling has been shown to be required for IL-1 β production in DCs [189] and MHCII expression in macrophages [192]. NF- κ B signaling has been shown to epigenetically regulate the promoter of the chemokine eotaxin [190], and NF- κ B signaling has been linked to activities of the histone methyltransferase MLL1, which forms a multiprotein complex instrumental for IL-12p40 epigenetic regulation in DCs [193] and at numerous other promoters in myeloid cells [194]. Our studies showing that cryptococcal clearance is impaired by TNF- α signaling blockade during the first week postinfection highlight the critical importance of early TNF- α signaling in the transition between innate and adaptive immune responses mediated by DCs to the organism. Our yet unpublished results suggest that this transition may be highly influenced by TNF- α -induced epigenetic modifications to key DC1 and DC2 genes. Taken together, these studies suggest that TNF- α signaling can epigenetically modify DCs at critical regions of DC1 and DC2 genes during cryptococcal infection, and is a potential mechanism behind the long-term effects of short-term TNF- α depletion in cryptococcal infection.

Epigenetic modifications induced by TLR signaling may also contribute to the paramount importance of PAMP/TLR signaling in cryptococcal infection. TLRs signaling through the MyD88 adaptor protein use the NF- κ B and MAPK pathways, similar to TNF- α signaling (above), and affect the epigenetic regulation of inflammatory genes in monocytes [195–197]. In work by Weinmann *et al.*, chromatin remodeling and subsequent transcription factor

binding to the promoter region of IL-12p40 in murine macrophages depended upon TLR stimulation [198,199]. Further, *M. tuberculosis* induced robust IL-12 production from DCs specifically through ligation of TLR9, but not macrophages, in which signaling occurs chiefly through TLR2. This was due to chromatin remodeling around the IL-12p40 promoter downstream of TLR9, but not TLR2, signaling [119]. As discussed above, CpG DNA signaling through TLR9 critically contributes to IL-12 production in mouse models of cryptococcosis [111], and the loss of TLR9 impairs DC responses during cryptococcal infection [64]. Thus, similar to TNF- α , TLR signaling may epigenetically regulate pro-Th1 gene expression in DCs in response to *C.neo*. Other factors acting similarly are likely to be identified in the near future.

Conclusion

Protective immunity to *C.neo* requires robust T cell responses, and this is preceded by the production of key protective proinflammatory cytokines TNF- α , IL-12, GM-CSF and IFN- γ . These responses are primed by classically activated DCs in the node and in the infected lung tissue where effector DCs also play a role in phagocytosis and killing of *C.neo*. DCs in cryptococcal infection must overcome alternative activation or immaturity, which is promoted by a vast repertoire of cryptococcal factors. It is possible that epigenetic training of DC during cryptococcal infection aids DCs in maintaining DC1 programming in the infected lungs, lymph nodes and any sites of dissemination in order to prime effective T cell responses.

Future perspective

In this review, we have highlighted the evidence defining a critical role for DCs in orchestrating successful adaptive immune defenses against cryptococcal infections. Yet many patients do not develop effective immunity and either succumb to cryptococcosis or require chronic suppressive antifungal therapy, which is expensive and toxic. Furthermore, we suspect that the 'arms race' ongoing in the fields of organ transplantation, autoimmune disease and cancer therapy will continue to increase numbers of patients with temporary or lasting immunosuppression. Novel studies seem to emphasize quite uniformly that suppression of DC specifically makes humans and other mammalian hosts more susceptible to cryptococcal infections. As the novel therapeutic approaches are required to combat this deadly disease the advancements in our understanding of DC biology and their role in antifungal host defenses is likely to offer opportunities to develop new, immune-based, treatments for infected patients.

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Executive summary

Protective immunity to cryptococcal pathogens requires robust T cell responses

- *C. gattii* and *C. neoformans* cause disease in immunocompromised and immunocompetent patients alike.
- Both CD4⁺ and CD8⁺ T cells are required for anticryptococcal immune responses.
- Macrophages and DCs are also required for the generation of anticryptococcal immune responses.

Multiple DC subsets are involved in anticryptococcal immune responses

- Lung-resident CD11b⁺ or CD103⁺ DC phagocytose and kill the yeasts.
- Lung DC traffic to the lymph nodes to present antigen and induce T-cell proliferation in antigen-specific T cells.
- Monocytes are recruited from the bone marrow via CCR2-dependent mechanisms and are necessary for protective immunity.
- T-cells interact with DC in the lung and secrete cytokines to direct effector cell anticryptococcal responses.
- Plasmacytoid DC are emerging players in these immune responses.

PRR–PAMP interactions can modulate DC activation

- TLR9 appears to be the most important TLR in anticryptococcal immune responses, while TLR2 and TLR4 are of lesser importance.
- The mannose receptor CD206 plays roles in pathogen uptake and in modulation of cytokine secretion, and may be beneficial or detrimental depending on context.
- Multiple cryptococcal factors promote alternative activation or immature DCs, including GXM, mannoprotein and urease.

TNF- α is critically important in generation of protective immunity

- Highly virulent cryptococcal strains are capable of suppressing host DC-derived TNF- α production in humans, and human patients with disrupted TNF- α signaling are at increased risk of *C.neo* infection.
- TNF- α depletion in mouse models results in persistent infection.
- TNF- α depletion alters cell recruitment to infected tissues, promotes extrapulmonary dissemination and skews the cytokine profile away from potent Th1 immunity.

Epigenetic modification to DC may promote the generation of stable protective immunity

- There is evidence for epigenetic modification to DC1 and DC2 genes in other models of disease and during other fungal and bacterial infections.
- TNF- α signaling through MAPK and NF- κ B pathways can result in epigenetic modification of pro- and antiinflammatory genes in myeloid cells.
- TNF- α may exert some of its effects on DC polarization in *C.neo* infection through epigenetic activation of DC1 genes and suppression of DC2 genes.

Figure 1. Mobilization and trafficking of myeloid cells in different compartments during cryptococcosis

An increase in DC numbers in the regional thoracic lymph nodes 3 days post infection (dpi; top panel) suggests the immediate migration of resident DC from the lung. A second peak at 10 dpi coincides with a marked accumulation of Ly-6C^{high} monocytes and monocyte-derived CD11b⁺ DC in the lung (2nd panel), some of which may migrate to the node. The observed peak of Ly-6C^{high} monocytes in the lung (2nd panel), which coincides with similar peaks in the blood (3rd panel) and bone marrow (4th panel), suggests that inter-compartmental communication between lung, blood and marrow occurs to meet the need for additional lung DC in response to cryptococcal infection.

Data were generated from published [43,89] and unpublished *in vivo* studies from the Osterholzer and Olszewski labs.

Figure 2. Key interactions of immune cells and *C.neo* during cryptococcal infection

(Ai) DCs recognize and phagocytose *C.neo* in an infected lung and process the antigen, triggering DC maturation and migration to the lung-associated lymph nodes (LALN) through CCR7-CCL19/21 interactions (afferent phase immune response). (ii) In the LALN, naive T cells interact with mature DCs. DCs present antigen on MHCII, express costimulatory molecules (CD40/CD80/CD86) and secrete pro-Th1 cytokines such as IL-12. Cognate T cells become Ag-sensitized and proliferate. They do not yet produce cytokines. (iii) Ag-sensitized T cells traffic to the lungs (efferent phase immune response), and they interact once again with DCs in the lung. They do not proliferate at this stage, but they do begin to secrete cytokines, directing the immune cells in the lungs. (B) CD4⁺ T cells within the lungs do not proliferate, but do make cytokines such as IFN γ . (C) CD4⁺ T cells within the LALN are actively proliferating, but do not upregulate cytokine production until they get to the lungs. (B & C) are adapted from previously published work [9].

Figure 3. Host and pathogen-derived factors can skew DCs to DC1 or DC2 polarization

Activated DCs are capable of becoming DC1 cells, which promote protective Th1 T cell responses in *C.neo*-infected mice, or DC2 cells, which promote nonprotective Th2 T cell responses in *C.neo*-infected mice. Note that this schematic is over-simplified, as many DCs, particularly in human disease, exist on a spectrum between DC1 and DC2. The relative strength of DC1 and DC2 pathways is influenced by factors derived both from host and from pathogen, and the relative balance between these pathways determines the cumulative DC phenotype. DC1s express high iNOS, MHCII, costimulatory molecules and pro-Th1 cytokines whereas levels of Arginase, Fizz1 and other DC2 markers are low. Conversely, DC2 express a reciprocal pattern and are more associated with pro-Th2 cytokine expression.

Table 1

Summary of DC subsets in afferent and efferent immune responses to pulmonary cryptococcosis discussed in this review.

Surface markers	Common name	Equivalent human subset	Importance to <i>C. neo</i> immunity	Reference
Lung-resident populations				
CD11b ⁺ /CD11c ⁺ /CD103 ⁻	Conventional DCs	CD1c ⁺ /Dectin1 ⁺ /Decin2 ⁺	Initial fungal containment and T-cell priming	Osterholzer 2009 I&I, Traynor 2002, Collin 2013
CD11b ⁻ /CD11c ⁺ /CD103 ⁺	CD103 ⁺ DCs	CD141 ⁺ /CLEC9A ⁺ /SICR1 ⁺	Unclear	Osterholzer 2009 II, Collin 2013
CD11b ⁻ /CD11c ⁺ /autofluorescence ^{hi} /FSC ^{hi}	Alveolar macrophages	CD11c ⁺ /autofluorescence ^{hi} /CD206 ⁺ /SSC ^{hi} /FSC ^{hi}	Initial fungal containment	Osterholzer 2009 I&I, Shao 2005, Mody 1991, Feldmesser 2000, Jambo 2014
Recruited populations				
CD11b ⁺ /CD11c ⁺ /Ly6c ^{hi} /autofluorescence ^{lo}	Inflammatory DCs	CD1c ⁺ /CD16 ⁻	T-cell restimulation	Lin 2008, Collin 2013, Osterholzer 2011
CD11b ⁺ /CD11c ^{hi} /Ly6c ^{hi} /autofluorescence ^{hi}	Exudate macrophages	CD14 ⁺ /CD16 ⁺ /CCR2 ⁺	Phagocytosis	Lin 2008, Osterholzer 2011, Kawakami 1999
CD11b ⁺ /CD11c ^{int} /TNF-α ⁺ /iNOS ⁺ /autofluorescence ^{lo}	Tip-DCs	CD11c ⁺ /CD206 ⁺ /CD14 ⁺ /CD163 ⁺ /CD209 ⁺	Unclear	Osterholzer 2009 II, Serbina 2003, Boltjes 2014
Node populations				
CD11b ⁻ /CD11c ^{int} /CD4 ⁺ /CD8 ⁻ /Dec205 ⁻	CD4 ⁺ /CD8 ⁻ /Dec205 ⁻	CD11c ^{lo} /CD141 ⁺ /CD11b ⁻ /XCR1 ⁺	Unclear	Henri 2001, Shortman 2002
CD11b ⁻ /CD11c ^{int} /CD4 ⁻ /CD8 ⁻ /Dec205 ⁻	CD4 ⁻ /CD8 ⁻ /Dec205 ⁻		Unclear	Henri 2001, Shortman 2002
CD11b ⁻ /CD11c ^{int} /CD4 ⁻ /CD8 ⁺ /Dec205 ⁺	CD4 ⁻ /CD8 ⁺ /Dec205 ⁺	CD11c ^{lo} /CD141 ⁺ /CD11b ⁻ /XCR1 ⁺	Unclear	Henri 2001, Shortman 2002
Other populations				
CD11b ^{lo} /CD11c ⁺ /CD8 ^{lo} /Dec205 ^{lo}	Langerhans DCs	CD141 ⁺ /CLEC9A ⁺ /XCR1 ⁺	Unclear	Bauman 2000, Collin 2013
CD11b ⁻ /CD11c ⁺ /B220 ⁺ /Gr1 ⁺	Plasmacytoid DCs	CD303 ⁺ /CD304 ⁺ /CD123 ⁺	Unclear	Bauman 2000, Hole 2014, Collin 2013