Early TNFα Supports Sustained Th1 T Cell Polarization by Promoting Stability of Dendritic Cells in *C. neoformans* Infected Lungs

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Abstract

Cryptococcus neoformans (*C. neoformans)* is a major opportunistic fungal infection of immunocompromised populations. Robust Th1 immune polarization is required for clearance of *C. neoformans.* We hypothesized that TNFα promotes classical activation of dendritic cells (DC), which prime protective Th1 immunity. CBA/J mice were infected intratracheally with 10^4 CFU of *C. neoformans* strain 52D. Selected mice were given TNFα blocking antibodies (αTNFα) intraperitoneally just before infection. Mice were euthanized and evaluated for pulmonary and splenic fungal burden through a Colony Forming Unit assay. We observed a stark decrease in fungal burden in the lungs of CBA/52D mice in contrast to fungal persistence in αTNFα-CBA/52D mice. Immune polarization was assessed through isolation of T cell and DC RNA and assessment of mRNA expression of selected immune genes. In DCs and $CD4^+$ T cells, single administration of αTNFα profoundly down-regulated DC1 or Th1 genes, respectively, but up-regulated DC2 or Th2 gene expression. $CD4^+$ and $CD8^+$ T cell gene marker expression was also evaluated through flow cytometry. αTNFα decreased accumulation of pulmonary $CD4^+$ T cell and $CD8^+$ T cells. Differential cell counts were performed on lung leukocytes; administration of αTNFα decreased neutrophil accumulation. To assess the impact of TNFα on DC1 phenotype stability, mouse bone marrow-derived DCs (BMDCs) were given IFNγ with or without TNFα treatment and cultured, then washed and treated with IL-4 and assessed for key DC1/2 gene expression by qPCR. Following IL-4 exposure, TNFα/IFNγ-pretreated BMDCs continued to show high DC1 gene and low DC2 gene expression. Together these data revealed that early induction of TNFα is critical for generation of a protective immune response to *C. neoformans* infection. We propose that TNFα induces stable DC1 polarization in the infected lungs, which supports sustained Th1 T cell polarization, leading to fungal clearance.

Introduction

I. *Cryptococcus neoformans*

Cryptococcus neoformans is a major opportunistic fungal pathogen that leads to severe mycoses worldwide (1). A saprophytic fungus, *C. neoformans* can be found most commonly in soils, especially those associated with trees or contaminated with avian feces, in which the yeast externally digests and feeds on decaying animal and plant matter (2). As a versatile yeast, *C. neoformans* can cause disease in a wide range of hosts including protozoa, plants, insects, birds and mammals. In humans, the main route of infection is inhaling the desiccated yeast and/or blasidiospores and therefore primary cryptococcosis is usually localized in the alveolar spaces (2). Following infection, pathology ranges from subclinical to severe pulmonary pneumonia (2). Systemic dissemination is possible for severe, uncontrolled cryptococcal infections and the main site of dissemination is the central nervous system; cryptococcal meningoencephalitis is often a fatal condition (2). Current treatment for cryptococcosis requires the extended use of highly toxic antifungal drugs and often results in poor outcomes (3, 4).

As an opportunistic yeast, *C. neoformans* often infects immunocompromised humans (1, 4, 5). In the case of *C. neoformans*, the most commonly associated cause of immunodeficiency is in HIV positive patients where the viral infection has rendered the T cells ineffective (2). Consequently, *C. neoformans* is among the major causes of opportunistic infections in AIDS patients and cryptococcal meningitis is categorized as one of the AIDS-defining infections (6). In 2009, the CDC estimated that there were 1 million new cases of HIV-associated cryptococcosis per year, amounting to 680,000 deaths per year (7). Cryptococcosis is also common among other

immunocompromised patients including solid organ transplant recipients, those undergoing immunosuppressive therapies, and patients with hematological malignancies (2, 4, 8, 9). Interestingly, clinical reactivation or revertance has been shown to be responsible for 52% of cryptococcosis in both solid organ recipients (10) and in AIDS patients (11). Five different stages have been suggested for the pathogenesis of *C. neoformans* infections: initiation, dormancy, reactivation, dissemination, and systemic proliferation (12). These findings indicate that *C. neoformans* likely persists in a latent infectious stage in both immunocompromised and immunocompetent individuals. There have also been reported outbreaks of cryptococcosis where the afflicted patients and animals showed no detectable deficiency in immune function (13). These outbreaks among immunocompetent individuals demonstrate that highly virulent Cryptococcus strains can evade host immune defenses and interfere with the development of protective immunity even in healthy individuals. Therefore, a deeper understanding of *C. neoformans* and its interaction with the host immune system is needed to ensure that all the mechanisms necessary for the development of protective immunity against *C. neoformans* are recognized.

Much research over the past few decades has demonstrated that the development of an immune response to *C. neoformans* requires interaction of multiple arms of the immune system. It has been shown that in order for the host to achieve clearance of *C. neoformans*, optimal innate and adaptive immune responses are necessary (reviewed in (8). While it has been shown that the innate immune system is insufficient on its own in achieving clearance (14), its proper function is required for generation of an optimal adaptive immune response; thus, defects in either the innate or adaptive immune responses can lead to insufficient clearance of *C. neoformans* (9).

These findings demonstrate the importance of interplay between the innate and adaptive immune components for effective elimination of cryptococcal infection.

II. Innate Immunity in *Cryptococcus neoformans* Infection

Innate Immunity

The innate immune response is the subsystem of immunity composed of the cells and mechanisms that defend the host from infection. The innate immune response responds to pathogens in a generic way and is a first line of response; it does not confer long-lasting immunity. (15)

Innate Immune Recognition by Phagocytes

The principal innate immune cells involved in recognition of *C. neoformans* are phagocytes, which internalize fungal cells and products (15-21). Phagocytes include macrophages and dendritic cells (DCs) and their precursor cells, monocytes. These phagocytes recognize and respond to infections (22). Upon infection, large numbers of DC precursors are recruited into the lungs of infected mice (60-62). Disruption of events leading to DC recruitment lead to the development of non-protective Th2 responses and prolonged pulmonary infection (63, 64). DCs are clearly crucial to the development of a protective immune response to *C. neoformans.* DCs must not only be recruited in substantial numbers but must also be optimally polarized to generate an effective immune response against *C. neoformans*. Although the antimicrobial defenses initiated by innate recognition of the fungus are insufficient to clear the infection, the innate immune system is vital to the development of a protective, antigen-specific adaptive immune response (22). These immune signals recruit more leukocytes (immune cells), such as lymphocytes, to the lungs.

III. Dendritic Cells in *Cryptococcus neoformans* Infection

Adaptive immune responses depend crucially on DCs, the antigen presenting cells that stimulate T cells (32, 34). DCs are present in most organs/tissues and they recognize signs of invading pathogens in local environments, respond to pro-inflammatory stimuli by up-regulation of co-stimulatory molecules and various chemokine receptors, and migrate to the lymph nodes to stimulate T cells to eliminate the infection (32, 34). The majority of DCs recruited into sites of infection serve primarily to direct local adaptive immune responses (59). DCs are most associated with a protective phenotype when they take on a "DC1" phenotype, in which they maintain high expression of genes that promote DC maturation and the generation of a Th1 immune response such as iNOS, MHC class II, IL12b, and CCR7 (65). DCs are more associated with a non-protective phenotype when they take on a "DC2" phenotype, in which they promote Th2-skewed immune responses by maintaining lower expression of the DC1 genes and higher expression of DC2 genes such as IL10, Arg1 and Fizz (65).

The Role of TNFα in Dendritic Cell Polarization

TNFα has been shown to be crucial in the generation of a protective host response to *C. neoformans*. Sufficient TNFα expression has to been shown to correlate strongly with positive clinical outcomes, while lowered expression correlates with negative clinical outcomes (66). Increased TNFα expression has been associated with a lower fungal burden, high T cell numbers, and more likely survival in HIV patients with cryptococcal meningitis (67). Diminished TNFα levels in AIDS patients prior to antiretroviral therapy during *C. neoformans* infection is associated with increased mortality (68). Virulent fungal strains such as *C. gatti* (R265), which is

causing major outbreaks in the Pacific Northwest and Canada, suppresses TNFα production by DCs (69).

Patients with autoimmune diseases such as inflammatory bowel disease and rheumatoid arthritis are often treated with anti-TNFα antibodies. These antibodies play a crucial role in diminishing inflammation but leave the host susceptible to *C. neoformans* infection (70-73). Furthermore, Cryptococcal strains that weakly induce TNFα demonstrate increased virulence relative to strains that strongly induce TNFα (74, 75). Conversely, mouse strains that typically induce weaker TNFα response during *C. neoformans* infection have decreased resistance to the infection (74).

IV. Adaptive and T cell Mediated Immunity to *Cryptococcus neoformans*

Adaptive Immunity

Adaptive immunity is a subsystem of the immune response composed of specialized, systemic, cells that eliminate pathogen growth. (16) Adaptive immunity creates immunological memory after an initial response to a specific pathogen. The adaptive system includes both humoral and cell-mediated immunity. (16)

Adaptive immunity is characterized by antigen-specific responses by activated T cells (39-42). T cells are the most important adaptive immune cells during cryptococcal infection, as is clear by the susceptibility of T cell deficient HIV/AIDS patients to *C. neoformans* and by acquisition of anti-cryptococcal immunity through adoptive transfer of T cells in mice, but not through transfer of B cells (7, 39-41, 43, 44). Optimal adaptive immunity ultimately is necessary to stimulate macrophages to eliminate the cryptococcal infection (45, 46-51).

T cell Mediated Responses

For effective clearance of *C. neoformans*, activated T cells must be recruited to the lungs where they can coordinate protection (Fig. 1, 46, 52, 53). T cells mostly direct the responses of other immune cells, including effector (or killing) functions of macrophages (45, 46, 54). These protective responses by T cells depend on their activation or polarization phenotype. Th1 polarization leads to protection against infection while Th2 polarization is non-protective.

Protective Th1 immunity is characterized by the up-regulation of a variety of immune signals, including IFNγ, TNFα, and IL-12 (24, 45, 55-57). In contrast, non-protective Th2 responses are driven by up-regulation of the cytokines IL-4 and IL-10 (24, 45, 55-57). Th17 responses are characterized by IL-17 production, and are protective and associated with Th1 responses (24, 45, 55-57). In general, Th1/Th17 versus Th2 activation is not an either-or process; rather the overall balance of Th1-type and Th2-type cytokine expression places T cells along a continuum of Th1/Th7 versus Th2 responses (43, 51, 54). Th1/Th17 responses help recruit and activate macrophages to aid in fungal clearance (43, 45). Conversely, clearance is less successful during Th2 responses (43, 45). Thus, the development of protective immunity to *C. neoformans* requires not only the presence of T cells also their optimal polarization.

T cell Stability In *C. neoformans* Infection

In addition to the development of a protective immune response, stability of the effector phenotype is required to complete fungal clearance. T cells demonstrate plasticity; that is, they can change their cytokine production over time when environmental signals are changed (58). Thus a major question is how a protective immune response remains stable despite the plasticity of its main effector cells.

Role of TNFα in T Cell-Mediated Responses

Signaling by TNFα is associated with a protective T cell-mediated response, and this is true of TNFα in cryptococcal infection (32). First of all, TNFα signaling is required for robust T cell recruitment to the lungs during the adaptive phase of the immune response (Fig. 1, 32). TNFα deficiency causes a shift from a Th1-biased cytokine profile to a Th2-biased cytokine profile, including reduced IFNγ expression and increased IL-4 expression (32). This effect is associated with lower clearance of the fungus in TNFα-depleted mice as compared to control mice (32). We propose that the mechanisms by which TNFα achieves its effects on Th polarization and cytokine expression occur through TNFα-mediated modulation of DC polarization. (Fig. 1)

Priming of Adaptive Responses by Innate Immunity

Recognition of pathogens by phagocytes also initiates priming of adaptive immune responses. In this respect, there are two major inflammatory functions that innate immunity serves—recruitment of leukocytes to the site of infection and stimulation of T cells, the directors of adaptive immunity. These processes involve the production of chemokines and other cytokines by innate immune cells and require the optimal polarization of DCs (Fig.1, 22). Cytokines are small proteins involved in cell signaling and immuno-modulatory functions; chemokines are a family of cytokines that can induce directed chemotaxis of immune cells. Upon recognition of pathogens, innate immune cells produce inflammatory cytokines that attract more leukocytes to the lungs and induce changes in the capillary endothelium that enhances the ability of leukocytes to transmigrate into the lungs from the bloodstream (23-25). Recruited leukocytes

enable greater surveying of the infection and many will ultimately migrate to the lymph nodes to activate T cells and the adaptive response.

Cytokines also direct the stimulation of T cells with antigen. The primary innate immune cells that stimulate T cells in cryptococcal infection are DCs (26-28). For optimal stimulation of T cells to occur, DCs must be polarized correctly. Migration and correct polarization enables DCs to migrate to lung-associated lymph nodes, where T cells reside, and to present antigen and stimulate these T cells. These responses are achieved through recognition pathways, including direct recognition of pathogens by the DCs as well as indirect effects of the optimal chemokines and cytokines produced by other innate immune cells upon recognition of pathogens (29).

Optimal DC polarization induces changes in chemokine receptor expression by DCs that enhance their propensity to migrate from the lungs to the lymph nodes (30-33). Once in the lymph node, DCs present antigens to T cells via direct cell-to-cell contact (21, 28, 29). MHC class I and II are the proteins used by DCs for antigen presentation (21, 28, 29). For a robust T cell response to occur, DCs need to also express co-stimulatory molecules, including CD40, CD80, and CD86 and produce inflammatory cytokines, such as IFNγ, IL-12, and TNFα, which further activate the T cells (21, 24, 25, 28, 34, 35). Such factors are the major determinants of the type of T cell-mediated response that will develop (22, 29, 34, 36-38). In summary, recognition of *C. neoformans* by phagocytes leads to production of chemokines and inflammatory cytokines, leading to leukocyte recruitment and DC polarization. These processes enable priming of the adaptive responses and are crucial for the type of adaptive response that will develop, ultimately determining the outcome of infection.

Materials and Methods

Mice. Female wild type CBA/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were aged to 8–10 weeks at the time of infection or 12 weeks at the time of bone marrow isolation. Mice were humanely euthanized by CO_2 inhalation at 1, 2, and 4 weeks post-infection (wpi) for *in vivo* experiment sample collection as described below. Uninfected mice were euthanized humanely for *in vitro* dendritic cell experiments as described below. The University Committee on the Use and Care of Animals and the Veterans Administration Institutional Animal Care and Use Committee approved all experiments.

C. neoformans. *C. neoformans* strain 52D (ATCC 24067) was recovered from 10% glycerol frozen stocks stored at -80°C and grown to log phase at 37°C in Sabouraud dextrose broth (1% neopeptone, 2% dextrose; Difco, Detroit, MI) on a shaker. The cultures were then washed in non-pyrogenic saline (Travenol, Deerfield, IL), counted on a hemocytometer, and diluted to 3.3 $\times 10^5$ yeast cells/ml in sterile non-pyrogenic saline.

Intratracheal inoculation with *C. neoformans.* Mice were anesthetized via intraperitoneal injection of Ketamine and Xylazine (100 and 6.8 mg/kg body weight, respectively) and were restrained on a foam plate. A small incision was made through the skin covering the trachea. The underlying salivary glands and muscles were separated. Infection was performed by intratracheal injection of 30 µl (10^4 CFU) via 30-gauge needle actuated from a 1-ml tuberculin syringe with *C. neoformans* suspension (3.3 $\times 10^5$/ml). After inoculation, the skin was closed with cyanoacrylate

adhesive and mice were monitored during recovery from anesthesia. Following infection the inoculi were plated and cultured to confirm the number of organisms injected into the mice.

Anti-TNFα Injection. Before infection, selected mice were treated with TNFα antibody (100 ng/mouse, Taconic, Hudson, NY) or non-specific IgG (vehicle) in a volume of 20 µl via intra-peritoneal route at day 0.

Lung Leukocyte Isolation. The lungs from each mouse were excised, washed in RPMI, minced with scissors, dissociated by gentleMACS tissue dissociator (Miltenyi Biotec, Cambridge, MA). Processed lung tissues were digested enzymatically at 37°C for 30 min in 10 ml/mouse of digestion buffer [RPMI, 5% FBS, penicillin and streptomycin (Invitrogen, Grand Island, NY); 1 mg/ml collagenase A (Roche Diagnostics, Indianapolis, IN); and 30 µg/ml DNase (Sigma, St. Louis, MO)], and dissociated a second time. 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 NH_4Cl buffer (0.829% NH_4Cl, 0.1% $KHCO_3$, and 0.0372% Na_2EDTA, pH 7.4) for 3 min followed by a 10-fold excess of RPMI. Cells were pelleted, resuspended, and a second cycle of syringe dispersion and filtration through a sterile 100-µm nylon screen (Nitex, Kansas City, MO) was performed. The filtrate was centrifuged for 30 min at 1500 RPM 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 following dilution in Trypan Blue (Sigma, St. Louis, MO).

Colony-Forming Unit Assay. For determination of microbial burden, small aliquots of dispersed lungs or spleens were collected following the digest procedure. Serial 10-fold dilutions of the samples were plated on Sabouraud dextrose agar plates in duplicates of 10-μl aliquots and incubated at room temperature for 3 days. *C. neoformans* colonies were counted 2 days later and the number of colony-forming units (CFU) was calculated on a per-organ basis.

Visual identification of leukocyte populations. 50,000 leukocytes from the end of the lung digest were cytospun onto charged microscope slides. Samples were fixed and pre-stained for 2 min in a one-step methanol based Wright-Giemsa stain (Harleco, EM Diagnostics, Gibbstown, NJ) and stained using steps two and three of the Diff-Quik stain procedure. A total of 300 cells were counted for each sample from high power microscope fields. Macrophages and lymphocytes were visually counted in Wright-Giemsa-stained samples of lung cell suspensions cytospun onto glass slides. The percentages of leukocyte subsets were multiplied by the total number of leukocytes to determine the absolute number of specific leukocyte subsets in each sample.

Flow Cytometry. All staining reactions were performed according to the manufacturers' protocols. Data were collected on a FACS LSR II flow cytometer using FACSDiva software (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Tree Star, San Carlos, CA). A minimum of 200,000 cells were analyzed per sample. Initial gates were set based on light-scatter

characteristics followed by gating on $CD45^+$ population, then T cells were separated from myeloid cells by expression of CD3, CD4, CD8, CD11b, CD11c. Macrophages were distinguished from DCs by autofluorescence and CD11b expression. The total number of cells within each mouse tissue was calculated by multiplying the frequency of this population by the total number of leukocytes in each sample (percentage of cells multiplied by the original hemocytometer count of total cells).

Pulmonary T cell and Dendritic Cell Isolation. 10^8 lung leukocytes from each sample were exposed to a PE labeling reagent, incubated, exposed to EasySep PE Selection Cocktail, and incubated further (Stem Cell, Vancouver, BC). EasySep magnetic nanoparticles were added to attach to $CD4^+$ cells and eluent was poured off. Cells were washed with 2% FBS in PBS 3 times, resuspended in buffer, centrifuged, and resuspended in Trizol. Then, CD11c cells were isolated from the eluent via the same procedure, pelleted, and resuspended in Trizol.

Quantitative Real-Time PCR (qRT-PCR). RNA was extracted from Trizol using phenol chloroform purification followed by isopropanol precipitation, quantified by spectrophotometer, and first-strand cDNA was synthesized using RNeasy Plus Mini Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions using 500ng total mRNA per sample. qPCR was performed using an MX 3000P system (Stratagene, La Jolla, CA) according to the manufacturer's protocols. Forty cycles of PCR (94°C for 15 seconds followed by 60°C for 30 seconds and 72°C for 30 seconds) were performed on a cDNA template. The mRNA levels were

normalized to beta-acctin levels and the ratio of sample to uninfected-baseline expression level (fold induction) was calculated.

Isolation & Culture of Bone-Marrow Derived Dendritic Cells (BMDCs). Bone marrow cells from mice were harvested by removing the ends from the femurs and tibias (after removal of skin and muscle) and flushing the marrow with 1 ml of RPMI medium using a 1 ml syringe and a 25½-guage needle. Cells were cultured in 100 × 15-mm dishes in complete DMEM medium with GM-CSF (20ng/ml, PeproTech, Rocky Hill, NJ). After 7 days, the loosely adherent BMDCs were pipetted off. BMDCs were then plated at a density of 1×10^6 cells/ml in 2 mls in a non-tissue-culture-treated 6-well dish and exposed to primary cytokine stimulations for 24 hours, then washed, and treated secondary cytokine stimulations for a subsequent 24 hours. Following these sequences, BMDCs were removed via Trizol and processed for RNA and qRT-PCR as described above.

Calculations and statistics. Statistical significance was calculated using Student's t-test for individual paired comparisons or t-test with Bonferoni adjustment, whenever multiple groups were compared. Means with *P* values of <0.05 were considered significantly different and were represented by *. All values are reported as means ± standard errors (SEM). Calculations were performed using Primer of Biostatistics software (McGraw-Hill, NY).

Results

Figure 1

Mechanism of clearance of *Cryptococcus neoformans*

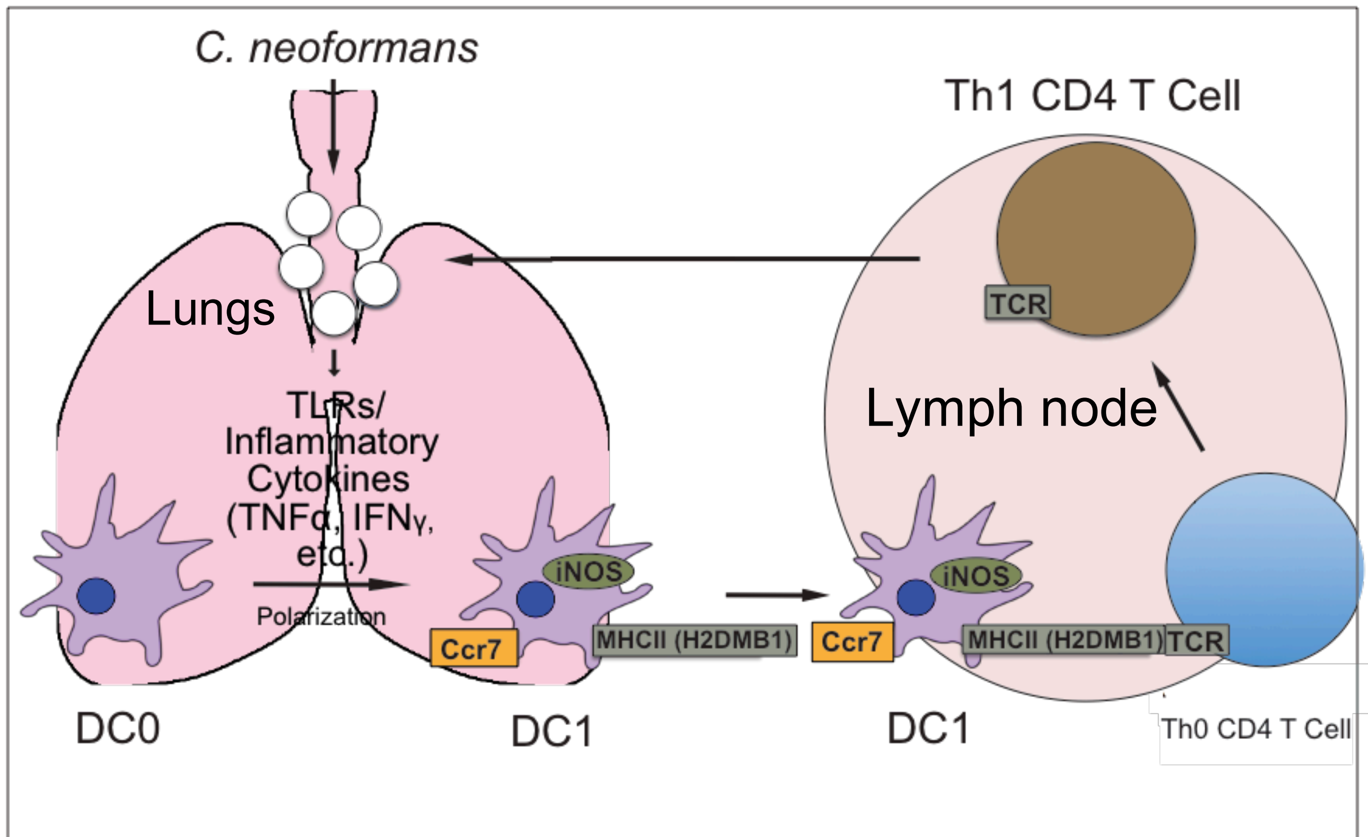

Figure 1: *C. neoformans* infects the lungs. Immature DCs recognize *C. neoformans* and are stimulated by the fungus to DC1 maturation and expression of MHC markers. DCs are also stimulated by the fungus to express chemokine receptor Ccr7, which promotes migration of DCs to the lymph nodes. In the lymph nodes, DCs activate T cells promoting their expansion and polarization. Antigen-specific effector T cells developed and primed for Th1, Th2 or Th17 polarization migrate to the lungs to mount and orchestrate the adaptive immune response which then develops into a robust Th1 response which eradicates the infection.

TNFα is Required for Pulmonary Fungal Clearance and Preventing Systemic Dissemination of *C. neoformans*

To determine the importance of TNFα to the pulmonary clearance of *C. neoformans* in infected mice during the "early" - innate and subsequently developing the adaptive phase of the immune response, CBA/J mice were intratracheally inoculated with *C. neoformans* strain 52D and intraperitoneally injected with either an anti-TNFα antibody or a control antibody. Pulmonary fungal burden was determined at 1, 2, and 4 weeks post infection (wpi). In both mice groups given the control antibody (CBA/52D) and the anti-TNFα antibody (αTNFα-CBA/52D), fungal burden increased during the "early/innate" phase to the same extent (Fig. 2A). In contrast, while the fungal load began to decrease from 2 wpi on in CBA/52D group, resulting in a 200-fold decrease in fungal burden between the peak at the 1 wpi and the 4 wpi time point, it remained high in αTNFα-CBA/52D (Fig. 2A). Thus indicated protective adaptive immune response the development in CBA/52D but not αTNFα-CBA/52D mice.

Next, extra-pulmonary/systemic dissemination was evaluated by measuring splenic fungal burden. During the "early" phase, low but detectable ($<10^2$ CFU) fungal burdens were observed in spleens of CBA/52D mice, and decreased to a minimal level at 4 wpi on (Fig. 2B), suggesting that the dissemination was minimal and self-limited in these mice. This contrasted with increased ($<10^3$ CFU) and persistent fungal dissemination in αTNFα-CBA/52D; a significant increase in dissemination in αTNFα-CBA/52D relative to CBA/52D was found at both 2 at 4 wpi, reaching 3-orders of magnitude difference at 4 wpi (Fig. 2B). Thus, early TNFα signaling is required for pulmonary fungal clearance and to prevent systemic dissemination during *C. neoformans* infection.

Figure 2

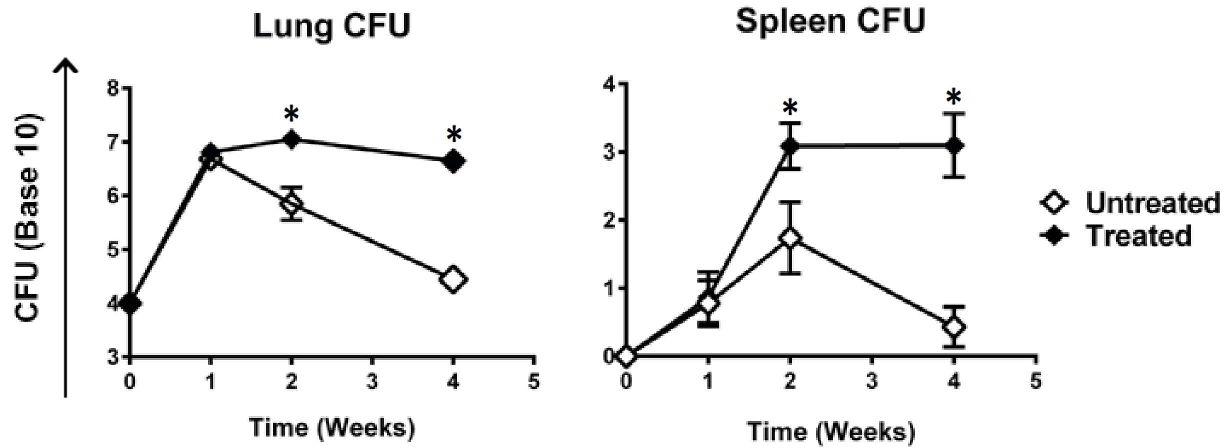

Figure 2: Fungal burden was evaluated in the lungs and spleens. Mice were inoculated intratracheally with 10^4 *C. neoformans*. Lungs and spleen were harvested at 1, 2, and 4 wpi for analysis of fungal burden. We observe an over 200-fold decrease in fungal burden in the lungs of CBA/52D mice between 1 and 4 wpi, which contrasts with persistence of high fungal burden in αTNFα-CBA/52D mice. There is continued fungal growth in the spleens of CBA/52D mice, whereas splenic fungal burden in αTNFα-CBA/52D remains at a lower level throughout the experiment. Data represent mean pooled from 3 separate matched experiments, N=4 for each of the analyzed parameters; * $p<0.05$ in comparison between CBA/52D and αTNFα-CBA/52D.

Early TNFα Plays a Transient Role in Pulmonary Recruitment of Leukocytes Throughout the Time-Course of Infection.

To assess whether the observed deficiency of αTNFα-CBA/52D mice in pulmonary clearance was associated with changes in magnitude of the cellular inflammatory response or a change in composition of leukocyte population, pulmonary leukocytes from digested lung tissue samples were enumerated and then differential cell count analysis was conducted at 1, 2 and 4 wpi. We focused on lymphocyte and mononuclear myeloid cell subsets, as they are essential for adaptive clearance. Similar numbers of total lung leukocytes were observed through most time points of the immune response (Fig. 3A). αTNFα-CBA/52D mice had statistically significantly fewer total leukocytes compared to CBA/52D mice at 2 wpi, suggesting that TNFα was important for the peak inflammatory response observed at that time point (Fig. 3A). At 4 wpi, however, both CBA/52D and αTNFα-CBA/52D mice showed elevated leukocyte numbers in the lungs, no longer significantly different from each other (Fig. 3A). αTNFα-CBA/52D mice had significantly down-regulated macrophage and lymphocyte numbers at 2 wpi (Fig. 3B-C), indicating that TNFα is necessary for a robust early immune response and that that early TNFα signal contributed to the recruitment of lung leukocyte subsets important for successful pulmonary fungal clearance. To assess whether the observed deficiency of αTNFα-CBA/52D mice in pulmonary clearance was associated with a change in T cell subset numbers, T cells (which are most crucially required subset for anti-cryptococcal defenses) were enumerated via flow cytometry. $CD4^+$ and $CD8^+$ T Cell numbers were similar throughout the experiment, except at 2 wpi, when αTNFα-CBA/52D mice carried fewer $CD4^+$ T cells which appears to be compensated by greater number of $CD8^+$ T cells when compared to CBA/52D mice (Fig. 4D-E). These data suggest that TNFα signaling was required for optimal pulmonary recruitment of inflammatory cells, but did not dramatically affect T cell numbers.

Figure 3

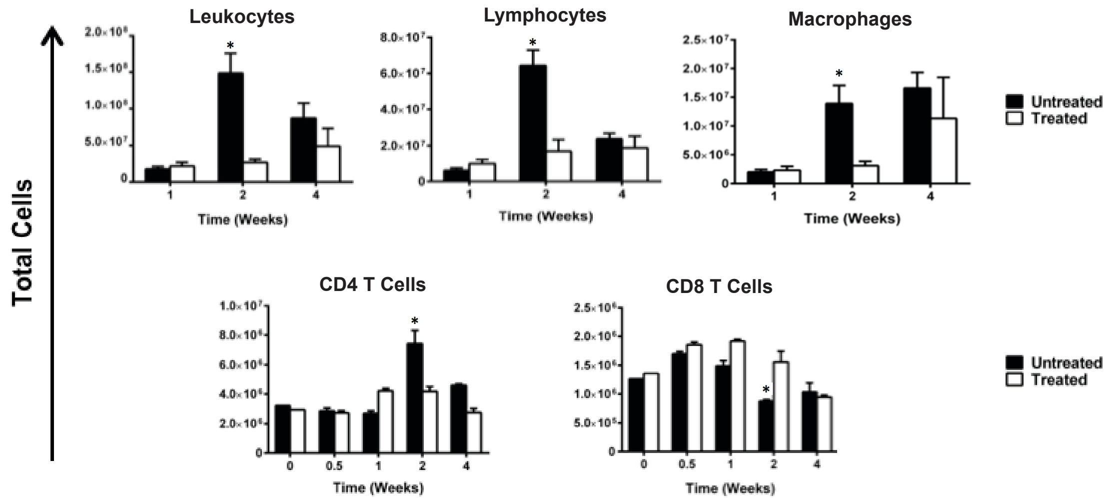

Figure 3: The effect of TNFα on pulmonary leukocyte accumulation. Lung leukocytes were isolated from infected mice at 1, 2, and 4 wpi. Cells were plated, stained, and counted for percent composition by leukocyte subset, which were multiplied by total leukocyte number. Treatment decreased lymphocyte number, macrophage number, and total leukocyte number at 2 wpi. Treatment also decreased $CD4^{+}$ T cell number and increased $CD8^{+}$ T cell number at 2 wpi. Data was pooled from 2 separate matched experiments, N=4 for each of the analyzed parameters; * $p< 0.05$ in comparison between CBA/52D and αTNFα-CBA/52D.

TNFα Increases Pulmonary CD4$^+$ T Cell IFNγ Production and Decreases CD4$^+$ and CD8$^+$ T Cell IL-10 Production

Since, the effects on recruitment were transient and were not likely to account entirely for such a major and prolonged difference in clearance, we examined T cell polarization readouts. T cells were isolated analyzed via flow cytometry for mean fluorescence intensity of selected gene markers. Treatment down-regulated, by more than half, production of the "protective" Th1 cytokine IFNγ in CD4$^+$ T cells at 2 and 4 wpi, but transiently up-regulated production of IFNγ in CD8$^+$ T cells at 2 wpi (Fig. 4A, 3B). At 2 wpi, treatment significantly up-regulated production of non-protective regulatory cytokine IL-10 in CD4$^+$ T cells by up to 3-fold and in CD8$^+$ T cells (Fig. 4B, 4D). Since, the effects on T cell polarization were more profound and lasting than the changes in leukocyte recruitment, our data demonstrate that early TNFα signaling is required for optimal, protective, T cell polarization, providing an explanation for the profound and long-lasting effect of early TNFa on pulmonary clearance of *C. neoformans*.

Figure 4

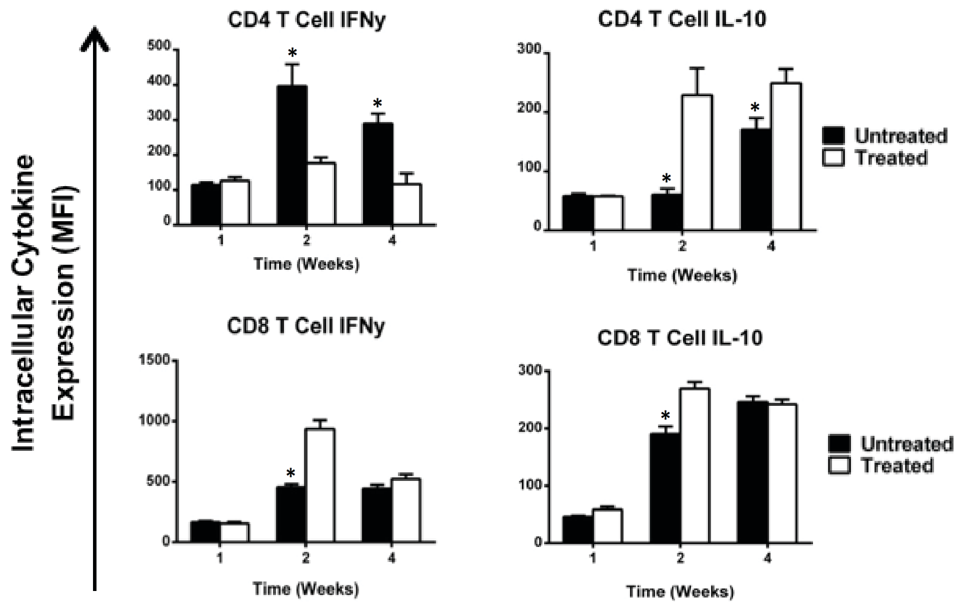

Figure 4: The effect of TNFα on T cell intracellular cytokine expression. T cells were isolated and analyzed by flow cytometry following extracellular stain with CD4 and CD8 antibodies and intracellular antibody staining for IFNγ and IL-10. Bars show mean fluorescence intensity for these cytokines in $CD4^+$ and $CD8^+$ T cell subsets. Treatment down-regulated expression of the Th1 cytokine IFNγ in $CD4^+$ T cells and up-regulated expression of the Th2 cytokine IL-10 in $CD4^+$ and $CD8^+$ T cells at 2 wpi. Interestingly, at the same time point, treatment up-regulated expression of IFNγ in $CD8^+$ T cells. Data was pooled from 2 separate matched experiments, N=3 for each of the analyzed parameters; *$p < 0.05$ in comparison between CBA/52D and αTNFα-CBA/52D.

TNFα is Required for Optimal Th1 Cytokine Expression and Prevention of Th2 Cytokine Induction in $CD4^+$ T Cells during Cryptococcal Infection

To assess whether the observed deficiency of αTNFα-CBA/52D mice in pulmonary clearance was associated with broader changes in $CD4^+$ T cell polarization, cytokine and cytokine transcription factor mRNA expressions were evaluated in sorted lung $CD4^+$ T cells at 1, 2 and 4 wpi. The effects were variable based on the group of cytokines analyzed. TNFα expression was suppressed early in αTNFα-CBA/52D, but increased at the later time points (Fig 5A). Similarly, the Th17 cytokine IL17a expression levels were down regulated in the mice at 1 wpi (Fig 5C). IFNγ expression was significantly down-regulated in the αTNFα-CBA/52D mice relative to the CBA/52D mice at 4 wpi (Fig 5B), indicating suppression of protective Th1 response resulting from early TNFα depletion. In contrast, we observe a strong component of non-protective Th2 immune response with GATA4 transcription factor highly up-regulated through 1-2 wpi and the IL4 cytokine upregulated at 2-4 wpi in TNFα depleted mice (5D, 5E). Finally, T-regulatory cell transcription factor Foxp3 in the αTNFα-CBA/52D mice is dramatically unregulated at 2 wpi compared to control mice, further indicating dysregulated T cell polarization following early TNFα depletion (Fig. 5F). Thus early TNFα signaling has a profound effect on T cell polarization, and its depletion results in broad alteration in $CD4^+$ T cell polarization status for up to 4 wpi.

Figure 5

TNFα is Required for Optimal Th1 Cytokine Expression and Prevention of Th2 Cytokine Induction in CD4$^+$ T Cells during Cryptococcal Infection

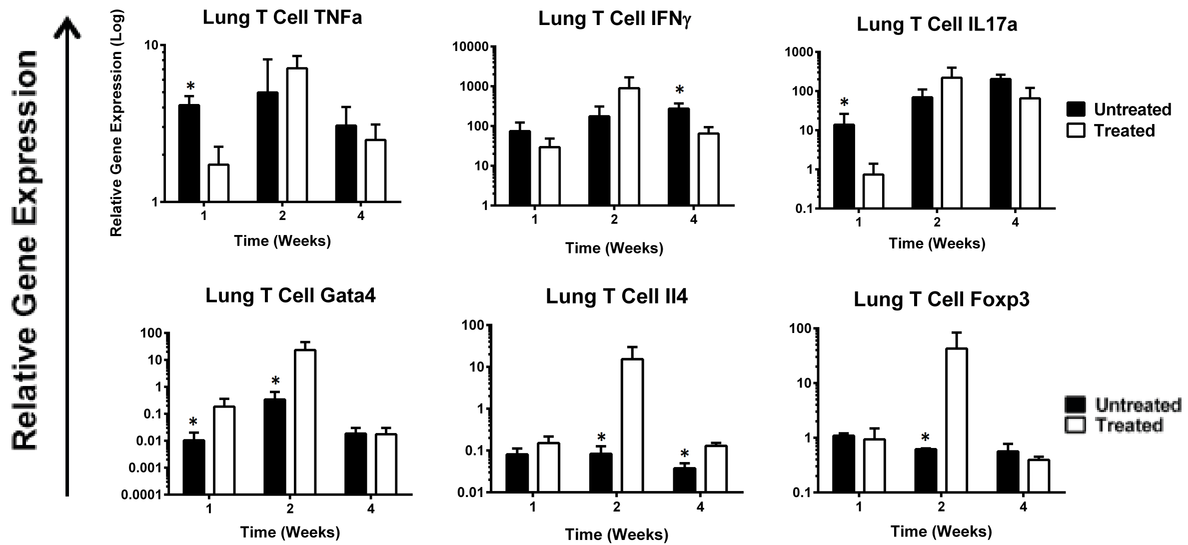

Figure 5: The effect of TNFα on gene expression in CD4$^+$ T Cells. Lung leukocytes were isolated from infected mice at 1, 2, and 4 wpi and CD4$^+$ T cells were sorted using Robosep magnetic bead/antibody selection system to 99% purity. RNA was isolated from CD4$^+$ cells and converted to cDNA for evaluation of gene expression through qPCR arrays. Bars represent mean gene fold expression relative to uninfected control mice ±SEM. Treatment up-regulated the Th2 cytokine IL-4 at 1, 2, and 4 wpi. Treatment also up-regulated the Th2 transcription factor GATA4 and the T regulatory gene Foxp3 at 2 wpi. Treatment down-regulated expression of the Th1 cytokines TNFα and IL17a at 1 wpi and down-regulated IFNγ at 4 wpi. Data was pooled from 2 separate matched experiments, N=4 for each of the analyzed parameters; * $p < 0.05$ in comparison between CBA/52D and αTNFα-CBA/52D.

TNFα Regulates Expression of H2-DMb1, CCR7, and Cytokines by Pulmonary Dendritic Cells

Dendritic cells (DCs) play an important role in directing T cell polarization. To determine whether the observed dysregulation in T cell polarization of αTNFα-CBA/52D mice was associated with a change in DC expression of genes relevant to DC effects on T cell polarization, the enriched lung DC population was obtained by magnetic sorting using CD11c antibodies, RNA isolated and assessed by qPCR. We evaluated expression of major histocompatibility complex (MHC) II, CCR7 (DC1/maturation factor), Arg1 (DC2 marker), and T cell polarizing cytokines IL12 and IL10 expression, at 1, 2 and 4 wpi (Fig. 6). Following TNFα depletion, we observed lasting down-regulation of H2-DMb1, the gene encoding for the DC maturation marker MHCII (Fig. 6A) and lymph node migration receptor CCR7 (Fig. 6C), suggesting that the DC maintained a less mature and less DC1-like phenotype following early TNFα depletion. In contrast, the hallmark of DC2, Arginase1, was up regulated in the αTNFα-CBA/52D mice at 1 and 2 wpi (Fig. 6B), suggesting that DC1/DC2 balance was shifted towards DC2. Consistently, expression of the Th1 driving cytokine, IL12b, was diminished in αTNFα-CBA/52D mice at 1 and 2 wpi while regulatory and Th2 driving cytokine IL-10 was strongly upregulated at 2 wpi in these mice (Fig. 6D, 6E). Thus, early TNFα depletion in *C. neoformans* infected mice caused a shift in pulmonary DC phenotype away from DC1 towards a DC2 phenotype, explaining diminished Th1 and Th2 response in these mice.

Figure 6

TNFα Regulates Expression of H2-DMb1, CCR7 and Cytokines by Pulmonary Dendritic Cells

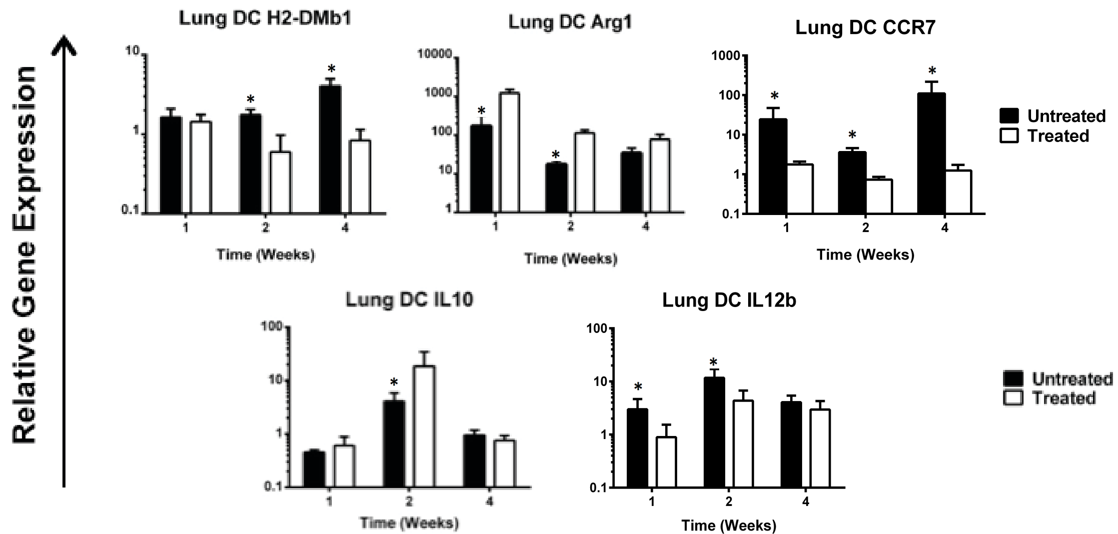

Figure 6: The effect of early TNFα signaling on dendritic cells gene expression profile in *C. neoformans* infected lungs. Lung leukocytes were isolated from infected mice at 1, 2, and 4 weeks post-infection. RNA was isolated from $CD11c^+$ cells (DC) and converted to cDNA for evaluation of gene expression through qPCR arrays. Bars represent mean gene expression change relative to uninfected control mice ±SEM. Treatment down-regulated the Th1 cytokine IL12b, the Th17 cytokine IL17a, the co-stimulatory molecule H2-DMb1, and the migration stimulatory molecule CCR7, at 1, 2, and 4 wpi. Treatment up-regulated the Th2 cytokines IL10 and the hallmark of Alternative Activation of macrophages, Arg1, at 1 and 2 wpi. Data was pooled from 2 separate matched experiments, N=4 for each of the analyzed parameters; * $p < 0.05$ in comparison between CBA/52D and αTNFα-CBA/52D.

TNFα Stimulation Stabilizes IFNγ-Induced Dendritic Cell DC1 Gene Expression Despite Subsequent IL-4 Exposure

Early TNFα was essential to the generation of a robust, immune response against *C. neoformans* and induced profound long-term effects on T cell and DC polarization. To assess whether TNFα contributed to robust and stable DC1 programing of DC, thereby promoting long-term protective effects on T cell polarization in *C. neoformans* infected lungs, we tested the effects of TNFα to modulate DC polarization *in vitro*. Bone Marrow-derived DCs (BMDCs) were exposed to IFNγ, a cytokine that normally causes DC1 polarization, ± TNFα, followed by 24 hours of either stimulation with IFNγ or IL-4, a cytokine that normally causes DC2 polarization. RNA was then extracted and evaluated via qPCR for expression of the DC1 gene for Nitric Oxide Synthase (iNOS) and the DC2 gene factor Fizz. When DCs were initially stimulated with and maintained in IFNγ, they showed high iNOS expression, which was diminished when they were stimulated with IFNγ followed by IL-4 (Fig. 7A). Importantly, there was no significant difference in iNOS expression between DCs given IFNγ + TNFα and subsequently IFNγ and DCs given IFNγ + TNFα and subsequently IL-4 (Fig. 7A), suggesting that TNFa stabilized DC1 phenotype in DC, making them less susceptible to the DC2 switch caused by IL-4. When DCs were initially stimulated with IFNγ and it was maintained, they showed baseline expression of DC2 marker, Fizz, which became profoundly upregulated when these DCs were stimulated with IL-4 (Fig. 7B). However, when DCs were co-stimulated with IFNγ + TNFα and subsequently stimulated with IL-4, they could no longer up-regulate Fizz (Fig. 7B). These data demonstrated that DCs exhibit DC1 to DC2 plasticity in the absence of TNFα, but became stable DC1 following the initial pulse with TNFα.

Figure 7

Early TNFα Stabilizes Dendritic Cell DC1 Gene Expression Despite Late IL4 Exposure

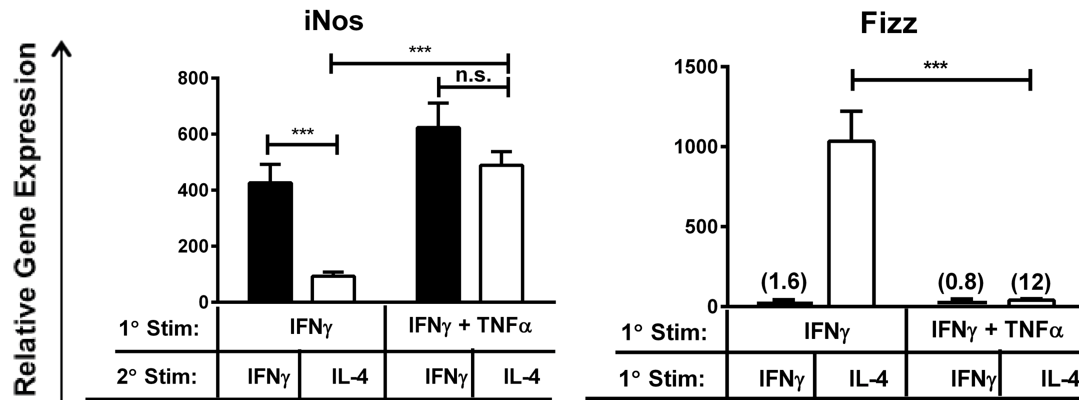

Figure 7: The effect of TNFα on DC gene expression. DCs were derived from mice bone marrow as described in "Methods." BMDCs were cultured and exposed to 24-hour stimulation of IFNγ ± TNFα before 24 hours of IL-4 stimulation. BMDCs were then harvested for RNA, which was converted to cDNA and evaluated for gene expression through qPCR. Bars represent mean gene expression ±SEM. Replacement of IFNγ with IL4 resulted in significant suppression of DC1 gene iNOS up-regulation, with consecutive up-regulation of DC2 gene Fizz, indicating that the "naïve DC" can readily switch from DC1 to DC2 following the change in cytokine environment. Priming of DC with TNFα entirely removed the effect of IFNγ replacement by IL4 on iNOS expression and severely blunted up-regulation of Fizz. Data was pooled from 3 separate matched experiments, N=18 for each of the analyzed parameters; * $p < 0.05$ in comparison between IFNγ+TNFα and IFNγ.

Initial TNFα Stimulation Fails to Stabilize Dendritic Cell DC2 Phenotype Following Subsequent IFNγ Exposure

Because TNFα strongly stabilized the DC1 phenotype in BMDC we sought to determine if TNFα would also induce DC2 phenotype stabilization. To test this BMDC were stimulated with IL-4 ± TNFα followed by 24 hours of either IFNγ or IL-4 stimulation. DC1/DC2 phenotypes were analyzed as above. DC stimulated and maintained in IL4 did not express iNOS, but when switched to IFNγ they rapidly upregulated iNOS, which indicated DC1 phenotype (Fig. 8A). The addition of TNFα did not prevent up-regulation of iNOS following addition of IFNγ; DCs given IL-4+ TNFα and subsequently IFNγ robustly expressed iNOS (Fig. 8A). DCs maintained in IL-4 showed robust expression of the DC2 marker Fizz and addition of IFNγ showed a strong decreasing trend in expression of this gene (Fig. 8B). Addition of TNFα did not significantly alter Fizz expression in IL-4 maintained condition, and subsequent IFNγ stimulation dramatically down-regulated Fizz expression (Fig. 8B). Thus in contrast with strong DC1 phenotype stabilization, TNFα failed to induce to stabilize DC2 phenotype in pre-polarized DC2 as they remained susceptible to DC1 switch.

Figure 8

Initial TNFα Stimulation Fails to Stabilize Dendritic Cell DC2 Phenotype Following Subsequent IFNγ Exposure

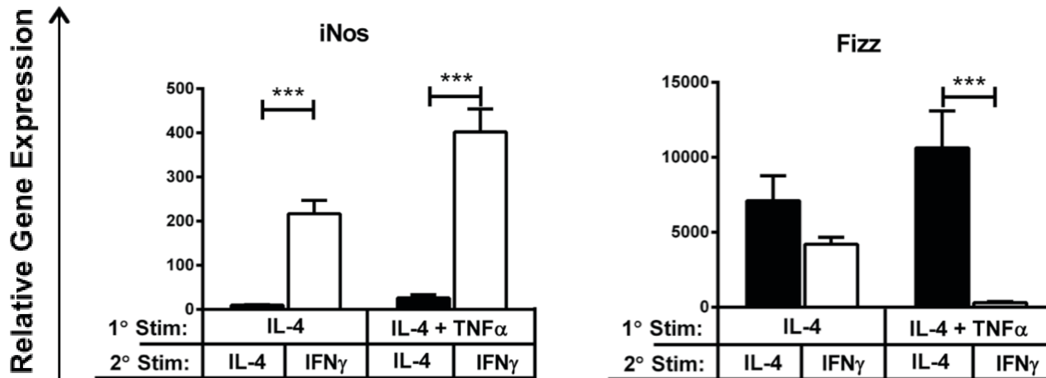

Figure 8: The effect of TNFα on DC gene expression. DCs were isolated from mice bone marrow. BMDCs were cultured and exposed to 24-hour stimulation of IL-4 ± TNFα before 24 hours of either IL-4 or IFNγ stimulation. BMDCs were then harvested for RNA, which was converted to cDNA and evaluated for gene expression through qPCR. Replacement of IL-4 with IFNγ resulted in significant up-regulation of the DC1 gene iNOS, with consecutive down-regulation of the DC2 gene Fizz, indicating that the "naïve DC" can readily switch from DC1 to DC2 following the change in cytokine environment. Priming of DC with TNFα made no significant difference to DC polarization plasticity. Bars represent mean gene expression ±SEM. Expression of both iNOS and Arginase were similar regardless of TNFα stimulation. Data was pooled from 3 separate matched experiments, N=18 for each of the analyzed parameters; * $p < 0.05$ in comparison between IL-4 and IL-4+TNFα.

Discussion

This study examined the role of early TNFα signaling (at the onset of infection with *C. neoformans*) in modulation of the development of protective immunity to this fungal pathogen in a mouse model, using transient depletion of TNFα with a neutralizing antibody. Our study shows that transient TNFα depletion resulted in: 1) impaired fungal clearance during the adaptive but not innate phases of the immune response; 2) diminished accumulation of leukocytes including macrophages and lymphocytes in the lungs; 3) an altered DC activation profile, specifically skewing from DC1 to DC2 activation profile; 5) an altered T cell activation profile mixed Th1/Th2 with variable activation at different time points. Our study further established that TNFα's effect as a DC1- (but not DC2-) –phenotype stabilizer is the likely mechanism by which TNFα promotes protective Th1 response.

Our data show that early TNFα depletion results in a stark impact on pulmonary clearance of *C. neoformans* during the adaptive phase of the immune response. Specifically, cryptococcal clearance that occurs following the development of adaptive immunity (after 1 wpi) is disrupted in animals in which TNFα was transiently depleted by a single dose of anti-TNFa antibody at the onset of infection. This finding is consistent with what has been shown before (32, 83, 87, 90). The fungal burden in αTNFα-CBA/52D relative to CBA/52D was significantly elevated at 2 and especially 4 wpi, indicating that transient, early depletion of TNFα induced a long-term downstream effect on the ability of the immune system to clear *C. neoformans* infection.

Our study also confirms the previously reported deficiencies in total pulmonary leukocyte population and specific leukocyte subsets including lymphocytes and macrophages. Leukocytes

play an essential role in the cell-mediated immune response to invading pathogens including viruses, mycobacteria and fungi in the pulmonary environment (76). In the case of *C. neoformans*, the kinetics and magnitude of recruitment of various leukocyte subsets critically affects pulmonary clearance (reviewed in (77, 78, 82, 92, 93)). Our study showed the onset cryptococcal clearance corresponded with peak inflammatory response in the lungs of CBA/52D mice and that the 50% reduction of pulmonary leukocyte recruitment in αTNFα-CBA/52D mice corresponded with the absence of clearance at that time (Figure 2). Lymphocyte and macrophage accumulation is especially crucial for clearance of *C. neoformans* in the infected host (94). Analysis of leukocyte subsets identified both defects in lymphocyte and macrophage recruitment in anti-TNFα treated mice, providing additional evidence that the adaptive immune response was profoundly distorted by the absence of early TNFa signaling. Thus early TNFa signaling is required for subsequent generation of optimal and sufficiently robust immune response that is required for the progressive clearance of pathogen from the infected lungs.

Among lymphocyte subsets, T cells have been shown to be vital to clearance in cryptococcosis and recruitment of the appropriate subsets of leukocytes into the lungs (81, 74). While we observed a transient decrease in $CD4^+$ T cells in αTNFα-CBA/52D (only at 2 wpi), we also observed an increase in $CD8^+$ T cells at this time point (Figures 4D, 4E). Since $CD8^+$ T cells have been reported to be an important contributor to anticryptococcal cellular immunity and can compensate for absence of $CD4^+$ (96), we believe that the transient decrease in $CD4^+$ T cell recruitment was not the predominant mechanism that led to long-term defect in fungal clearance in αTNFα-CBA/52D mice. However, the successful clearance of *C. neoformans* relies not only on the presence of T cells but also on their proper (Th1-type) polarization (74, 82). Our findings support that early TNFα-induced signals have a profound and lasting effect on cytokine

production by T cells and are required for optimal Th1 response. Flow cytometry demonstrated that the detrimental Th2 cytokine IL-10 is up-regulated in both $CD4^+$ and $CD8^+$ T cells in αTNFα-CBA/52D mice at 2 wpi (Figure 4A,C), while clearance-promoting IFNγ is down-regulated in $CD4^+$ T cells at 2 and 4 wpi. Consistently, mRNA expression data in $CD4^+$ T cells corroborated with these results, showing clear down-regulation of IFNγ at 4 wpi. (Figure 5B). Early TNFa signaling also affected early but not late IL17a expression and strongly suppressed expression of key factors associated with a non-protective response, GATA4, IL-4, and Foxp3, which were all up-regulated in TNFα depleted mice at 2 wpi (Figure 5D, E, F). Thus TNFα is clearly an important regulator of T cell cytokine production that overall promotes a Th1 bias of T cell polarization.

DCs are the primary phagocytic cells that present antigen to T cells during cryptococcal infection and are a major innate determinant of the Th1/Th2 polarization phenotype that will develop during the transition between innate and the adaptive immune response (84, 85, 94). The effects of DC on T cell polarization occurs through the types of cytokines DC express, as well as through expression of various co-stimulatory molecules which act on T cells during direct DC/T cell contact (80, 89, 94, 95). TNFα is known to be an important factor for DC activation and maturation. Thus, we further tested if changes in dendritic cell phenotype occurred upstream from the dysregulated T cell polarization in TNFα depleted mice. DC activation status was assessed to be dramatically changed by TNFα depletion, as demonstrated by mRNA expression data. Decreased expression of mature DC1 genes, such as Ccr7, IL-12b, and H2-DMb1, and IL12b and increased expression of DC2 genes by pulmonary $CD11c^+$ cells, such as Arg1 and IL10, occurred in αTNFα-CBA/52D mice (Figure 6). TNFα signaling causes DC1 bias at every time point. Thus it is clear TNFα plays a crucial role in promoting stable DC1 phenotype in the

infected lungs during both the innate and adaptive phases of infection. Because the effects of transient TNFα depletion on T cell signatures and gene expression were not identical across all the time points, perhaps due to the highly dysregulated response, it is likely that early DC1 bias promotes T cell Th1 polarization during the later phases of infection.

To determine if TNFα directly affects DC1 activation status, DC1 stability experiments were performed. Initially DC1 activated Bone Marrow-derived DCs maintained high expression of iNOS, a DC1 gene, when exposed to TNFα, regardless of what cytokine they were subsequently exposed to (Figure 7A). Similarly, BMDCs maintained low Fizz expression when initially DC1 activated and exposed to TNFα, regardless of what cytokine they were subsequently exposed to (Figure 7B). This is in contrast to initially DC1 BMDCs not exposed to TNFα, which displayed polarization plasticity. So, the early effects of TNFα were to stabilize DCs in a protective activation status. The DC-1 activated DCs most likely promoted long-term protective effects on T cell polarization in *C. neoformans* infected lungs. This model could account for the long-term effects caused by initial, transient depletion of TNFα, and has been postulated before (91).

To determine if TNFα directly affects DC2 activation status, DC2 stability experiments were performed. Initially DC2 activated Bone Marrow-derived DCs differentially expressed iNOS, a DC1 gene, when subsequently exposed to a different cytokines, regardless of if they were exposed to TNFα (Figure 8A). Similarly, initially DC2 BMDCs differentially expressed Fizz, a DC2 gene, when subsequently exposed to a different cytokines, regardless of if they were exposed to TNFα (Figure 8B). So, TNFα does not stabilize BMDCs in a non-protective activation status. They remain susceptible to a DC1 switch. Thus, because TNFα only maintains a DC1 bias it is most likely only instrumental in promoting a Th1 T cell polarization in a *C.*

neoformans infection.

Together these data revealed that early TNFα is critical for generation of a protective immune response to *C. neoformans* infection. We propose that TNFα induces stable DC1 polarization in the infected lungs, which supports sustained Th1 T cell polarization and prevents non-protective Th2 bias, paving the way for the effective clearance of the infection. Together, our T cell and DC analysis suggest that the crucial interaction between DCs and T cells is impaired in αTNFα-CBA/52D mice.

Understanding more completely the mechanisms behind these effects could create a new opportunity for immunotherapy intended to enhance the immune response during cryptococcal infection. Our study shows that TNFα could be potentially beneficial to improve clearance of *C. neoformans* in infected patients.

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