The Role of CC Chemokine Receptor 7 During Pulmonary Invasive Aspergillosis

by

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Abstract

Invasive aspergillosis (IA) is a serious, life-threatening disease caused by the mold *Aspergillus fumigatus* (*A. fumigatus*). IA primarily affects immunocompromised individuals, and is especially common among hematopoietic stem cell transplantation (HSCT) patients. Immune strategies directed at promoting antifungal responses following HSCT hold promise. In particular, alteration of chemokines and their receptors have been shown to markedly modulate the immune response against *A. fumigatus.* Examples include chemokine receptor 1 (CCR1), CCR2, CCR4, CCR6, CXCR2, and their associated chemokine ligands. However, more recently it was shown that *Aspergillus* infection upregulates CC chemokine receptor 7 (CCR7) expression on dendritic cells, a major effector cell mediating antifungal immune responses in the lung. CCR7 via activation by chemokine ligand 19 (CCL19) and chemokine ligand 21 (CCL21) is a key trafficking receptor found on naïve lymphocytes and mature dendritic cells. In addition, CCR7 gene transcripts are found in hematopoietic stem cells and myeloid progenitor cells. Given the role of CCR7 for immune cell trafficking, we hypothesized that CCR7 would be an important chemokine receptor during IA. To test our hypothesis, we utilized two distinct murine models of immunosuppression. In our first model, we describe a potentially deleterious role for CCR7 during primary immune responses directed against *A. fumigatus* following antibody-induced neutropenia. CCR7-deficient chimeric mice displayed significantly reduced mortality, which correlated with

rapid fungal clearance and increased effector responses by myeloid dendritic cells compared to wild-type chimeras. In our second model, we demonstrate a novel inhibitory role for CCR7 on hematopoietic stem cell and myeloid progenitor cell proliferation following HSCT. Mice reconstituted with CCR7-deficient hematopoietic stem cells and myeloid progenitors had greater numbers of immune cells in the lung 14 days after transplantation, compared with mice receiving wild-type hematopoietic stem cells and myeloid progenitors, and this resulted in accelerated fungal clearance in *A. fumigatus* challenged mice. Collectively, our results demonstrate a detrimental role for CCR7 during primary immune responses against *A. fumigatus* and following HSCT, and suggest CCR7 as a new target for the treatment and prevention of IA.

Chapter 1

Introduction

Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is used to treat and cure a variety of hematologic and nonhematologic diseases, both malignant and nonmalignant. The first successful HSCT was performed between identical twins in 1956, by E. Donnall Thomas, for the treatment of leukemia (1). Since 1956, the number of patients undergoing HSCT has risen worldwide to over 50,000 individuals a year (2). In 2005, it was reported that 37% of all transplants were from allogeneic donors and 63% were of autologous origin, including identical twins (3). HSCT, both allogeneic and autologous, is currently being used to treat a significant number of diseases, most commonly hematologic malignancies, including acute myeloid leukemia, acute lymphoblastic leukemia, chronic myeloid leukemia and other myeloproliferative disorders, myelodysplastic syndromes, chronic lymphocytic leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, myeloma, AL amyloidosis, and severe aplastic anemia (4). HSCT has also been used for the treatment of solid tumors, including breast, renal, and colon cancer (3, 5-8). Recently, interest has been generated in the use of HSCT for the treatment of severe autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease (9-18). There has also been recent clinical data to suggest that, in certain cases, HSCT could be used as a treatment for HIV (19, 20).

Regardless of the underlying disease, it is clear that over the last 50 years HSCT has become an effective clinical therapy for many devastating diseases.

Though HSCT appears to have an enormous clinical benefit, spanning a wide range of diseases, there are several drawbacks to the therapy. The most prominent complication is the development of graft-versus-host disease (GVHD). During an allogeneic HSCT, GVHD develops when donor T cells recognize the host's major histocompatibility complex (MHC) molecules as foreign. The resulting donor T cell activation causes massive cytokine production, leading to the activation of additional innate and adaptive immune responses, all of which results in severe tissue and organ damage (21). Current clinical guidelines stipulate that stem cell donor and recipient match HLA-A, HLA-B, and HLA-DR genes, with a preference to also match HLA-C genes (22). Unfortunately, even with careful genotyping of donor and recipient, GVHD continues to be the most common cause of mortality associated with HSCT (23, 24). Treatment for GVHD exists, and generally involves suppression of the immune system with the use of steroids, in addition to other immunosuppressants such as tacrolimus and cyclosporine (25). Despite the complications of GVHD, individuals undergoing HSCT for solid tumors experience a benefit of GVHD, as donor T cells can attack the malignant tissue, leading to a decrease in tumor relapse (26).

Another drawback to HSCT, whether autologous or allogeneic, is the requirement for the host hematopoietic system to be partially or completely ablated prior to transplantation. The time between the elimination of the host immune system, and engraftment and reconstitution from the donor hematopoietic system, is a period when the patient is severely immunocompromised. The combination of the myeloablation

conditioning regiment and any use of immunosuppressants leaves the patient highly susceptible to opportunistic infections. Predominate viral infections following HSCT include cytomegalovirus, Epstein-Barr virus, pneumocystosis, respiratory syncytial virus, and reactivation of varicella zoster virus (27-32). An exceptionally wide array of bacteria also pose a significant risk for HSCT patients, with gram-positive infections occurring more commonly early after transplant and gram-negative infections occurring later (33). Fungal infections, particularly *Candida* and *Aspergillus* species, also play a significant role in post-transplant-related infection (32, 34). A recent study found that 44% of patients undergoing allogeneic transplantation were colonized with at least one species of *Candida*, with *Candida albicans* being the most common isolate (35). Though *Candida* remains a serious cause of morbidity and mortality in HSCT patients, it is treated prophylactically, since *Candida* is endogenous, and this greatly reduces serious complications from the yeast (32, 35). Unlike *Candida*, *Aspergillus* is an exogenous mold that is not treated prior to suspected infection. Consequently, the incidence of invasive aspergillosis (IA), an acute, life-threatening disease, ranges from 5%-10% in allogeneic stem cell recipients, and related mortality is as high as 80%. Recent experimental data has shown that an infusion of hematopoietic progenitor cells during HSCT provides significant protection against opportunistic infections, especially IA (36-39).

All cells in the hematopoietic system originate from hematopoietic stem cells (HSCs). HSCs are self-renewing cells that provide lifelong regeneration of all hematopoietic cells. It has been reported that HSCs retain their capacity for self-renewal and hematopoietic reconstitution even after repeated stem cell transplantations (40). In

the process of becoming a fully differentiated cell, HSCs must first differentiate into precursor cells committed to either a myeloid cell lineage or a lymphoid cell lineage. All myeloid cells originate from a common precursor cell known as the common myeloid progenitor (CMP) (41). CMPs give rise to granulocyte monocyte progenitors (GMPs) and megakaryocyte erythrocyte progenitors (MEPs). GMPs ultimately differentiate into monocytes, macrophages, dendritic cells (DCs), neutrophils, eosinophils, and basophils, while MEPs are responsible for the generation of red blood cells and platelets (**Figure 1.1**). Although exogenously added CMPs and GMPs have a limited self-renewal capacity in vivo, they differentiate into functional leukocytes in less than 6 days (7-8 days faster than HSCs) and they are largely absent by four weeks following HSCT (41, 42). The rapid expansion and differentiation of CMPs and GMPs into effector cells has been shown to provide protection against IA in a murine model of HSCT (38). It was shown that 10^4 CMPs and 2.0 x10^4 GMPs provided significant protection against IA as early as 7 days after HSCT. Infusion of CMPs and GMPs during HSCT significantly shortened the period of neutropenia in these mice, leading to accelerated fungal clearance and a decrease in morbidity and mortality.

In conclusion, although there is an enormous risk associated with HSCT, new research and clinical technologies will enhance the usefulness and safety of this therapy, which holds the promise of a cure for a myriad of diseases ranging from autoimmunity to cancer.

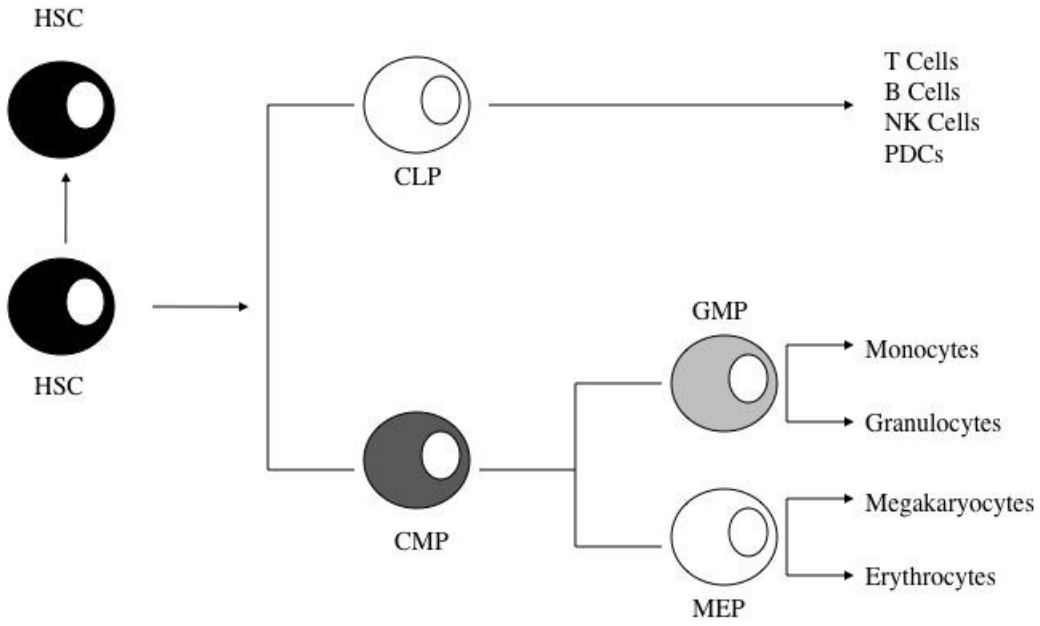

FIGURE 1.1. Hematopoietic lineage tree. HSCs have the ability to self-renew or differentiate into CMPs or CLPs. CMPs reconstitute all myeloid cells through their differentiation into GMPs and MEPs. CLPs further differentiate into additional lymphoid progenitors, including pro-B and pro-T cell progenitors, to reconstitute the lymphoid compartment.

Aspergillus Fumigatus

Aspergillus fumigatus (*A. fumigatus*) is a saprophytic fungus that is found ubiquitously in the environment, leading to the inhalation of hundreds to thousands of conidia (spores) every day (43). Normally innocuous, *A. fumigatus* is known to cause a wide range of diseases, from allergic airway disease to invasive aspergillosis, which is a life-threatening infection mostly observed in immunocompromised patients. Interestingly, *A. fumigatus* comprises only a small portion of airborne fungus, but is responsible for the majority of clinical fungal complications (44). For example, in a 1990 study of hospital air, it was found that *A. fumigatus* made up 0.3% of all fungal isolates in the air, but was discovered in 44% of patients (45). These observations identify *A. fumigatus* as a significant pathogen and opportunistic mold that is in need of further clinical and basic research.

Reproduction and Growth

A. fumigatus is normally found in the soil and plays a vital role in recycling carbon and nitrogen from decaying matter (46). Of the over 250 different species of *Aspergillus*, the vast majority are known to reproduce asexually (47). *A. fumigatus* is a haploid organism that begins its life cycle as a spore (known as conidia), which germinates to form hyphae. The hyphae elongate and branch, resulting in the formation of a conidiophore. The conidiophore contains a single layer of cells, known as phialide, from which individual conidia bud following contraction of the phialide (48, 49). Thousands of conidia, 2-3mm in diameter, are released from every conidiophore during

sporulation, and this is the form of fungus inhaled by individuals daily. Recent evidence has suggested that *A. fumigatus* has the ability for sexual reproduction, although asexual means appear to be the main form of reproduction (47, 50). In 2008, O'Gorman et el. published a report showing that *A. fumigatus* has a functioning sexual reproductive cycle that includes the production of ascomata and ascospores (51). In addition to the recent discovery of a sexual cycle, the entire genome of *A. fumigatus* has been decoded (52). The genome includes eight chromosomes containing 9,926 genes, 700 of which appear to be unique to *A. fumigatus*. The discovery of a sexual cycle and the completion of the *A. fumigatus* genome should increase our basic understanding of the fungus and its associated pathogenicity.

Transition of *A. fumigatus* conidia into its invasive hyphal form is regulated by environmental factors such as temperature, stress, and nutrient availability (44). *A. fumigatus* is a relatively thermotolerant fungus, adding to it's pathogenic potential. Conidial growth can occur from below 22^0C to 55^0C, while conidia can survive temperatures as high as 70^0C (48, 53). Though the mechanism of thermotolerance has not been discovered, studies have shown that mutating the *crgA* gene and deleting the *thtA* gene affect fungal virulence and growth, respectively, in a temperature-dependent manner (54, 55). Environmental stress also contributes to *A. fumigatus* growth. A recent study highlighted the importance of calcineurin, which controls several stress responses in eukaryotic cells, in *A. fumigatus* growth. Here, *A. fumigatus* lacking the calcineurin catalytic subunit was found to be hypovirulent and had delayed hyphal growth (56). Also contributing to *A. fumigatus* growth is nutrient uptake and availability. One group found that as nitrogen levels decreased in the environment, the expression of a nitrogen-

regulated gene, *rhbA,* was dramatically increased (57). The importance of this gene was demonstrated in a model of invasive aspergillosis, where *A. fumigatus* lacking a functional *rhbA* gene showed decreased fungal virulence (58). Further understanding of the growth requirements of *A. fumigatus* may provide new insights into fungal survival and virulence, thus offering new treatment strategies for *A. fumigatus*-related diseases.

Physical Characteristics

A. fumigatus is a relatively complex organism, with mechanisms that allow for conidial dispersion, growth and reproduction, and immune cell evasion. One of the predominate features of the conidial cell surface is the presence of rodlets (44). Rodlets are hydrophobic by nature, and their removal hampers the dispersion of conidia (59, 60). There are two major rodlets on the outer cell wall of conidia: RodA and RodB (61). Unlike the disruption of the *RodB* gene, mutation of the *RodA* gene makes conidia more hydrophilic. This enhances macrophage phagocytosis of conidia, indicating that rodlets are important for both dispersion and survival (61). Interestingly, it has recently been shown that during germination, the rodlet layer is disrupted, revealing a hydrophilic surface that allows for hyphal growth (62). The cell wall of *A. fumigatus* conidia is made up of polysaccharides, glycosylphosphatidylinositol (GPI)-linked proteins, melanin, and chitin (63-65). Polysaccharides on the cell wall include a-(1,3) glucan, b-(1,3), (1,4), and (1,6) glucan, and galactomannan, and are useful in the diagnosis of *A. fumigatus*-associated diseases. Two genes synthesize alpha-glucan, AGS1 and AGS2. When these genes were mutated, hyphal formation was altered and there was a decrease in conidia production; however, these alterations did not affect the pathogenic potential of *A.*

fumigatus (66). Melanin is a very important feature of the conidial cell wall, helping to protect many fungi against ultraviolet radiation. *A. fumigatus* appears a dark green due to the production of 1,8-dihydroxynaphthalene-melanin, which is able to scavenge reactive oxygen intermediates and thus reduce fungal phagocytosis by immune cells (67). Polyketide synthase activity is responsible for the generation of melanin, and mutations in the gene result not only in white conidia, but enhanced immune activation against the transformed conidia (68). Chitin production is also important following the germination of conidia into hyphal growth. Several studies have shown that deletion of chitin synthase or the use of nikkomycin Z, a chitin synthase inhibitor, prevents the appropriate development of hyphae (69, 70). Together, these various physical characteristics of *A. fumigatus* provide the mold with tools to enter favorable environments and enhance its growth and survival.

Associated Diseases

Over the past few decades, *A. fumigatus* has become an increasingly problematic pathogen. It is responsible for a wide range of diseases that can be categorized as allergic, saprophytic, or invasive. Allergic diseases include *Aspergillus* sinusitis, bronchopulmonary aspergillosis (ABPA), and hypersensitivity pneumonias (71). ABPA is of particular clinical interest as it features type I, II, and IV hypersensitivity reactions, and because it predominately affects asthmatic and cystic fibrosis patients, occurring in 2% and 35% of patients, respectively (72, 73). Saprophytic aspergillosis, also known as aspergilloma or "fungal ball", is typically seen in patients with a pre-existing lung condition, such as sarcoidosis and tuberculosis, which leaves space for *A. fumigatus* to

grow (53, 74, 75). Aspergilloma is characterized by the presence of a spherical mass, consisting of hyphae surrounded by reproductive fungal units. Aspergilloma can ultimately lead to hemoptysis as blood vessels surrounding the fungal ball are invaded and disrupted, leading to both blood in the mucus and internal bleeding (76, 77). The diseases that comprise invasive aspergillosis, discussed below, include airway invasive aspergillosis, chronic necrotizing apergillosis, and invasive aspergillosis (71). Given the wide range of diseases that are caused by *A. fumigatus* and the variety of susceptible patients, it is clear that new therapies and treatments directed against *A. fumigatus* are required.

Invasive Aspergillosis

Invasive aspergillosis (IA) is a serious, life-threatening disease that primarily affects immunocompromised individuals, including solid organ transplant recipients, leukemia and bone marrow failure patients, AIDS patients, and recipients of HSCT (78) (**Figure 1.2**). The majority of IA cases are a result of *A. fumigatus*, with the exception of IA in patients with chronic granulomatous disease (CGD), who appear to be more susceptible to *Aspergillus nidulans* (44, 79). Individuals suffering from CGD lack functional NADPH oxidase and are unable to mount an appropriate innate immune response to many pathogens; these patients are thus highly susceptible to the morbidity and mortality associated with IA (80). The major risk factors for developing IA generally include pre-existing conditions, such as fungal infection in lung transplant recipients (74, 75), and duration and dose of immunosuppression, such as chemotherapy regimens in leukemia patients (81).

TABLE 1.1. Predisposing factors for patients populations at risk for developing invasive aspergillosis

Patient Population	**Risk Factors**
Solid organ transplant recipients	Immunosuppression to prevent graft rejection and preexisting fungal infection (lung transplant)
Leukemia and bone marrow Failure patients	Chemotherapy and/or radiotherapy induced immunosuppression
AIDS patients	Diminished CD4+ T cell numbers, neutropenia in 50% of patients
Hematopoietic stem cell transplant recipients (primarily allogeneic)	1) Immunosuppression from myeloablative conditioning regimen prior to HSCT 2) Acute GVHD 3) Chronic GVHD
Chronic granulomatous disease patients	Non-functional NADPH oxidase

Though the above-mentioned patient groups are at risk, HSCT recipients represent the most susceptible group to developing IA; indeed, as many as 10% of individuals undergoing allogeneic HSCT develop IA (82). The primary reason for the increased susceptibility in HSCT recipients is due to the three stages of immunosuppression during and following an allogeneic transplant. The first stage of immunosuppression occurs during the myeloablative conditioning regimen prior to transplantation (83). Typical ablation is achieved using high levels of chemotherapy, which has replaced traditional full body irradiation. This is a period of profound neutropenia, making the host highly susceptible to IA. The second period of immunosuppression occurs during acute graft-versus-host disease (GVHD) (84, 85). Even if GVHD does not develop, the condition is prophylactically treated using immunosuppressants, again making the patient susceptible to infection. The third stage of immunsuppression occurs during chronic GVHD, which can last more than 100 days post-HSCT. Not surprisingly, the more severe the GVHD is, the more susceptible the patient is to developing IA, likely as a result of the enhanced immunosuppressive therapy that is required for control of the graft-versus-host reaction (86). A recent clinical report further highlighted the impact of GVHD on IA in a large, multicenter investigation (87). It was found that individuals with no risk of GVHD, i.e., autologous transplant recipients, had only a 0.5% chance of developing IA. In patients with a low risk of developing GVHD, i.e., those with partial HLA gene matching, such as between a related donor and recipient, the incidence for developing IA was 2.3%. In patients who received HLA mismatched HSCT, and thus at high risk for development of GVHD, the occurrence of IA was up to 4% following transplantation. Similarly, mortality associated with IA was

approximately 30% lower in patients undergoing an autologous HSCT when compared to allogeneic transplant recipients.

There are several risk factors associated with the development of IA following HSCT (86). These include patients that are older, those with an underlying hematologic malignancy, such as aplastic anemia, and patients who have received a cord blood or T cell-depleted transplantation. Viral infections can also play a significant role in susceptibility to IA, such as transplant recipients with a concurrent cytomegalovirus infection, or who contract a respiratory viral infection. As might be expected, patients receiving escalating doses of corticosteroids are at an increased risk for developing IA. The risk is 8-fold higher when the dose of steroids is 2-3 mg/kg/day, and escalates to a 15-fold higher risk in those receiving more than 3mg/kg/day (86). Finally, it has been documented that certain genetic polymorphisms of the IL-10 promoter actually decrease susceptibility to IA, regardless of other risk factors, such as GVHD (88).

Diagnosis of IA in HSCT patients is often a very complicated and difficult task, as symptoms are not always suggestive and current diagnostic tools do not unequivocally predict many cases of IA. In fact, a recent study found that only 40% of IA cases were diagnosed prior to death, leaving 60% of individuals' cause of death unknown or misdiagnosed (89). Symptoms of IA are very general and include chest pain, dyspnea, fever, cough, and malaise (90). The absence of clear symptoms for this disease has led to the clinical use terms such as "highly probable", "probable", "possible", and "suspected" to indicate the certainty of IA. Patients with a suspected case of IA frequently undergo a CT scan, which may show the common "halo" image caused by hemorrhagic necrosis around a fungal lesion (53, 91, 92). The halo sign is of significant clinical importance

because it is seen in more than 80% of HSCT patients who develop IA, but unfortunately, other fungal and bacterial pathogens can have similar CT images (92). Bronchoalveolar lavage (BAL) is another technique that is useful in the diagnosis of IA. The BAL fluid is examined microscopically and can be cultured for the presence of *A. fumigatus*, though it is only accurate in 50% of cases (93, 94). BAL fluid can also be used for a serum galactomannan assay (95), which is a double-sandwich ELISA that has a sensitivity of 71% and a specificity of 89% (96). Problems arise with the glactomannan assay because of false positive tests resulting from antibiotic use, and a decrease in assay sensitivity due to prophylactic anti-fungal use (97, 98). Another diagnostic test used for the detection of IA and other invasive fungal diseases is the (1→3)- β- d- glucan (BG) assay. A recent multicenter study showed the BG assay to have a sensitivity of 69% and a specificity of 89% for detecting invasive fungal diseases, including IA (99). Yet a final technique for the detection of IA is the use of PCR for the detection of fungal genes. Though this technique is still under development, current approaches use primers to amplify the internal transcribed spacer (ITS) regions of *Aspergillus*. Here, serum detection of *A. fumigatus* had a sensitivity and specificity of 91.6% and 94.4% respectively (100). Though IA is difficult to detect, using a combination of tools is extremely useful and informative for the diagnosis of this disease.

Treatment of IA is complex, is associated with high cytotoxicity, and is rarely successful. Though antifungal therapy has led to increased rates of survival over the past 10 years, there is still a greater than 60% mortality rate associated with IA following allogeneic HSCT (101, 102). The current standard in antifungal therapy is the use of voriconazole, which has been shown to be more effective in the clinic than amphotericin

B (103). In a large study of patients with suspected IA, 52.8% of patients responded to voriconazole and had a survival rate of 70.8% at 12 weeks, whereas the patient group receiving amphotericin B had a 31.6% response rate and a 12-week survival of 57.9%. Equally as impressive, the voriconazole-treated group had significantly fewer toxicity-related side affects, as demonstrated by a 9-fold reduction in patients suffering from renal dysfunction. Newer antifungal drugs, known as echinocandins, such as Caspofungin, inhibit the synthesis of glucan, thereby inhibiting fungal growth (104). When these first-line drugs are not tolerated or ineffective, salvage therapy is required for any chance of survival. Salvage therapy involves the use of combination therapy, where several different antifungals, with distinct mechanisms of action, may be used (105). However, combination therapy is still an evolving clinical option; with only a small number of retrospective studies, it has yet to become a standard of practice, much less a first-line option. This lack of effective, non-toxic treatment for IA highlights the need for better therapies. The primary reason a patient develops IA is due to a lack of a functional immune system, thus, future treatment must address the cause of the problem, immunosuppression. If therapies can reduce periods of immune deficiency and instead enhance immunity toward *A. fumigatus* the use of antifungal drugs will become the second line of defense against IA.

Immunity to *Aspergillus fumigatus*

A. fumigatus is a complex pathogen, but rarely does it cause problems for immunocompetent individuals. The innate immune response is both adequate and appropriate for the prevention of growth and infection from inhaled conidia. The adaptive immune response is highlighted by a protective T-helper type 1 (Th1) response, leading to the production of associated pro-inflammatory cytokines, which likewise promote fungal clearance.

Innate Immunity

The innate immune response to *A. fumigatus* occurs in three steps: exposure, recognition, and resolution. Exposure is not as simple as the presence of *A. fumigatus*. Different innate immune responses are required depending on the maturation state of *A. fumigatus*, i.e., whether it is resting conidia, germinating conidia, or growing hyphae. Recognition of *A. fumigatus* is dependent on soluble receptors, Toll-Like Receptors (TLR), and other surface receptors such as dectin-1. Resolution of infection occurs by phagocytosis and the production of antimicrobial products.

The first line of defense from inhaled conidia is alveolar macrophages, which are able to phagocytose and kill conidia using reactive oxidant intermediates (106, 107). Conidia phagocytosed by macrophages begin to swell as they germinate. This activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase production in macrophages, likely due to the presence of β-glucan revealed during conidial swelling in the phagosome (106, 108). NADPH converts oxygen into superoxide anion and

downstream reactive oxidant metabolites (106). Downstream reactive oxygen intermediates can result in the production of acids, such as hypochlorous acid, which is able to kill conidia in the phagolysosome following the fusion of the phagosome and endosome (109). Of clinical interest are reports showing that corticosteroids inhibit macrophage killing of conidia, leading to rapid germination and hyphal growth (106, 107).

Neutrophils were once regarded as a second line of defense in the innate immune response to *A. fumigatus*, as they were thought to primarily respond to hyphae, which develop upon conidia germination. In recent years, however, the anti-conidial activity of neutrophils has been well established. In a study using nitrogen mustard-induced neutropenia, it was found that mice were highly susceptible to germinating conidia, but not resting conidia, indicating a preferential ability of macrophages to kill resting conidia and neutrophils to kill germinating and growing *A. fumigatus* (107). Like macrophages, neutrophil detection of *A. fumigatus* activates NADPH oxidase, which is able to release reactive oxygen species to induce conidial damage (110, 111). In addition, more recent bacterial studies have shown that NADPH oxidase activity is coupled with the activation and release of cationic granule proteins, which are ultimately, or in conjunction with reactive oxygen species, responsible for the antimicrobial effect of neutrophils (112). Another enzyme required for proper antifungal responses by neutrophils is myeloperoxidase (MPO). MPO, an oxidant-producing enzyme, produces hydrogen peroxide and hypochlorous acid, which is used in the phagolysosome to kill conidia (113). Interestingly, it has been reported that patients lacking functional NADPH oxidase are highly susceptible to IA, while patients deficient in MPO activity are not (114). This

observation suggests that NADPH oxidase is required for neutrophil antifungal responses, but MPO is not. Additional work with human neutrophils revealed that neutrophils are able to secrete lactoferrin, which binds available iron, thus limiting availability to *A. fumigatus* and inhibiting fungal growth (115). Recently, work by Bonnett et al. showed a new role for neutrophils during initial conidia exposure (116); in this study, neutrophils responded to conidia and formed "oxidative-active" aggregates around the fungus, thereby preventing its germination.

In addition to macrophages and neutrophils, natural killer (NK) cells have also been found to play an important role during *A. fumigatus* infection. Using a mouse model of cyclophosphamide-induced neutropenia, Morrison et al. demonstrated that antibody depletion of NK cells resulted in a two-fold greater mortality and reduced fungal clearance during IA (117). More recently, is has been shown that NK cells mediate their effect by the early production of IFN-γ: depletion of NK cells in a neutropenic mouse model of IA resulted in reduced IFN-γ levels, which in turn diminished the antimicrobial effects of macrophages and decreased pro-inflammatory cytokines (118).

Myeloid dendritic cells (DCs) are an additional population of innate immune cells capable of producing a strong, anti-fungal response. A recent study found that if DC trafficking to the lung is inhibited during IA, neutropenic mice had significantly decreased survival rates and higher fungal burden (119). Additional evidence suggests that DCs are able to clear *A. fumigatus* conidia and hyphal elements, indicating their utility at the onset of, and following, IA (120). Maturation of DCs by conidia and hyphae results in the production of pro-inflammatory cytokines, such as TNF-α, IL-12, IL-6, and

IL-10, as well as upregulates the chemokine receptor CCR7 (121-123). In addition to the innate immune roles of DCs during infection, it has been documented that DCs participate in the adaptive immune response: when conidia-activated DCs were adoptively transferred into mice, they were able activate fungal-reactive, IFN-γ producing (Th1) T cells (120).

Molecular Recognition

Recognition of fungal elements is essential for the innate immune response directed against *A. fumigatus.* Fungal recognition by effector cells leads to phagocytosis, cytokine and chemokine production, and to the release of antimicrobial molecules such as reactive oxygen species and cationic granular proteins (**Table 1.2**). One of the initial receptors for *A. fumigatus* conidia is the soluble receptor, pentraxin-3. Pentraxin-3 is produced by several effector cells including macrophages, endothelial cells and myeloid DCs, and binds galactomannan on the surface of conidia. This serves to enhance phagocytosis by DCs and macrophages (124, 125). The importance of pentraxin-3 was demonstrated using pentraxin-3 knockout mice, which were highly susceptible to IA, even without additional immunosuppression (124). In addition, when pentraxin-3 was administered following murine HSCT, it was found to be as effective or more effective than antifungal therapy against IA (126).

Several pathogen recognition receptors, including Toll-Like Receptors (TLRs), are associated with cellular recognition and activation. TLR2 and TLR4 have both been shown to recognize conidia and hyphal elements (127-129). Expression of TLR2 and TLR4 on human neutrophils, monocytes, and macrophages is linked to an antifungal

response. It has been reported by Bellocchio et al. that cyclophosphamide immunosuppressed mice lacking functional TLR4 are significantly more susceptible to mortality following a conidial challenge than wild-type mice (130). Interestingly, mice lacking the common TLR adaptor protein, MyD88, also show reduced survival following conidia challenge, but TLR2 deficient mice are not more susceptible than wild-type mice, indicating that TLR2 may provide a protective effect during conidial germination or later. Additional work with TLR2-deficient mice has shown that these mice secrete significantly less TNF-α, IL-12, and macrophage inhibitory protein-2 alpha, which are potent antifungal cytokines, during conidial challenge (131). Contrary to the report by Bellocchio, this study found that TLR2 knockout mice had decreased survival during conidial challenge, again suggesting that TLR2 differentially recognizes resting and swollen conidia. Though the pathogen-associated molecular pattern required for TLR activation to *A. fumigatus* has not been fully elucidated, it is known that TLR2 recognizes phospholipomannan while TLR4 binds to O-linked mannosyl residues, which are constituents of different fungal cells (132, 133).

TLR-independent recognition of *A. fumigatus* is mediated through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) and dectin-1 (134-137). DC-SIGN is a type II C-type lectin found on macrophages and myeloid DCs. This receptor has been shown to bind conidia and likely leads to its internalization, as surface levels of DC-SIGN increase once conidia is recognized and phagocytosed (137). Dectin-1 is known to bind β-(1,3) glucan, which is present on the surface of germinating conidia and hyphae, but not on resting conidia (135, 138). Activation of dectin-1 leads to the production of pro-inflammatory cytokines, chemokines, and reactive oxygen

intermediates (135, 139). The importance of dectin-1 was highlighted in a recent study, which demonstrated that dectin-1-deficient mice had significantly higher mortality and substantially less pro-inflammatory cytokine production during a conidial challenge than wild-type mice (136). This study was particularly interesting because no immunosuppression was needed to make conidia lethal to dectin-1 deficient mice.

TABLE 1.2. Innate Immune Recognition of *A. fumigatus*

Pathogen Recognition Receptor	**Pathogen associated molecular pattern**	**Effector Cell Expression**
Pentraxin-3	Galactomannan	Macrophages, endothelial cells, DCs
TLR2	phospholipomannan	Neutrophils, monocytes, macrophages, DCs
TLR4	O-linked mannosyl residues	Neutrophils, monocytes, macrophages, DCs
DC-SIGN	fucosylated glycans and subsets of oligomannose- and complex-type N-glycans	Macrophages and DCs
Dectin-1	β-(1,3) glucan	Neutrophils, monocytes, macrophages, DCs

Role of Cytokines, Chemokines, and Chemokine Receptors

Cytokine and chemokine expression following *A. fumigatus* recognition is essential to the host immune response. Pro-inflammatory, or Th1-type cytokines, are required for an appropriate anti-fungal immune response, while anti-inflammatory and Th2 cytokines are associated with diminished fungal clearance and high rates of mortality. Key effector cytokines include, but are not limited to, TNF-α, IFN-γ, IL-12, IL-18, IL-17 and IL-6 (121, 140, 141). TNF-α, secreted by macrophages, monocytes, and DCs, has been shown to be critical during antifungal immune responses (142-144). When TNF-α was neutralized in a murine model of IA, there was a significant increase in mortality associated with increased fungal burden when compared to control mice (142). TNF-α appears to have two functions during fungal infections: first, the cytokine is involved in the recruitment of mononuclear and polymorphonuclear cells to the site of infection (142). Second, TNF-α enhances neutrophil and macrophage activation against *A. fumigatus* conidia by enhancing the production of superoxide anion by neutrophils, and increasing the ability of alveolar macrophages to phagocytose conidia. (143). A major complication of TNF-α occurs because it is produced at very high levels during GVHD, thus, a common therapy for prevention and maintenance of GVHD is the use of anti-TNF antibodies, such as infliximab (21). Unfortunately, anti-TNF antibodies have been associated with a significant number of invasive pulmonary aspergillosis cases (145).

Several other cytokines have been shown to influence the immune response to *A. fumigatus*. The importance of IFN-γ was demonstrated clinically when a patient with chronic granulomatous disease was administered recombinant human IFN- γ, which

vastly improved her symptoms (146). Additional data in a murine model of IA showed that the administration of IFN-γ was highly protective and significantly reduced mortality (147). INF-γ is also important in skewing effector CD4 T cells toward a pro-inflammatory, Th1-type cell and preventing the generation of Th2 cells. In a study examining the role of IL-4 during IA, it was found that IL-4 deficient mice were much less susceptible to fungal infection than wild-type mice (148). In agreement with these findings, IFN-γ knockout mice had a substantially reduced ability to clear *A. fumigatus*, as they initiated a detrimental Th2 type response. Recently, interest has been generated in the role of IL-17 during IA. IL-17 has been shown to regulate neutrophil recruitment to sites of inflammation, as well as to modulate granulopoiesis (149). Interestingly, it was found that the IL-23/IL-17 pathway was a negative regulator of Th1-associated anti-fungal immunity (150). Accordingly, mice lacking the ability to produce IL-23 had lower levels of mortality, fungal burden, and higher conidial killing by neutrophils, suggesting that IL-23/IL-17 play a pathogenic role during fungal infection. Two additional cytokines of importance during *A. fumigatus* infection are IL-12 and IL-10. IL-12 deficient mice are highly susceptible to IA, while IL-10 knockout mice are highly resistant to IA, further highlighting the importance of Th1-associated pro-inflammatory cytokine production during IA (148, 151).

In addition to cytokines, chemokines and their associated receptors play a critical role in host defense during IA. Chemokines regulate the influx of innate immune cells, such as neutrophils, monocytes, macrophages, NK cells, and DCs, to sights of infection and inflammation. Chemokines also direct the homeostatic migration of lymphocytes – T and B cells – to secondary lymphoid organs for priming by antigen presenting cells. This

in turn leads to lymphocyte activation, clonal expansion and initiation of adaptive immunity.

Neutrophils express the chemokine receptor CXCR2 and respond to its ligands, CXCL1 and CXCL2, which are produced during infection with *A. fumigatus*. When CXCR2 was depleted using antibody neutralization, it was found that immunocompetent mice were highly susceptible to IA compared with control mice (152). This study further demonstrated that mortality was associated with impaired neutrophil recruitment, though neutralization of CXCR2 may have depleted neutrophils in the mice, making them susceptible to IA because they were neutropenic. Another study showed that CXCL1 overexpression during *A. fumigatus* infection led to enhanced recruitment of neutrophils, macrophages, and monocytes, as well as increased production of Th1-associated cytokines, all of which led to a decrease in mortality and fungal burden (153). These studies confirm the important role of neutrophils and their associated chemokine receptors that permit migration of this immune cell to the lung upon *A. fumigatus* infection.

Other chemokines found to be important during IA are associated with monocyte, macrophage and DC migration, including CCL3 and CCL20, which bind CCR1 and CCR6, respectively. In a model of IA induced in neutropenic mice, it was found that neutralization of CCL3 reduced the recruitment of monocytes and macrophages to the lung, leading to a six-fold increase in mortality and a 12-fold increase in fungal burden compared to control mice (154). Another study investigated the role CCR6 on DCs, again using a murine model of IA (119). Here it was found that CCR6-deficient DCs were unable to traffic to sights of fungal infection, leading to high levels of mortality.

Additionally, it was found that if CCL20, the ligand for CCR6, was neutralized, DCs were again unable to migrate to the lung of infected animals, resulting in increased fungal growth. Interestingly, recent clinical studies demonstrated CXCL10 to be an important immune immune mediator during IA (155), which is a chemokine produced by DCs and mediates migration of effector T cells. Here, DCs in patients with a polymorphism in the CXCL10 allele were found to produce significantly less of this protein, which was associated with an increased risk of developing IA.

Though many questions remain regarding the role of NK cells during IA, especially clinical data, it is clear that in neutropenic mice, NK cells have a beneficial antifungal effect. To further highlight the importance of these cells during IA, it was shown that neutralization of the chemokine CCL2 led to high rates of morbidity and mortality in neutropenic mice (117).

Although several chemokines and their receptors play a significant role in antifungal responses to *A. fumigatus*, there have been reports indicating possible detrimental effects of some chemokines. In a murine model of pulmonary IA, it was found that CCR4-deficient mice were highly protected, having increased survival and decreased fungal burden, when compared to wild-type mice (156). In addition, it was found that CCR4 knockout mice had significantly higher levels of the beneficial chemokine, CCL2, and had greater lung recruitment of DCs and macrophages. The importance of many chemokines and chemokine receptors during IA still needs to be determined, but one chemokine receptor of current interest is CC chemokine receptor 7 (CCR7). It was recently reported that CCR7 was upregulated on DCs following infection

with *A. fumigatus* conidia, indicating a potential role for this receptor and its ligands, CCL19 and CCL21, during IA (122, 123).

CCR7, CCL19, and CCL21 Biology

CCR7 was first identified in 1994, in the laboratory of Patrick Gray, and was designated EBI1 (157). Though the ligand for this receptor was still unknown, it was realized that expression was prominent on B and T cell lines. The designation of CCR7 was first coined in 1997 in work done by Osamu Yoshie, where the chemokine ELC (CCL19) was first discovered (158). Since the discovery of CCR7 and its ligands, chemokine ligand 19 (CCL19) and CCL21, significant progress has been made in understanding the role of this receptor and its ligands during both innate and adaptive immune responses.

CCL19 and CCL21 are constitutively expressed and are involved in the homeostatic migration of CCR7-expressing hematopoietic cells to secondary lymphoid organs (159). CCL19 and CCL21 are expressed by fibroblastic reticular cells and by high endothelial venules (HEVs) (160, 161). CCR7 expression has been reported on naïve B cells, naïve T cells, regulatory T cells, central memory T cells, macrophages, and mature DCs, allowing for the trafficking and ultimately, the interaction of DCs with T cells (162-167).

T cell trafficking to the lymph node occurs via HEVs. Here, naïve T cells expressing CCR7 bind CCL19 or CCL21, which leads to CCR7 signaling, resulting in cellular arrest on ICAM1 and ICAM2, which is required for transendothelial migration into the lymph node (168, 169). The importance of CCR7 during T cell trafficking is seen in mice deficient for the receptor. Lymph nodes and Payer's Patches in CCR7 knockout mice are devoid of T cells, a result that is mimicked in paucity of LN T cells

(plt) mice, which lack functional expression of CCL19 and CCL21 in lymph nodes and Payer's patches (170, 171). Interestingly, though T cells in CCR7-deficient mice cannot traffic to secondary lymphoid organs, they form aggregates in the lung and gastrointestinal tract, referred to as bronchus-associated lymphoid tissue (BALT) and gut-associated lymphoid tissue (GALT), respectively (170, 171). To further highlight the importance of CCR7 expression on T cells, it was recently shown that regulatory T cells from CCR7 knockout mice were unable to function in vivo as they were unable to traffic into lymph nodes (172). Though CCR7-deficient regulatory T cells are functionally suppressive in vitro, their inability to inhibit autoreactive T cells in vivo leads to systemic autoimmune disease in CCR7 knockout mice (173).

Unlike naïve T cells, immature DCs do not express CCR7, but instead express CCR6, which allows DCs to migrate to sites of inflammation and infection. Once a DC becomes activated, and begins to mature, it downregulates the expression of CCR6 and increases CCR7 expression, allowing the antigen-loaded DC to traffic to the lymph node and prime naïve T cells (174). Though DC numbers in wild-type and CCR7 knockout mice are unchanged, the defective trafficking patterns seen in CCR7-deficient T cells are also apparent in knockout DCs. Although a typical lymph node is composed of 2-4% DCs, it was found that lymph nodes in CCR7 deficient mice lacked a definable population of DCs (163). Additionally, when the skin of CCR7 knockout mice was painted with FITC, a maturing stimulus, it was shown that DCs did not migrate to the draining lymph node, but rather remained in the dermis (170).

Though chemotaxis and migration appear to be the most important role of CCR7 on T cells and DCs, a recent review named 9 separate functions of CCR7 signaling,

which impacted over 12 different cell types including human, mouse, and cancer cells (175). These functions of CCR7 include chemotaxis, adhesion, proliferation, changes in cytoarchitecture, endocytosis, migratory speed, differentiation, survival, and invasive ability. Given the importance of CCR7 and its ligands in innate immunity, adaptive immunity, and autoimmunity, selective targeting of this receptor, and CCL19 or CCL21 may have a significant clinical benefit, leading to new therapies and treatments for a wide range of diseases.

Summary and Chapter Outline

Hematopoietic stem cell transplantation is an important clinical therapy that has the potential to cure a verity of hematologic and non-hematologic diseases. The expanded application of HSCT is limited by several adverse side effects, including GVHD and prolonged immunosuppression, leaving patients susceptible to pathogenic and opportunistic infectious including *A. fumigatus*. Over the past decade, it has been shown that chemokines and their receptors have a significant role in the host immune response during IA. CCR7 and its ligands CCL19 and CCL21 control the migration of T cells, B cells, and DCs to secondary lymphoid organs, thereby playing a significant role in the development of adaptive immunity. To examine the role of CCR7 during fungal infections, we used two distinct murine models of IA. In chapter 2, we examine the role of CCR7 on DCs during IA, and report that CCR7 deficiency enhances fungal clearance and enhances DC function. In chapter 3 we report a novel inhibitory role for CCR7 on HSC and myeloid progenitor cell proliferation, leading to prolonged immunosuppression and increased susceptibility to IA. In Chapter 4, we will discuss the relevance of our findings and the overall future directions of this project.

Chapter 2

Materials and methods

Mice

Wild-type female, C57BL/6 mice (6-8 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME). CCR7-/- mice, generated on a C57BL/6 background by Dr. Martin Lipp's group (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany), as previously described (170), were a kind gift from Sergio Lira (Mount Sinai School of Medicine, NY, NY) and breeding colonies of these mice were established and maintained in the University Laboratory of Animal Medicine facility. For all experiments, mice were sex-matched and used between 8-10 weeks of age. All animals were used in accordance with regulations mandated by the University Committee on Use and Care of Animals at the University of Michigan.

Generation of bone marrow chimeras

Wild-type (C57BL/6) mice were lethally irradiated with 1000 cGy using a cesium source. Within 12 hours of irradiation, recipient mice were reconstituted with 5.0×10^6 whole BM cells in PBS from WT or CCR7-/- mice, via tail vein injection. Mice were considered fully chimeric 8-10 weeks following BM transplantation.

Murine model of antibody induced invasive aspergillosis

Mice were depleted of neutrophils with an intraperitoneal injection of 100 μg of RB6-8C5 (anti-Gr-1) as previously described (152). One day after the induction of neutropenia, mice were anesthetized with a mixture of ketamine and xylazine and given an i.t. injection of 6.0 x 10^6 *A. fumigatus* conidia suspended in 30 μl of 0.1% Tween 80 in PBS. The highly virulent *A. fumigatus* strain 13073 (American Type Culture Collection, Manassas, VA) was used to elicit a reproducible form of invasive aspergillosis in anti-Gr-1-treated mice (152).

Isolation of hematopoietic stem cells (HSCs), common myeloid progenitors (CMPs), and granulocyte-monocyte progenitors (GMPs)

To assess the role of CCR7-/- on myeloid progenitor cells following HSCT, progenitor cells and HSCs were coinjected following irradiation. HSCs are required for long-term engraftment and survival, as myeloid progenitor cells have limited capacity for self-renewal. Hematopoietic cell isolation was performed as previously described, with a few modifications (**Fig 3.1**) (176). Briefly, bone marrow (BM) cells from WT or CCR7-/- mice were flushed from the femur and tibiae with sterile staining media (PBS with 5% FCS). A single cell suspension was generated by filtering BM cells through a 70-micron nylon mesh filter, which was followed by a single wash in staining media. Using biotinylated antibodies, BM cells were labeled with the lineage markers Ter119, Gr-1, B220, Mac-1, IL-7Ra and CD3 (BD Bioscience). The cells were again washed with staining media, and cells expressing lineage markers were depleted using anti-biotin beads (Miltenyi Biotech). The remaining cells were stained with FITC CD34, PE

CD16/32, PE-CY7 Sca-1, APC c-kit, and APC-CY7 streptavidin (BD Bioscience). Cells were sorted using a BD FACS Aria. HSCs were identified as lineage negative, c-kit positive, sca-1 positive, and CD34 negative. Collectively, CMPs and GMPs were defined as lineage negative, c-kit positive, sca-1 negative and CD34 positive. CMPs expressed low levels of CD16/32, while GMPs had high expression of CD16/32.

Hematopoietic stem cell transplantation (HSCT) and invasive aspergillosis (IA)

WT mice were irradiated with 1000 cGy using a cesium source. FACS sorted HSCs, CMPs, and GMPs from WT or CCR7-/- mice were resuspended in serum free media. Each recipient mouse received 600 HSCs with $1.0x10^4$ CMPs and $1.0x10^4$ GMPs within 6 hours of irradiation, via tail vain injection. Fourteen days after HSCT, mice were anesthetized with a mixture of ketamine and xylazine and given an i.t. injection of 2.0 x 10^6 *A. fumigatus* conidia suspended in 30 µl of 0.1% Tween 80 in PBS. *A. fumigatus* strain 13073 was used to elicit a reproducible form of invasive aspergillosis following HSCT.

Lung histology

Lungs were collected 2 days after intratracheal conidia challenge in WT or CCR7-/- chimeras. Whole lungs were excised, perfused with 10% formalin, and placed in fresh formalin for an additional 24-48 hours. Routine histologic techniques were used to paraffin-embed this tissue, and 5-µm sections of whole lung were stained with hematoxylin and eosin (H&E) or with Gomori methanamine silver (GMS) stain to visualize *A. fumigatus* conidia and hyphal elements (black staining). An Olympus

BX40F microscope equipped with 20x/0.5 and 100x/1.3 objective lenses and a 10x eyepiece (Olympus, Melville, NY) was used to capture digital images of lung sections. Digital photographs were obtained with a Sony 3CCD color video camera, model number DXC-960MD (Sony, Tokyo, Japan), and IP Lab Spectrum software was used for image acquisition (Scanalytics, Fairfax, VA).

RNA isolation and real-time TaqMan PCR analysis

Total RNA was isolated from homogenized mouse lungs or bone marrow derived DCs (BMDCs) with Trizol reagent (Invitrogen). Approximately 5 μg of purified RNA was reverse transcribed to yield cDNA. CCR7, TNF-α, INF-γ, IL-17, IL-10, IL-12, IDO, CXCL2, CXCL10, and CCL5 gene expression was analyzed by real-time quantitative reverse-transcriptase–polymerase chain reaction (RT-PCR) using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). For quantitative TaqMan analysis of CCL21, primers were custom made as previously described (177). Gyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as an internal control and gene expression was normalized to GAPDH. Fold changes in gene expression levels were calculated by comparison of the gene expression in unchallenged samples or mice, which were assigned an arbitrary value of 1. Additional analysis was performed by assigning WT challenged mice or BMDCs a value of 1 and comparing this with CCR7-/- challenged samples.

Protein analysis of lung cytokine levels

Whole-lung cytokine levels of TNF-α, IL-10, CCL3, CCL5, CCL2, CXCL9, and CXCL10 from wild-type or CCR7-/- chimeras were measured using a Bioplex bead-based cytokine assay purchased from Invitrogen or Bio Rad and analyzed on the Bio Rad Bioplex 200 system according to the manufacturer's protocol.

Flow cytometry

Whole lung samples were minced using surgical scissors and incubated in RPMI-1640 supplemented with 5% FCS, type IV collagenase, (Sigma Aldrich, St. Louis, MO) and DNAse for 45 minutes. Cells were flushed through a nylon mesh filter and washed with FACS buffer, consisting of Ca^{2+} and Mg^{2+} free PBS with 0.1% azide, 1% bovine serum albumin, and 5 mM EDTA as described (178). Prior to surface staining with labeled antibodies, nonspecific binding was blocked by incubating cells with purified rat anti–mouse CD16/CD32 (FcγIII/II receptor) monoclonal antibody from eBioscience. Flow cytometry analysis was performed with a Beckman Coulter Cytomics FC 500 and data was analyzed using the FlowJo 8.2 software.

Chitin assay

Chitin, a major constituent of the hyphal cell wall, is absent in mammalian tissue and thus can be used as a method to determine the fungal burden in the lungs of infected mice. The chitin assay was performed as described (117).

In vitro BMDC culture

Bone marrow (BM) cells were flushed from the femurs and tibiae of wild-type or CCR7-/- mice with sterile media. A single cell suspension was obtained by filtering BM cells through a 70-micron nylon mesh filter. BM cells were plated at a concentration of 3.0 x 10^6 cells/10mls in RPMI media containing 20 ng/ml murine granulocyte-macrophage colony-stimulating factor (GM-CSF, R&D systems, Minneapolis, MN). Media was replenished at three days and BMDCs were purified using anti-CD11c magnetic beads (Miltenyi Biotech). Cells were replated in fresh media, without GM-CSF, and incubated with live *A. fumigatus* conidia at a 1:5 ratio. Using the Trizol reagent (Invitrogen/ Life Technologies, Carlsbad, CA), RNA was extracted from the BMDCs after two hours of culture with conidia to prevent fungal overgrowth, and to prevent the use of antifungals, which can affect dendritic cell function and maturation (122).

DC transfer and tracking

In vitro-derived WT or CCR7-/- BMDCs were cultured as described, and in some experiments were labeled with carboxyfluorescein succinimidyl ester (CFSE). BMDCs (1.0×10^6) were injected 6 hours before conidia challenge or were co-injected with conidia (6.0×10^6 to 10^7) into WT mice one day after administration of 100 μg of anti-Gr-1. One set of mice was euthanized twenty-four hours after the injection, and lungs were analyzed for CFSE expression. The remaining mice were sacrificed at 48 hours for lung histology, protein, and chitin analysis.

Statistical analysis

All results are expressed as the mean +/- the standard error and are representative of 2 or more experiments. A Student *t* test or analysis of variance was used to determine statistical significance between WT and CCR7-/- mice or BMDCs. Survival rates were expressed as percentages, and a log-rank test (x^2 test) was used to detect differences in mouse survival. *P* values of <0.05 were considered statistically significant.

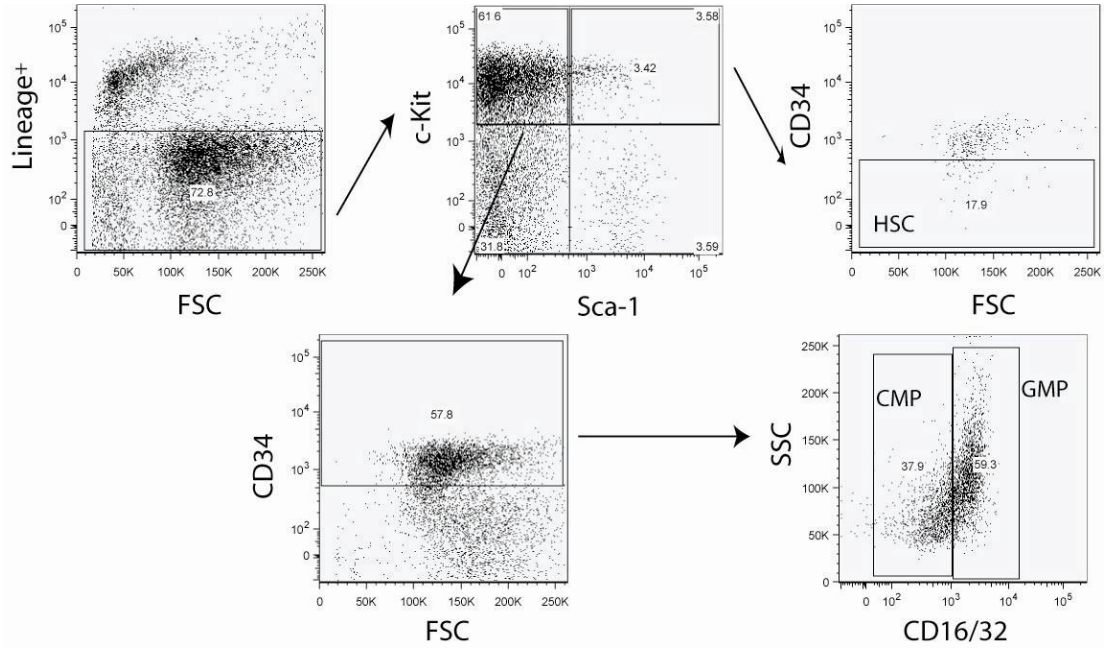

FIGURE 2.1. FACS sorting of HSCs, CMPs, and GMPs from the BM of naïve WT mice. HSCs, top, are classified as lineage negative, c-Kit^{+}, Sca-1^{+}, CD34^{-}. CMPs and GMPs, top and bottom, are defined as lineage negative, c-Kit^{+}, Sca-1^{-}, CD34^{+}. CMPs are distinguished from GMPs based on the expression CD16/32.

Chapter 3

CCR7 Deficiency on Dendritic Cells Enhances Fungal Clearance in a Murine Model of Pulmonary Invasive Aspergillosis

Summary

Aspergillus fumigatus (*A. fumigatus*) is a sporulating fungus found ubiquitously in the environment and is easily cleared from immunocompetent hosts. Invasive aspergillosis develops in immunocompromised patients, and is a leading cause of mortality in bone marrow transplant patients. Chemokine receptor 7 (CCR7) and its ligands, chemokine ligand 19 and 21 (CCL19 and CCL21), are responsible for the migration of dendritic cells from sites of infection and inflammation to secondary lymphoid organs. To investigate the role of CCR7 expression on dendritic cells during invasive aspergillosis, we used a well-characterized neutropenic murine model. During invasive aspergillosis, chimeric mice with a hematopoietic deficiency of CCR7 exhibited increased survival and decreased lung pathology compared with chimeric mice with wild-type bone marrow. Flow cytometric analysis of the chimeras revealed an increase in the number of dendritic cells present in the lungs of the knockout animals following infection with aspergillus conidia. An adoptive transfer of dendritic cells into neutropenic mice provided a protective effect during invasive aspergillosis, which was enhanced by deficiency in CCR7. Additionally, in vitro coculture data revealed an increase in TNF-α, CXCL10, and CXCL2 produced by CCR7-/- dendritic cells, indicating a more activated cellular

response to *A. fumigatus*. Our results suggest that the absence of CCR7 is protective during invasive aspergillosis in neutropenic mice. Together, these data demonstrate a potential deleterious role for CCR7 during primary immune responses directed against *Aspergillus fumigatus*.

Introduction

Aspergillus fumigatus (*A. fumigatus)* is a sporulating fungus present ubiquitously in the environment (179). Most individuals are not affected by exposure to Aspergillus, however, the fungus can cause a broad spectrum of diseases, ranging from hypersensitivity reactions, such as allergic bronchopulmonary aspergillosis, to serious opportunistic infections, including chronic pulmonary necrotizing aspergillosis and invasive aspergillosis (180). Invasive aspergillosis is a rapidly progressive disease, often originating in the pulmonary system, in which inhaled conidia from *A. fumigatus* germinate into hyphae and invade the lung parenchyma, leading to severe pneumonia and massive inflammation (181). This severe, usually fatal disease disproportionally affects immunocompromised individuals, and is a leading cause of mortality in bone marrow transplant recipients (102, 182-184). Neutropenia was initially described as the most significant risk factor for the development of invasive aspergillosis (185), but mounting evidence has shown the importance of several additional effector cell populations, most notably, myeloid dendritic cells (DCs) (117, 121, 186).

Dendritic cells play a crucial role during the immune response to *A. fumigatus*. Studies have shown that DCs become activated by *A. fumigatus* conidia and hyphae, leading to DC maturation and production of inflammatory cytokines, such as TNF-α, IL-12, IL-6 and IL-10 (121). Mature DCs have been shown to clear both *A. fumigatus* conidia and hyphal elements, indicating their importance prior to, and following, the onset of invasive disease (120). DCs also participate in the adaptive immune response: when conidia-activated DCs were adoptively transferred, they activated IFN-γ producing T cells, leading to fungal resistance.

Immature DCs migrate to sights of fungal infection using chemokine receptor 6 (CCR6) (187, 188). A recent study has shown that CCR6-/- mice fail to recruit adequate numbers of DCs to the lung following conidia challenge, and consequently had significantly enhanced morbidity and mortality compared to wild-type mice (119). Unlike CCR6, chemokine receptor 7 (CCR7) is not expressed on immature DCs, but is upregulated after exposure to a maturing stimulus, allowing DCs to migrate away from sites of inflammation to secondary lymphoid organs (189). In our studies, we wanted to examine whether eliminating CCR7 signaling during invasive aspergillosis would enhance fungal clearance by preventing the efflux of DCs from the site of conidial challenge. Additionally, we sought to determine whether CCR7 expression was required for the proper activation and maturation of DCs during a fungal challenge, as it has been shown that human DCs upregulate their expression of CCR7 upon internalization of *Aspergillus* conidia (122, 123).

Our results show that a deficiency of CCR7 in the bone marrow compartment markedly enhanced survival and fungal clearance in neutropenic mice. This protective effect was associated with a significantly higher number of DCs in the lungs of CCR7-/- bone marrow chimera mice than were in the lungs of CCR7-sufficient mice. Additionally, it was found that CCR7-/- bone marrow-derived DCs (BMDC) had a more activated cellular response to *Aspergillus* conidia than did wild-type BMDCs, and intratracheal instillation of CCR7-/- BMDCs more effectively protected neutropenic wild-type mice from invasive aspergillosis than did wild-type BMDCs. Thus, CCR7 expression negatively regulates the innate response to *Aspergillus* conidia, thereby permitting the development of invasive pulmonary aspergillosis.

Results

CCR7 and its ligands, CCL19 and CCL21, were highly expressed in the lung during invasive aspergillosis

During the past decade it has become increasingly clear that chemokines and their receptors play an important role in the antifungal response during invasive aspergillosis (117, 119, 152, 155, 156). To investigate whether CCR7 is involved in host defense to *A. fumigatus*, wild-type mice were first rendered neutropenic by the administration of 100μg of anti-Gr-1 approximately 24 hours prior to an i.t. challenge with 6.0 x 10^6 conidia (**Fig. 3.1A and 2.1B**). Lungs from infected mice were examined 48 hours after infection by flow cytometry. Dendritic cell numbers significantly expand in the lungs of challenged mice 2 days after infection (**Fig. 3.1C**). CCR7 expression is not limited to DCs, but is highly expressed on activated DCs during invasive aspergillosis (**Fig. 3.1D**). Lungs of the infected mice also have dramatically increased gene expression of CCR7 and its ligand CCL19 at 48 hours post-infection (**Fig. 3.1E**). Interestingly, gene expression of CCL21a and CCL21b, the other ligands for CCR7, are dramatically downregulated in the lungs of infected mice. Taken together, these results suggest that CCR7 and its ligands play an important role in the immune response to *A. fumigatus*.

CCR7-/- mice are protected from invasive aspergillosis

To test the effect of CCR7 during invasive pulmonary fungal disease, we infected wild-type and CCR7-/- mice with 6.0 x 10^6 conidia. Uninfected CCR7-/- mice have significant cellular infiltrate and lymphoid aggregates in the lungs prior to the fungal

challenge, but these mice appear to be protected from invasive aspergillosis as demonstrated by the decrease in fungal elements in the lung (**Fig. 3.2A**). CCR7-/- mice also have significantly less TNF-α and IL-12 than wild-type mice as determined by real time PCR (**Fig 3.2B**). Together these data indicate that CCR7-/- mice are protected during invasive aspergillosis, and have a lower inflammatory response due to a more rapid clearance of *A. fumigatus*.

Deficiency of CCR7 on hematopoietic cells significantly enhanced survival and decreased fungal burden following A. fumigatus conidia challenge

Previous studies, and work in our laboratory, have demonstrated that CCR7-/- mice exhibit anatomical abnormalities, such as alterations in secondary lymphoid organs, and generalized autoimmunity (170, 173). Given this feature of intact CCR7-/- mice, we elected to make bone marrow chimeras to examine the effect of CCR7 solely on the hematopoietic system during invasive aspergillosis. CCR7-/- chimeras had significantly lower mortality (10%) than their wild-type counterparts (66%) at day five post-infection with *A. fumigatus* (**Fig. 3.3A**). Analysis of whole lung chitin levels revealed that CCR7-/- chimeric mice had a significantly lower fungal burden compared to wild-type chimeras (**Fig 3.3B**). Consistent with these data, GMS staining of whole lung sections showed significant fungal growth and hyphal formation in the wild-type chimera lung, which was widely absent in the CCR7-/- chimeras (**Fig. 3.3C-3.3H**). The lungs of uninfected CCR7-/- chimeras exhibited some lymphoid aggregates made up predominantly of B cells and T cells as well as additional cell infiltrate, which was not observed in the wild-type chimeras (**Fig. 3.3C and 3.3D**). Interestingly, this infiltrate was markedly less than what

was observed in intact CCR7-/- mice of the same age. Two days after conidia challenge, H&E staining showed significant cell infiltrate in the lungs of both mice, but to a greater extent in the wild-type chimeras, when compared to CCR7-/- chimeras and to the unchallenged controls (**Fig. 3.3C to 3.3F**). This cellular infiltrate corresponded to a massive inflammatory response to *A. fumigatus*. Despite the influx of cells in both groups of mice, wild-type chimeras were unable to control the growth of the fungus, while the knockout animals did not develop invasive aspergillosis (**Fig. 3.3G and 3.3H**). These data clearly demonstrate that neutropenic CCR7-/- chimeric mice are protected from invasive aspergillosis.

Lung leukocyte cell recruitment following invasive aspergillosis challenge

Since treatment of mice with anti-Gr-1 eliminates neutrophils, a major effector cell type in the aspergillus-infected lung, we examined whole lung samples by flow cytometry to identify the cells providing the protective effect observed in CCR7-/- chimeras. We saw a dramatic increase in myeloid DCs and immature myeloid cells (monocytes and recruited macrophages) at 48 hours after aspergillus challenge in both the wild-type and CCR7-/- groups (**Fig. 3.4**). Conversely, the numbers of T cells, B cells, and NK cells were decreased two days after conidia challenge. While immature myeloid cell numbers were elevated in both groups, flow cytometric data revealed higher percentages of myeloid DCs in the lungs of CCR7-/- chimeric mice, which, in combination with a higher absolute number of cells in the lungs of the knockout animals, resulted in a significantly greater number of DCs present (**Fig. 3.4**). It has been previously reported that DCs provide protection against invasive aspergillosis and that mice lacking adequate numbers

of DCs are susceptible to disease (119). Therefore, antifungal responses in CCR7-/- chimeras appear to be the result of an enhanced accumulation of DCs.

Cytokine and chemokine production 48 hours after onset of invasive aspergillosis

Inflammation is required for an appropriate antifungal immune response, but it has been reported that an uncontrolled inflammatory response is detrimental to the host (150, 190). We found that protein levels were significantly higher in both groups of mice two days after infection when compared to uninfected controls (**Fig. 3.5**). Interestingly, infected wild-type chimeras show significantly higher expression of TNF-α, CCL2, CCL5, and CCL3, but lower levels of CXCL9 and CXCL10, the ligands for CXCR3, when compared to CCR7-/- chimeras. While CCR7-/- chimeras have lower levels of inflammatory mediators, both groups produce the same amount of the anti-inflammatory cytokine, IL-10. Together these findings suggest that the inflammatory response in the lung of CCR7-/- mice is appropriately regulated for the rapid clearance of *A. fumigatus*.

Characterization of wild-type and CCR7-/- dendritic cells following aspergillus challenge in vitro

Survival, histological, and fungal growth differences between wild-type and CCR7-/- chimeras appear to be the result of the accumulation of DCs in the lungs of the knockout mice. However, these differences could also be the consequence of differential activation and maturation by CCR7-/- DCs, or due to a difference in fungal recognition by these chemokine receptor deficient DCs. To test bone marrow derived dendritic cell (BMDC) activation and maturation, wild-type or CCR7-/- bone marrow was grown in vitro for

eight days with GM-CSF and the resulting BMDCs were challenged with Aspergillus conidia. Two hours after fungal challenge, CCR7-/- and wild-type BMDCs had very similar gene expression of the co-stimulatory molecules MHC II, CD86, and CD40 (**Fig. 3.6A**). When RNA was analyzed for expression of antifungal, pro-inflammatory cytokines at two hours, CCR7-/- BMDCs showed higher levels of TNF-α, CXCL10, CCL2, CXCL2 and similar levels of CCL5, when compared to wild-type uninfected control BMDCs (**Fig. 3.6B**). Given that it has been shown that a TLR2/dectin-1 complex, and TLR4 recognize *A. fumigatus* (129, 135, 191), we examined wild-type and CCR7-/- BMDCs for these receptors. We found that significantly more TLR2 gene expression was present 2 hours after conidia challenge, but dectin-1 levels were decreased. TLR4 levels were almost identical between the two groups (**Fig 3.6C**). When we cultured our cells for 24 hours in the presence of Amphotericin B, we observed that CCR7-/- DCs had slightly higher expression of CD86 than wild-type DCs (**Fig 3.6D**). Additionally CCR7-/- DCs appeared to be functionally more mature, as they were able to stimulate T cell proliferation in a mixed leukocyte reaction (MLR) better then wild-type DCs (**Fig. 3.6E**). Together these data indicate that CCR7-/- DCs might provide protection via a more activated and mature phenotype, characterized by the higher expression of co-stimulatory molecules, and by the production of greater amounts of pro-inflammatory cytokines compared to their wild-type counterparts.

Adoptive transfer and tracking of wild-type and CCR7-/- BMDCs

Dendritic cells appear to be critically important in the host immune response to invasive aspergillosis in our model. To determine whether CCR7 deficiency on DCs alone was

adequate to provide protection against *A. fumigatus*, we labeled 1.0 x 10^6 wild-type or CCR7-/- BMDCs (**Fig 3.7A**) with CFSE and injected them i.t. into neutropenic wild-type mice, which were challenged with 6.0 x 10^6 conidia. Twenty-four hours after the transfer, the lungs were analyzed for CFSE expression by flow cytometry. We found that mice receiving CCR7-/- BMDCs had significantly higher numbers of CFSE positive cells than mice receiving wild-type BMDCs (**Fig. 3.7B**). Upon histological analysis, at 48 hours, recipients of wild-type and CCR7-/- BMDCs both showed a decrease in fungal growth when compared to the chimeric mice without an adoptive transfer of BMDCs (**Fig. 3.7C and 3.7D and 3.3E**). Though fungal burden was diminished in both BMDC adoptive transfer groups, the histology indicates that mice receiving wild-type BMDCs had more fungal growth overall, especially in the air spaces. Additionally, protein data shows a similar trend to the wild-type and CCR7-/- chimeras infected with aspergillus (**Fig 3.7E**). TNF-α and CCL5 were statistically higher in the mice receiving wild-type BMDCs than in mice receiving CCR7-/- BMDCs. Since the adoptive transfer of BMDCs significantly decreased the fungal burden in both groups of mice, we increased the amount of conidia from 6.0 x10^6 to 10^7, to examine differences in lung chitin levels (**Fig 3.7F**). At 10^7 conidia, mice in both groups were susceptible to fungal growth, but mice receiving wild-type BMDCs had a decreased protective effect. Collectively these data indicate that CCR7-/- BMDCs provided better protection from invasive aspergillosis compared with wild-type BMDCs.

Discussion

Invasive aspergillosis represents a significant challenge in the clinic and remains a leading cause of death for immunocompromised individuals. While the highest incidence of disease is observed among bone marrow transplant recipients, invasive aspergillosis is also on the rise in solid organ transplant patients as well as in cancer and AIDS patients (87, 102, 182, 183, 192, 193). The present study investigated the role of the chemokine receptor, CCR7, in a well-established neutropenic murine model of invasive aspergillosis. We found that CCR7-/- chimeric mice had a distinct survival advantage, as well as decreased fungal burden in the lungs, after challenge with *A. fumigatus* conidia when compared to their wild-type counterparts. This was an unexpected observation given that we found a significant upregulation of CCR7 on dendritic cells, in addition to increased levels of CCL19, in wild-type mice challenged with *A. fumigatus* conidia, suggesting the importance of this chemokine-chemokine receptor interaction during fungal pathogenesis. Our data thus suggest that CCR7 induction on DCs during invasive pulmonary aspergillosis promotes a pathological response to *A. fumigatus.*

Dendritic cells are an important effector cell during invasive aspergillosis. The Mehrad group showed that CCR6-deficient mice were significantly more susceptible to invasive aspergillosis than CCR6-sufficient mice (119). CCR6 and its ligand, CCL20, are responsible for the migration of immature DCs to sights of inflammation, including the lung (187, 188, 194, 195). Consequently, DCs lacking the expression of CCR6 were unable to efficiently traffic into the infected lung, thus leaving a neutropenic host susceptible to invasive aspergillosis. As originally reported by Dieu et al. in human DCs, when DCs encounter a maturing stimulus, such as *A. fumigatus*, they downregulate their

expression of CCR6 and upregulate their expression of CCR7 (174). DC upregulation of CCR7 expression permits trafficking away from sites of infection and inflammation to secondary lymphoid organs, as the cells migrate in response to the chemokines CCL19 and CCL21 (196-199).

Based on these observations, we hypothesized that CCR7-/- DCs, with functional CCR6 expression, would be able to efficiently traffic to the lungs of infected mice; once there, however, the DCs would accumulate as they would be unable to respond to the ligands, CCL19 and CCL21, and effectively traffic out of the lung to the draining lymph node. Indeed, our results showed that 48 hours after infection with *A. fumigatus* conidia, there was a significant increase in the number of DCs in the CCR7-/- chimeras when compared to wild-type chimeric mice. We had similar findings in an adoptive transfer experiment where CCR7-/- or wild-type DCs are injected along with conidia into the lungs of wild-type neutropenic mice. Here we saw many-fold higher numbers of CCR7-/- DCs than wild-type DCs in the lungs of the neutropenic mice. These data support the hypothesis that CCR7-/- DCs remain in the lungs of infected animals, where they ultimately provide protection against invasive aspergillosis.

New evidence suggests that a hyper-inflammatory response in the lung during invasive aspergillosis is detrimental to the survival of the host (150, 190). When TNF-α, an important antifungal cytokine, was neutralized in a murine model of invasive aspergillosis, there was a significant increase in mortality associated with increased fungal burden (142). Consequently, significant levels of TNF-α are required for the clearance of *Aspergillus*, but dangerously high levels of TNF-α are implicated in severe inflammatory conditions such as sepsis (142, 147, 200, 201). Thus, a balance between

pro- and anti-inflammatory signals is required for appropriate fungal clearance, without excessive tissue injury. In our model, we found significantly higher production of TNF-α as well as the pro-inflammatory mediators, CCL2, CCL5, and CCL3, in wild-type chimeras. Conversely, CCR7-/- chimeric mice produced significantly higher CXCL10, a chemokine recently shown to render the host susceptible to invasive aspergillosis if expressed inappropriately (155). Additionally, both wild-type and knockout chimeras showed similar expression of the potent anti-inflammatory cytokine, IL-10, after conidia challenge. Taken together, the cytokine milieu demonstrated by the CCR7-/- chimeras may create a more appropriate balance between pro- and anti-inflammatory cytokines, which is required for appropriate fungal clearance. Alternatively, the CCR7-/- chimeras may simply have less inflammation: these animals had significantly less fungal growth, and so the resulting decrease in fungal burden eliminates the need to prolong an acute inflammatory response, leading to lower cytokine levels in the CCR7-/- mice.

While the accumulation of DCs in the lungs of CCR7-/- chimeras is likely playing an important role in the clearance of the fungus, it is possible that differences in DC phenotype or function also play a pivotal role during invasive aspergillosis. Previous reports have shown that human DCs exposed to *A. fumigatus* conidia in vitro upregulate their expression of co-stimulatory molecules and their production of proinflammatory cytokines (122, 123). In the present study, we observed a trend indicating greater expression of the costimulatory molecules, MHC II and CD86, on the CCR7-/- BMDCs following conidia challenge. Additionally, CCR7-/- BMDCs had statistically higher gene expression of TNF-α, CXCL10, CCL2, and CXCL2 compared with wild-type BMDCs. Due to the importance of proinflammatory cytokines in the antifungal response, the

protective phenotype provided by CCR7-/- DCs might derive not only from their accumulation in the lung, but also from their more robust cytokine response against *A. fumigatus*. A complication of these in vitro experiments is the two-hour time point, chosen to prevent the use of antifungal reagents, which have been shown to mature BMDCs in vitro (122). When Amphotericin B was used in the MLR, we observed maturation of our media control BMDCs, leading to significant T cell proliferation. Importantly, T cell expansion was always greater when CCR7-/- BMDCs were used as T cell stimulators, regardless of the maturing stimulus used. Collectively, this data indicates that CCR7-/- BMDCs have a more activated and mature phenotype then wild-type BMDCs during challenge with *A. fumigatus* conidia, which may augment the protective phenotype seen in vivo.

In summary, invasive aspergillosis primarily affects immunocompromised patients and is often fatal. New therapies are clearly required for effective treatment of this disease. The present study demonstrates a deleterious role for CCR7 in a murine model of experimental invasive aspergillosis, and so selectively targeting CCR7 might provide a new course of action. However, more study is required to fully understand the beneficial mechanism of CCR7 deficiency during invasive aspergillosis, and to determine whether this receptor is clinically relevant to human disease.

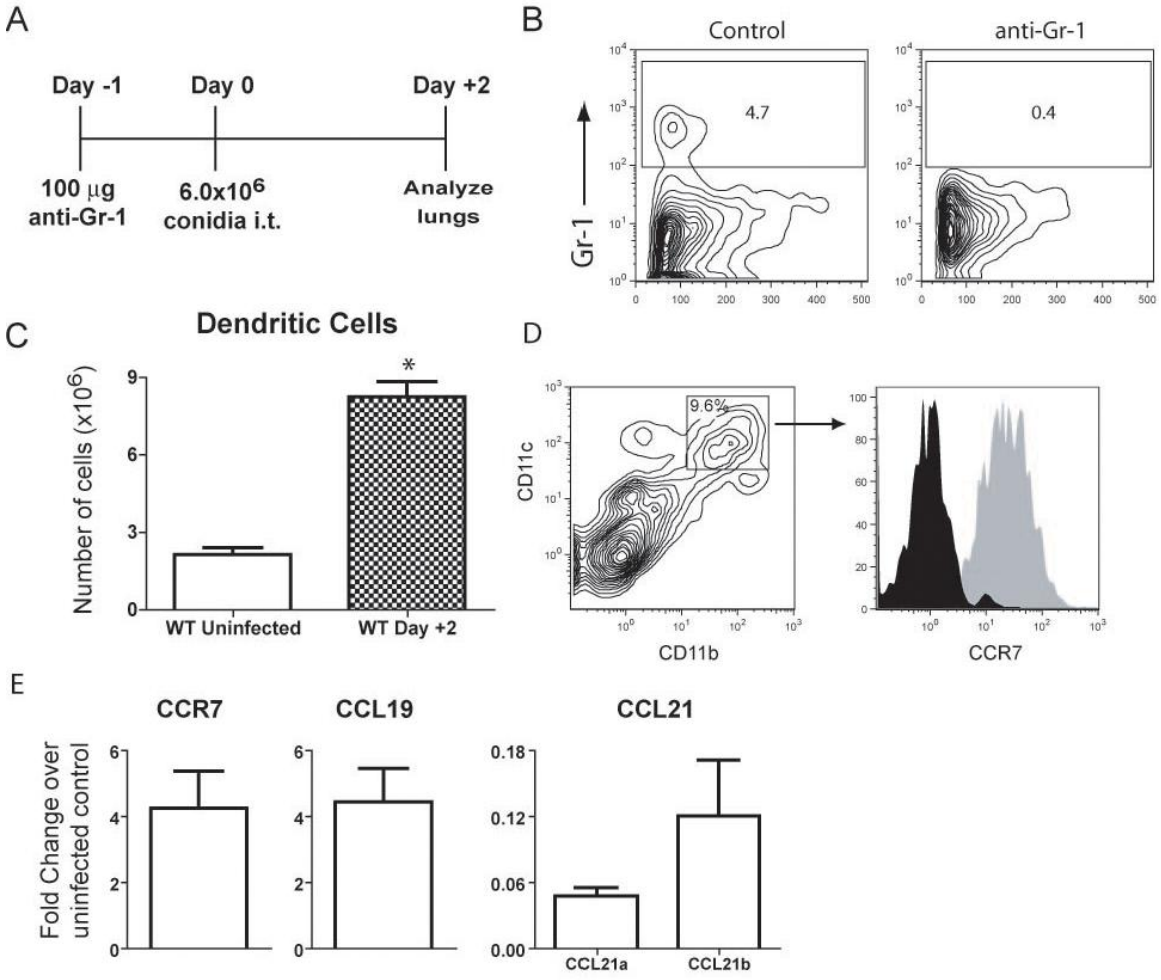

FIGURE 3.1. Whole lung analysis of neutropenic mice following conidia challenge. Experimental scheme in which wild-type, C57BL/6 mice, were administered of 100μg of anti-Gr-1 antibody 24 hours prior to an i.t. challenge with $6.0x10^6$ conidia *A*. Forty-eight hours after fungal challenge, lungs were harvested for flow cytometry and real-time PCR analysis. *B*, Gr-1 expression in wild-type mice, prior to (left) and after (right) the administration of anti-Gr-1 antibody. *C*, DC numbers, based on CD11c and CD11b expression, in the lungs of infected mice 48 hours after conidia challenge, and DC surface expression of CCR7, *D* (black histogram, isotype control). *E*, Real-time analysis of CCR7, CCL19, and CCL21a/b gene expression, 48 hours after conidia challenge. **P* <0.05 between DC numbers in the lungs before and after conidia challenge.

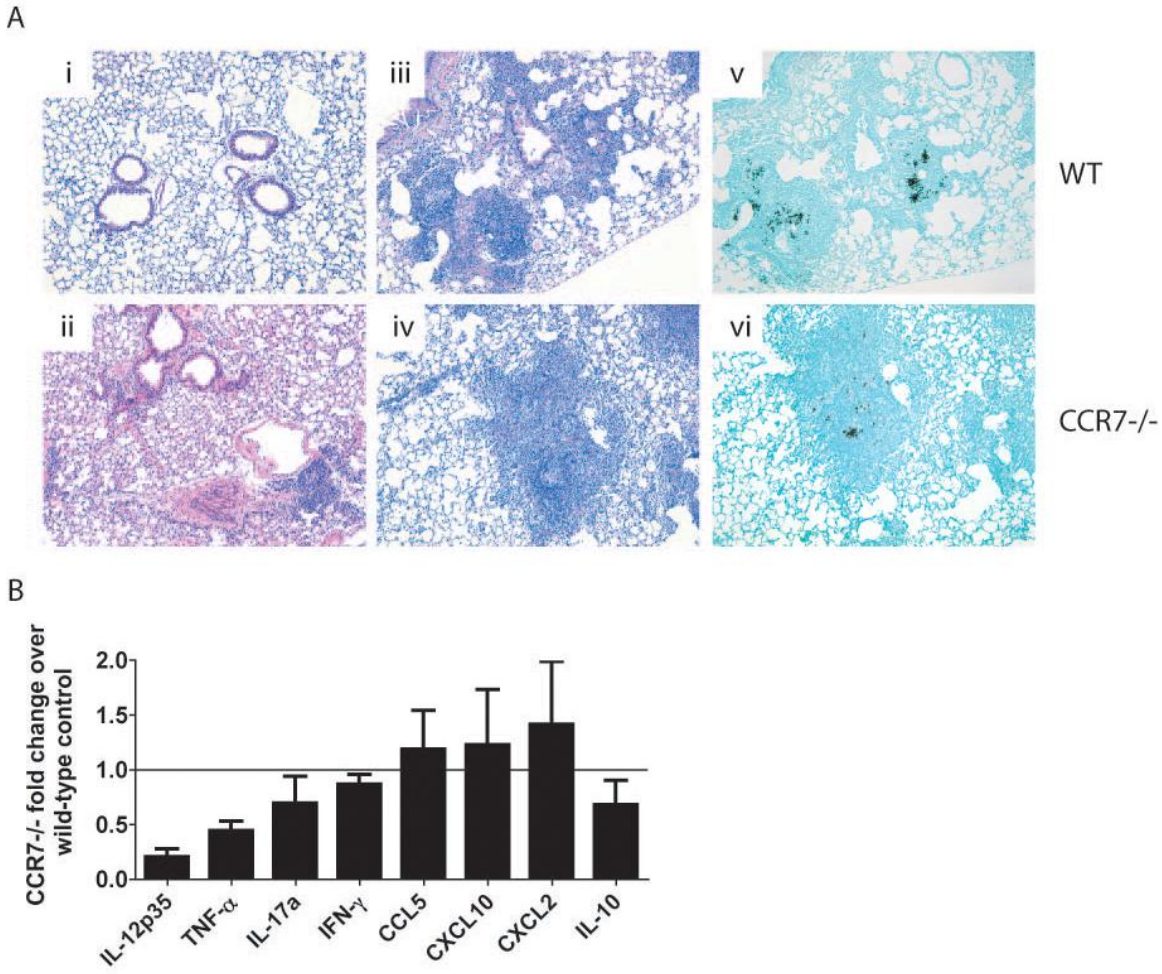

FIGURE 3.2. Conidia challenge in neutropenic wild-type and CCR7-/- mice. Twenty-four hours after the administration of anti-Gr-1 antibody, both groups of mice were given an i.t. challenge with $6.0x10^6$ conidia. Forty-eight hours after challenge with conidia, lungs were removed for histologic *A* and real-time analysis *B*. *A*, Results from wild-type mice are shown in the top panels and results from CCR7-/- mice are shown in the bottom panels. i, ii, iii, and iv represent H & E staining of uninfected (i, ii) and infected (iii, iv) lungs at 48 hours after conidia challenge, fungal elements are stained black . Panels v and vi are representative of GMS-stained lung at 48 hours after fungal infection. *B*, Real-time PCR analysis of whole lung cytokine and chemokine levels, the fold change represents CCR7 gene expression over wild-type gene expression. The black line represents the level of wild-type expression for each gene tested. Original magnifications were 10x.

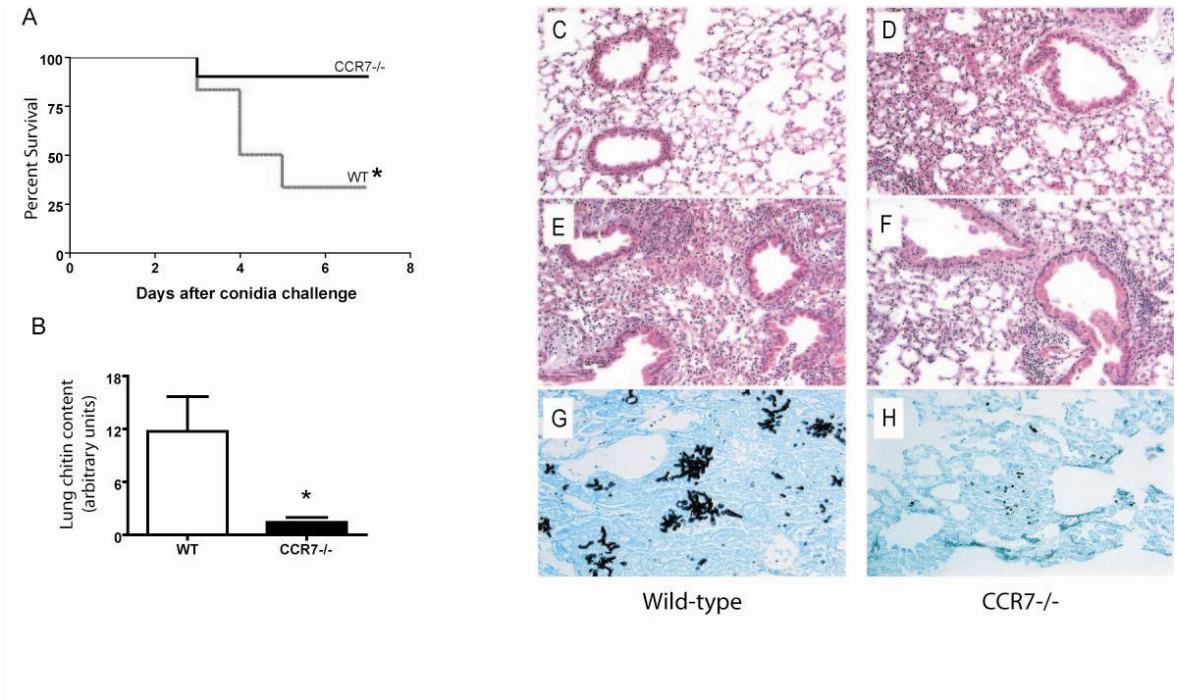

FIGURE 3.3. Survival, lung chitin content, and histology of wild-type and CCR7-/- bone marrow chimeras challenged with *A. fumigatus* conidia. Wild-type or CCR7-/- chimeric mice were given 100μg of anti-Gr-1 antibody 24 hours prior to an i.t. challenge with $6.0x10^6$ conidia and followed for survival or analyzed at 48 hours. *A*, survival of wild-type and CCR7-/- chimeras, n= 10 for wild-type and n=12 for CCR7-/- mice. *B*, Lung chitin content determined 48 hours after the onset of infection. *C-H*, Histologic analysis of lung tissue two days after conidia challenge. H & E staining of uninfected lungs (C and D) or 48 hours after conidia challenge (E and F). *G and H*, representative GMS stained sections from wild-type (G) or CCR7-/- (H) following conidial challenge, fungal elements are stained in black. Original magnification was 20x, for H & E stained sections and 40x for GMS sections. **P* <0.05 comparing WT and CCR7-/- chimera survival.

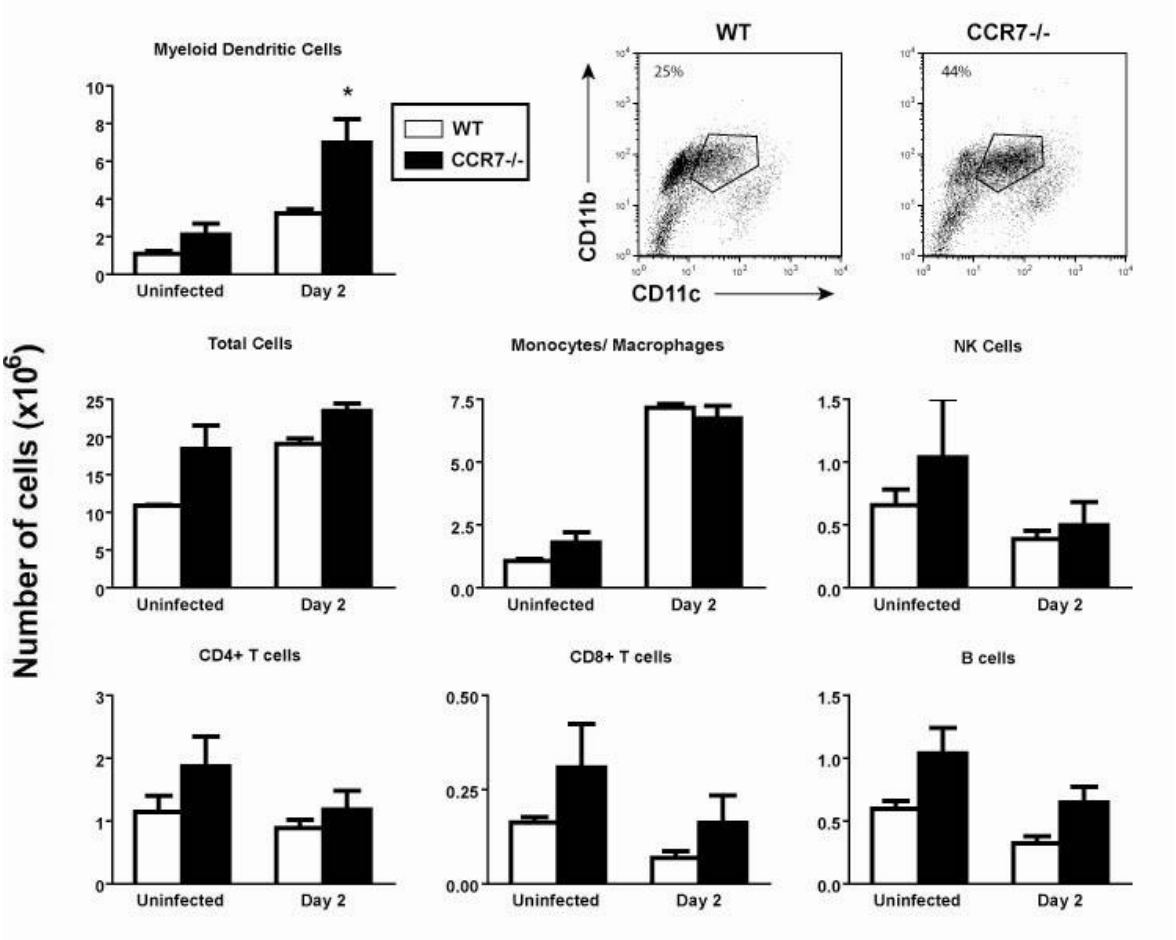

FIGURE 3.4. . The left lung lobe from wild-type or CCR7-/- chimeras was analyzed by flow cytometry 48 hours after fungal challenge. Myeloid DCs were CD11c high, CD11b high cells (as shown in the dot plots). Monocytes/macrophages were $CDllb^{+}$, $F4/80^{+}$, and $CD11c^{-}$. NK cells were $NK1.1^{+}$, T cells were $CD3^{+}$ and $CD4^{+}$ or $CD8^{+}$ and B cells were $CD19^{+}$. *P <0.05 when comparing DCs in wild-type and CCR7-/- mice on day 2 following infection.

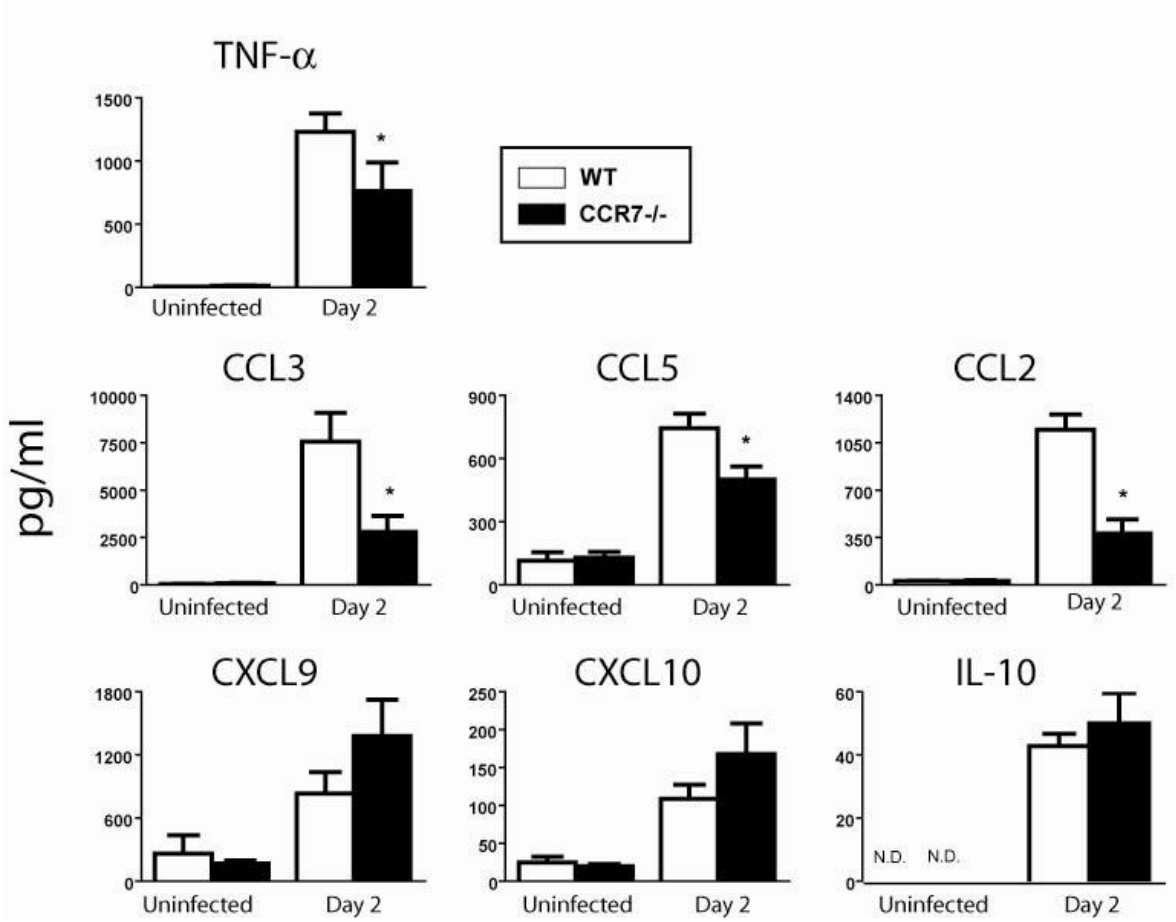

FIGURE 3.5. Whole lung protein levels 48 hours after conidial challenge in wild-type and CCR7-/- bone marrow chimeras. Cytokine and chemokine levels were determined using a Bioplex multiplex assay; all values are represented as pg/ml. **P* <0.05 when comparing wild-type and CCR7-/- chimeras following conidia challenge.

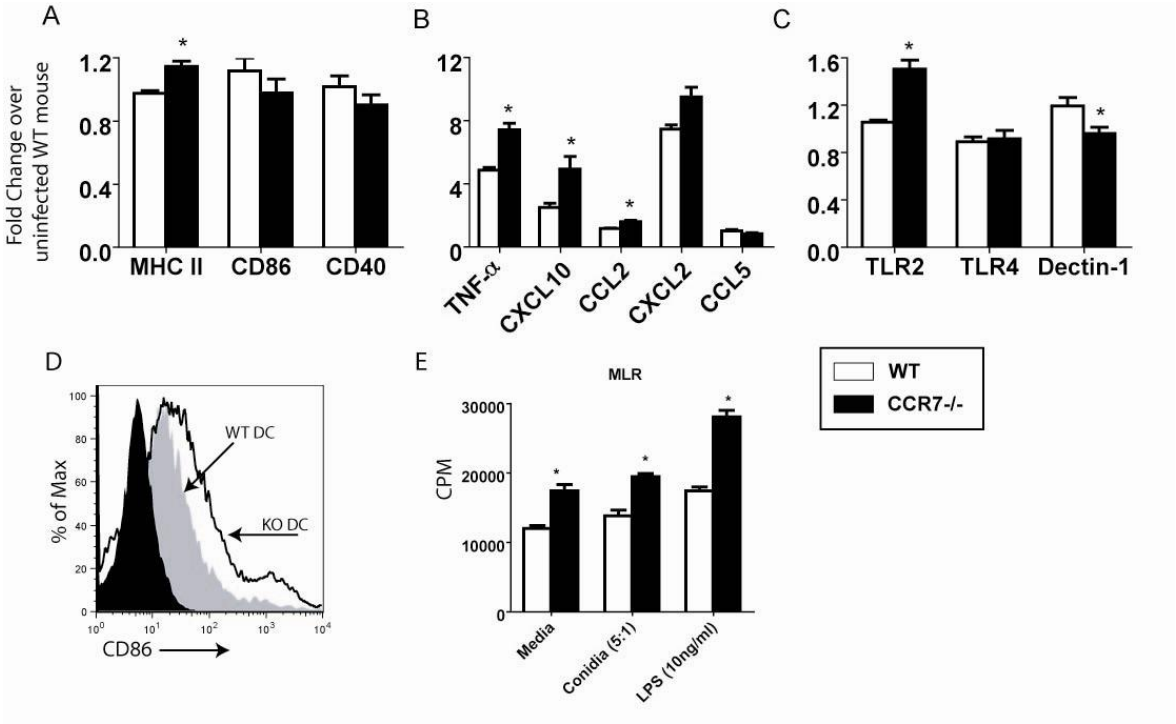

FIGURE 3.6. Phenotypic and functional comparison of wild-type and CCR7-/- BMDCs. *A, B, and C*, BMDCs were co-cultured in a 1:5 ratio with conidia for 2 hours, prior to analysis by real-time PCR. Data are fold change of CCR7-/- BMDC gene expression over wild-type BMDC transcript expression. *D and E*, BMDCs were cultured for 24 hours in a 1:5 ratio with conidia, Amphotericin B was added to culture after 2 hours. *D*, Relative expression of CD86 on wild-type (gray histogram) and CCR7-/- (solid black line) BMDCs (control is black histogram). *E*, Twenty-four hours after culture with conidia or LPS or in media, BALB/c T cells (allogeneic to DC) were added at a 10:1 ratio and allowed to proliferate for 72 hours before the addition of tritiated thymidine (^{3}H). CPM (counts per million) represents T cell proliferation. *P< 0.05, which compares wild-type or CCR7-/- BMDCs.

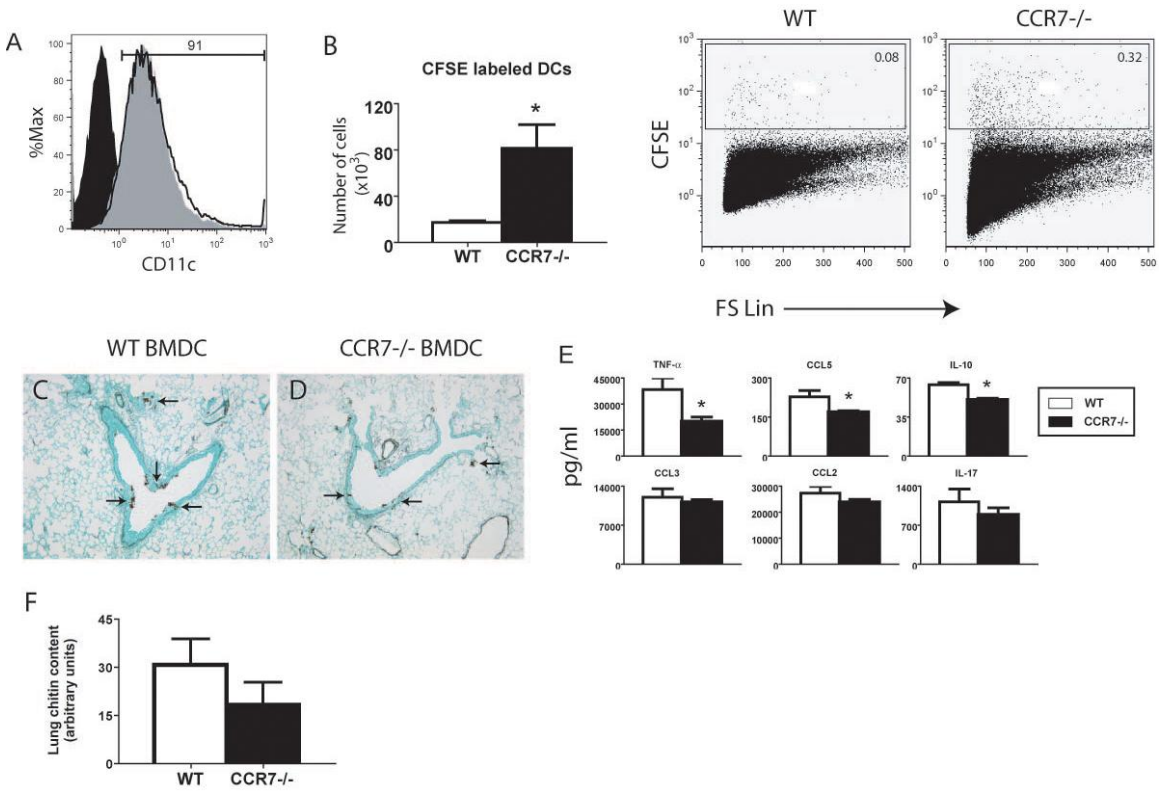

FIGURE 3.7. Adoptive transfer of wild-type and CCR7-/- DCs into neutropenic C57BL/6 mice. *A*, Percent of CD11c positive BMDCs used in the adoptive transfer, following in vitro culture and positive selection by magnetic bead selection. Black histogram indicated unstained, the grey histogram indicates wild-type BMDCs, and the solid black line indicates CCR7-/- BMDCs. *B*, BMDCs were labeled with CFSE and co-injected with $6.0x10^6$ conidia. Data represents the number of labeled DCs present in the lungs at 24 hours after injection of conidia. *C and D*, Lung histology 48 hours after infection. WT mice were given an adoptive transfer of $1.0x10^6$ WT (C) or CCR7-/- (D) BMDCs 6 hours prior to $6.0x10^6$ conidia. Black arrows indicate fungal growth. *E*, Whole lung protein levels from the mice in (C and D). *F*, lung chitin levels 24 hours following co-injection of $1.0x10^6$ BMDCs and 10^7 conidia. WT indicates an adoptive transfer of WT DCs into a WT mouse, CCR7-/- indicates CCR7-/- DCs transferred into a WT mouse. $*P < 0.05$ between wild-type and CCR7-/- groups.

Chapter 4

CCR7 deficiency enhances hematopoietic stem cell and myeloid progenitor cell proliferation and ameliorates susceptibility to invasive aspergillosis following hematopoietic stem cell transplantation

Summary

Invasive aspergillosis (IA) is a life-threatening disease caused by the ubiquitous fungus, *Aspergillus fumigatus*. IA primarily affects immunocompromised individuals, particularly those undergoing hematopoietic stem cell transplantation. Myeloid progenitor cells have been shown to mediate protection against IA, but little is known about the factors that regulate their expansion following transplant. CCR7, via activation by CCL19 or CCL21, is a key trafficking receptor found on naïve lymphocytes, mature dendritic cells, hematopoietic stem cells, and at least one subset of myeloid progenitor cells. In our studies, we investigated the role of CCR7 on hematopoietic stem cells and myeloid progenitor cells in a mouse model of IA following hematopoietic stem cell transplantation. We found that wild-type mice reconstituted with both CCR7-/- HSCs and myeloid progenitors showed a significant decrease in fungal burden as well as a modulation in proinflammatory cytokine production. Enhanced numbers of immune cells were found in spleen, lung and bone marrow of these CCR7-/- chimeric mice. Wild-type mice receiving an equal mix of wild-type and CCR7-/- stem cells and myeloid progenitor cells revealed a significant proliferative advantage for CCR7-/- hematopoietic cells. In

addition, wild-type chimeras treated with anti-CCR7 or anti-CCL19 had a similar cellular expansion to that observed in CCR7-/- chimeras. Thus, removal of the inhibitory effect of CCR7 by gene depletion or ligand immunomodulation enhances the expansion of myeloid cell populations, thereby accelerating fungal clearance in A. fumigatus challenged mice. Our data thus reveals a novel inhibitory role for CCR7 on HSC and progenitor cell proliferation.

Introduction

Invasive aspergillosis (IA) is an acute, life-threatening disease in which inhaled conidia from *A. fumigatus* are not adequately cleared from the lung. Consequently, conidia germinate into hyphae and invade the lung parenchyma and vasculature, leading to severe pneumonia, massive inflammation, and fungal dissemination (181). IA primarily affects immunocompromised individuals, including solid organ transplant recipients, leukemia and bone marrow failure patients, AIDS patients, and recipients of hematopoietic stem cell transplantation (HSCT) (78, 202). Invasive aspergillosis is surprisingly difficult to diagnose and treat, and new therapeutic options are needed, particularly in patients undergoing allogeneic HSCT, who have a 10% risk of developing IA (82). Neutropenia was originally recognized as a major risk factor in the development of IA, but recent work has demonstrated that several effector cell populations, including natural killer cells, T cells, macrophages and myeloid dendritic cells (DC), must function in conjunction with neutrophils in the clearance of this pathogen (106, 117, 121, 185, 186).

Enhancing myeloid cell function and presence during IA has been extensively studied in experimental settings with encouraging results (36-39). Common myeloid progenitors (CMP) and granulocyte monocyte progenitors (GMP) are able to reconstitute the entire myeloid compartment of cells derived from granulocytes and monocytes, respectively (41). A recent study showed that an infusion of myeloid progenitor cells, CMP and GMP, during HSCT provided protection from IA in a murine model (38). Protection was mediated by enhanced immune reconstitution, which included a

significant increase in the number of neutrophils present 7-14 days post-HSCT in mice receiving myeloid progenitor cells, compared with mice receiving only hematopoietic stem cells (HSCs). Additional studies revealed that the enhanced fungicidal activity of myeloid progenitor cells was present regardless of whether the transplant was syngeneic or allogeneic, and was not affected by different forms of myeloablative conditioning (36, 39). Thus, myeloid progenitor cell therapy appears to hold some promise in the prevention of IA following HSCT.

In the present study, we examined the role of CCR7 on HSCs and myeloid progenitor cells during IA in a murine model of HSCT. In the previous chapter, we found that CCR7 deficiency on DCs provided protection from IA during antibody-induced neutropenia. In order to utilize a more clinically relevant model of IA, which does not require the use of anti-Gr-1, we challenged mice with conidia two weeks after HSCT, during a period of severe immunosuppression. Additionally, our previous work demonstrated that the lungs of CCR7-/- mice and chimeras contained lymphoid aggregates prior to fungal challenge, which may have aided in the accelerated fungal clearance seen in those mice. To gain a better understanding of the role of CCR7-/- myeloid cells during IA, in the absence of lymphoid aggregates, we used a murine HSCT model in which the hematopoietic compartment of lethally irradiated mice was reconstituted with HSCs and myeloid progenitor cells from WT or CCR7-/- mice.

We found that wild-type (WT) mice receiving CCR7-deficient HSCs and myeloid progenitor cells were markedly less susceptible to IA than WT mice receiving WT HSCs and myeloid progenitor cells. This protective effect was a result of significantly enhanced immune reconstitution in mice receiving CCR7-deficient cells. The bone

marrow compartment, spleen, and lungs of irradiated mice receiving CCR7-deficient cells all showed increased myeloid cell numbers. A competitive reconstitution assay revealed enhanced proliferation of CCR7- deficient cells compared to WT cells. The binding of CCR7 to its ligands CCL19 or CCL21 was blocked using anti-CCR7, anti-CCL19, or anti-CCL21 antibody treatment during and following WT HSCT. Anti-CCR7 treated mice showed a significant increase in pulmonary effector cells during IA when compared to IgG treated mice. In addition, analysis of HSCs in naïve CCR7 knockout mice showed a significant increase in HSC numbers over WT mice. Our studies thus demonstrate that CCR7 negatively regulates HSC and myeloid progenitor cell proliferation, resulting in delayed immune reconstitution following HSCT and consequent susceptibility to IA.

Results

CCR7-/- mice show increased numbers of HSCs and GMPs in bone marrow

Previous studies have shown that CCL19 and CCL21 can inhibit the proliferation of myeloid progenitor cells in vitro (203, 204). Additionally, our lab and others have found the expression of CCR7 gene transcripts in HSCs, CMPs, and GMPs (**Fig. 4.1A**) (205). To examine the effect of CCR7 deficiency in the BM compartment, whole BM from CCR7-/- mice was analyzed to determine HSC, CMP and GMP cell populations in these animals. We found nearly a two-fold increase in the number of HSCs in the CCR7-/- mice compared with WT mice (**Fig. 4.1B**). In addition, there was a smaller percentage of CMPs in the CCR7-/- mice, but a significantly higher percentage of GMPs in these mice, indicating either retention of GMPs in the BM compartment or accelerated development of CMPs into GMPs (**Fig. 4.1C**). These results indicate that HSCs, and possibly GMPs, from CCR7-/- mice proliferate at a faster rate than WT cells, both in a homeostatic or HSCT setting.

CCR7 deficiency on hematopoietic cells is protective during IA following HSCT

To investigate the importance of CCR7 expression by myeloid progenitor cells during IA, we explored its role in a murine model of HSCT followed by an i.t. exposure to *A. fumigatus*. Analyses of histological sections of lungs taken from uninfected mice were similar in both groups. Notably absent from the lungs of CCR7-/- chimeric mice were lymphoid aggregates normally seen in naive CCR7 knockout mice (206) (**Fig. 4.2A and 4.2B**). Hematoxylin and eosin staining of both groups of infected mice reveled

significant cellular infiltration into the lungs, but the inflammatory response appeared to be more prominent in WT chimeras (**Fig. 4.2C and 4.2D**). Lastly, using GMS staining, WT chimeras were found to exhibit extensive fungal growth, which was mostly absent from the lungs of CCR7-/- chimeras (**Fig. 4.2E and 4.2F**). Thus, these results indicate that CCR7 expression on hematopoietic cells appears to impair the development of antifungal immunity following HSCT.

Lung leukocyte recruitment is enhanced in CCR7-/- chimeras during IA

Neutropenia and other immune cell deficiencies have been linked to the development of IA following HSCT. Complete engraftment and rapid proliferation of donor cells following HSCT is necessary to reduce this period of immunodeficiency and provide protection against opportunistic infections. To determine which cell types might be providing a protective effect in the lungs of CCR7-/- chimeras, we used flow cytometry to analyze immune cell subsets in both uninfected chimeric mice and after challenge with *A. fumigatus.* Surprisingly, uninfected CCR7-/- chimeras had significantly higher cell numbers in the lung, and this result was recapitulated in every cell population examined (**Fig. 4.3A**). In addition, 48 hours after *A. fumigatus* infection, effector cell numbers rose in both WT and CCR7-/- chimeras, but again remained statistically higher in the CCR7-/- chimeras, compared with WT chimeras (**Fig. 4.3A**). Of importance to the host immune response during IA, we found that neutrophils, monocytes/macrophages, and DCs were all significantly higher in the CCR7-/- chimeras compared with uninfected and infected WT chimeric mice. However, neutrophil and DC percentages in the lung were similar between CCR7-/- and WT chimeras (**Fig 4.3B**). Thus, these data indicate that the

antifungal protection observed in CCR7-/- chimeras was a consequence of increased effector cell numbers, not an overall skewing of the composition of the cells recruited to the lung.

CCR7-/- chimeras exhibit a modulated inflammatory response following conidia challenge

Proinflammatory cytokines and chemokines are required for an appropriate antifungal immune response in the lung. However, it has recently been reported that uncontrolled inflammation inhibits fungal clearance and enhances morbidity and mortality during experimental IA (150, 190). RNA analysis showed that WT chimeras had significantly higher expression of TNF-α, IFN-γ, and CXCL2, and higher amounts of CXCL10 and IL-12, when compared with CCR7-/- chimeras (**Fig. 4.4A**). Levels of the anti-inflammatory mediators indoleamine 2,3 dioxygenase (IDO) and IL-10 were similar between WT and CCR7-/- chimeras. Analysis of whole lung protein samples from WT and CCR7-/- chimeric mice demonstrated that CCR7-/- chimeras had lower levels of the proinflammatory cytokines TNF-α, CCL2, CCL5, and CCL3, but higher levels of CXCL10 and CXCL9 (**Fig. 4.4B**). Both groups of chimeras had similar levels of the anti-inflammatory cytokine, IL-10. These results suggest that CCR7-/- chimeras are able to modulate the inflammatory response to favor the efficient clearance of *A. fumigatus.*

CCR7-/- progenitor cells have an increased proliferative capacity following HSCT

To evaluate the proliferative advantage of CCR7-/- HSCs and progenitor cells, compared with WT HSCs and progenitors during IA, we generated mixed chimeras by combining

300 HSCs, 5,000 CMPs, and 5,000 GMPs from green fluorescent protein (GFP)-expressing WT mice with an equal number of the same cells from CCR7-/- mice. Recipient mice, expressing CD45.1, were congenic to the donor mice, which expressed CD45.2. This cell transfer approach allowed us to examine the role of donor cells as well as distinguish between WT and CCR7-/- cells during IA (**Fig. 4.5A**). We examined the lungs and spleen of infected mice and found that donor chimerism was greater than 75% in both organs (**Fig. 4.5B**). Interestingly, the percent of donor neutrophils and DCs originating from CCR7-/- mice was significantly greater in these organs than the percent of cells from WT GFP mice. In the lung alone, 75% of effector cells were of donor origin, and of these cells, CCR7-/- DCs were two-fold more prevalent than WT GFP expressing DCs (**Fig. 4.5C**). Thus, these data suggest that CCR7-/- HSCs and progenitor cells proliferate more robustly compared to these same cell populations from WT mice.

Antibody-mediated neutralization of CCR7 enhances immune cell reconstitution

Naïve CCR7-/- mice are known to have significant immune abnormalities, most notably the development of autoimmunity, which is partially a result of improper regulatory T cell function (172, 173). To address whether the results from CCR7 deficient HSCs and progenitors cells might be a consequence of this abnormal immune response, WT chimera mice were treated with either anti-CCR7 or control IgG for 14 days following HSCT. Upon challenge with *A. fumigatus*, mice treated with anti-CCR7 demonstrated increased numbers of effector cells in the lung, similar to that seen in CCR7-/- chimeras (**Fig. 4.6A**). In addition, CMP and GMP numbers were significantly increased in mice receiving anti-CCR7 antibody therapy, though HSC numbers were comparable to control

mice (**Fig. 4.6B**). To examine whether the hematopoietic expansion following the blockade of CCR7 was related to one of its ligands, we preformed a HSCT and treated the WT chimeras with IgG, anti-CCl9, or anti-CCL21 every other day for 14 days. Mice treated with anti-CCL19 exhibited a significantly greater hematopoietic expansion in the spleen and BM compartment, compared with IgG treated mice (**Fig. 4.6C**). These results indicate that blocking CCR7 or its ligands recapitulates the results seen with CCR7-/- HSCs and progenitor cells, and again confirms a regulatory role for CCR7 in HSC and progenitor cell proliferation.

Anti-Gr-1 treatment following HSCT enhances susceptibility to IA

Neutrophils are a major effector cell type during antifungal immune responses to IA, and have been shown to play a pivotal role in fungal clearance following HSC and myeloid progenitor cell transplantation (38, 185). To assess the contribution of neutrophils in our model of IA following HSCT, anti-Gr-1 was administered to WT and CCR7-/- chimeras 24 hours prior to an i.t challenge of *A. fumigatus* 14 days after transplantation. Both WT and CCR7-/- chimeras had substantial cellular infiltrate in the lungs 48 hours after fungal challenge (**Fig. 4.7A and B**). In addition, significant fungal growth was seen in both mice, but it was more pronounced in the WT chimeras (**Fig. 4.7C and D**). Analysis of lung leukocyte populations reveled higher number of total cells, DCs, and monocytes/ macrophages in the CCR7-/- chimeric mice, compared to WT chimeras (**Fig. 4.7E**). Taken together, these data indicate that neutrophils are required for proper fungal clearance following HSCT, but their depletion does not completely diminish the beneficial effects of CCR7 deficiency in the hematopoietic compartment during IA.

Discussion

HSCT patients and other immunocompromised individuals are highly susceptible to opportunistic infections, especially *A. fumigatus*, which can cause a serious, acute disease known as invasive aspergillosis (IA). *A. fumigatus* is normally cleared from the body by effector cells of the innate immune system, including neutrophils, macrophages and monocytes, and DCs. Elimination of these cell types during the pretransplantation myeloablative conditioning stage of HSCT renders the host highly susceptible to infection. Thus, one goal of HSCT clinical research has been to enhance engraftment and proliferation of hematopoietic cells to restore a functional immune system to the patient (207, 208).

The present study demonstrated a deleterious role for CCR7 expression during IA following HSCT in a murine model. When CCR7-/- chimeric mice were challenged with *A. fumigatus*, they showed a significantly reduced fungal burden at 48 hours after infection. Lung histology revealed very little fungal growth as determined by GMS staining in CCR7-/- chimeric mice, whereas significant hyphal growth and invasion into lung airways was observed in WT chimeric mice two days after fungal challenge. Flow cytometry data showed that there were significantly more cells present in the lungs of CCR7-/- chimeric mice compared to WT chimeras. The resultant increase in total number of lung cells in both uninfected and infected mice indicates that there was a proliferative advantage for HSCs and myeloid progenitor cells from CCR7-/- mice compared with WT mice. Of additional interest was the observation that both myeloid

and lymphoid cells were increased in the knockout chimeras, indicating that the CCR7-/- HSCs more rapidly reconstitute the entire immune system following HSCT.

The production of proinflammatory cytokines and chemokines required for antifungal immune responses has been well documented. One study found that neutralizing TNF-α led to rapid mortality in a murine model of IA (142). However, recent reports suggest that a hyper-inflammatory state results in decreased fungal clearance and enhanced mortality during IA (150, 190). These contrasting views may indicate that while inflammation is required for an appropriate immune response to *A. fumigatus*, an effective antifungal response requires a regulated production of cytokines. Our results showed that while there was a significant increase in proinflammatory cytokine production during *Aspergillus* challenge in both WT and CCR7-/- chimeras, the cytokine levels were significantly higher in the WT chimeras. Levels of the anti-inflammatory mediators IDO and IL-10 were at similar levels in both groups of mice. Though it is not immediately clear why CXCL10 and CXCL9 were increased in the CCR7-/- chimeric mice, it has been reported that high levels of CXCL10 production correlate with decreased susceptibility to IA (155). Thus, the balance of proinflammatory and anti-inflammatory mediators produced in the CCR7-/- chimeras appeared to promote a more favorable environment for fungal clearance.

There were several considerations to address in this study when using CCR7-/- mice for this model system. It has been reported that CCR7-/- mice have systemic autoimmunity, appears to impact the bone marrow compartment and affect the development of both HSCs and myeloid progenitor cells (173). Indeed, we saw increases in HSC and GMP numbers in naïve CCR7-/- mice compared with WT mice. These

differences could be the result of a bone marrow environment constantly inundated with inflammatory mediators, or may be the result of regulatory T cell deficiencies in the bone marrow, as these cells are unable to traffic properly (172). To address these concerns, we treated WT chimeric mice with an anti-CCR7 antibody for 14 days following HSCT. These mice had an identical phenotype to our CCR7-/- chimeric mice challenged with *A. fumigatus*, whereas control-treated mice looked no different than WT chimeric mice. In this experiment, it was also evident that while HSC numbers were not altered between the two groups, there was a significant increase in the number of myeloid progenitor cells in the anti-CCR7 treated group. These results indicate that depleting or blocking CCR7 enhances engraftment and leads to increased effector cell proliferation, resulting in enhanced immunity to *A. fumigatus*.

Our current working model for the role of CCR7 during IA, following HSCT, suggests that this chemokine receptor negatively regulates HSC and myeloid progenitor proliferation, resulting in a prolonged period of immunosuppression, which leads to increased susceptibility to IA. Studies from the laboratory of Hal Broxmeyer have shown that CCL19 and CCL21 inhibit the proliferation of human myeloid progenitor cells and chronic myelogenous leukemia progenitors in vitro (203, 204). Based on these results, and our observations, it appears that CCR7 deficient HSCs and myeloid progenitors do not interact with CCL19 and CCL21 and thus, are not subject to their anti-proliferative effects. Our transplant model requires 20,600 cells to reconstitute the entire hematopoietic system, thus, any anti-proliferative effects of CCL19 and CCL21 are greatly magnified following infection with *A. fumigatus*. Due to the increase in proliferation of CCR7-/- HSCs and progenitor cells, compared to WT cells, CCR7-/-

chimeric mice have greater numbers of effector cells, including DCs, neutrophils, and macrophages, in the lung prior to fungal challenge. Therefore, greater numbers of conidia are eliminated prior to germination in CCR7-/- chimeras than in WT chimeric mice. In addition, we have previously shown that CCR7 deficiency reduces the migration of DCs away from the lung during IA, thus providing a protective antifungal effect, which is also seen in CCR7-/- chimeras during IA, following HSCT. Due to the enhanced clearance of *A. fumigatus*, CCR7-/- chimeras also have a more regulated inflammatory response compared to WT chimeric mice. Recent studies have shown that excessive inflammation increases morbidity and mortality during a murine model of IA (150, 190). Thus, the CCR7-/- chimeras are protected from both invasive fungal disease and the accompanying hyper-inflammatory response that occurs during unresolved IA.

IA is a serious and highly lethal disease affecting immunocompromised patients, particularly HSCT recipients. IA is very difficult to diagnose and antifungal treatments remain highly cytotoxic. In this study, we demonstrate a novel, inhibitory role for the chemokine receptor CCR7, which is a key trafficking receptor for immune cells during homeostasis as well as upon pathogenic stimulation. During IA, CCR7 additionally impacts the proliferation of HSC and myeloid progenitor cells, acting to modulate the presence of these cells as well as their effectors, which inhibits an appropriate immune response to IA. CCR7 thus represents a potential new avenue of research in the effort to develop more effective treatments for IA.

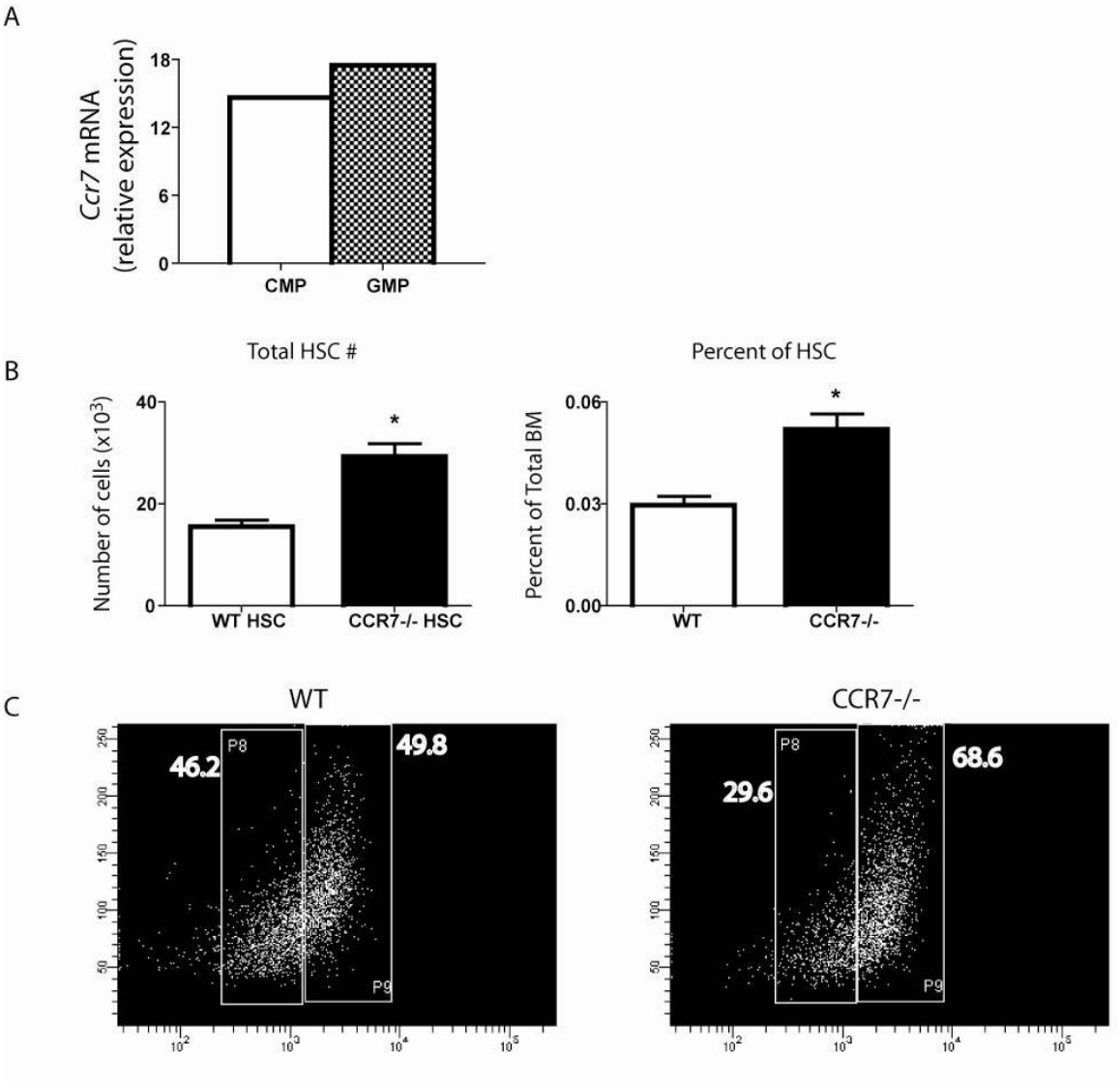

FIGURE 4.1. Analysis of HSCs, CMPs, and GMPs from the BM of naïve WT and CCR7-/- mice. *A*, CCR7 gene expression from FACS sorted WT CMPs and GMPs was analyzed by real-time PCR. *B and C*, Whole BM from naïve WT and CCR7-/- mice was stained for the presence of HSCs (B) and CMPs or GMPs (C) using cell surface markers for lineage positive cells, CD34, Sca-1, c-Kit, and CD16/32.

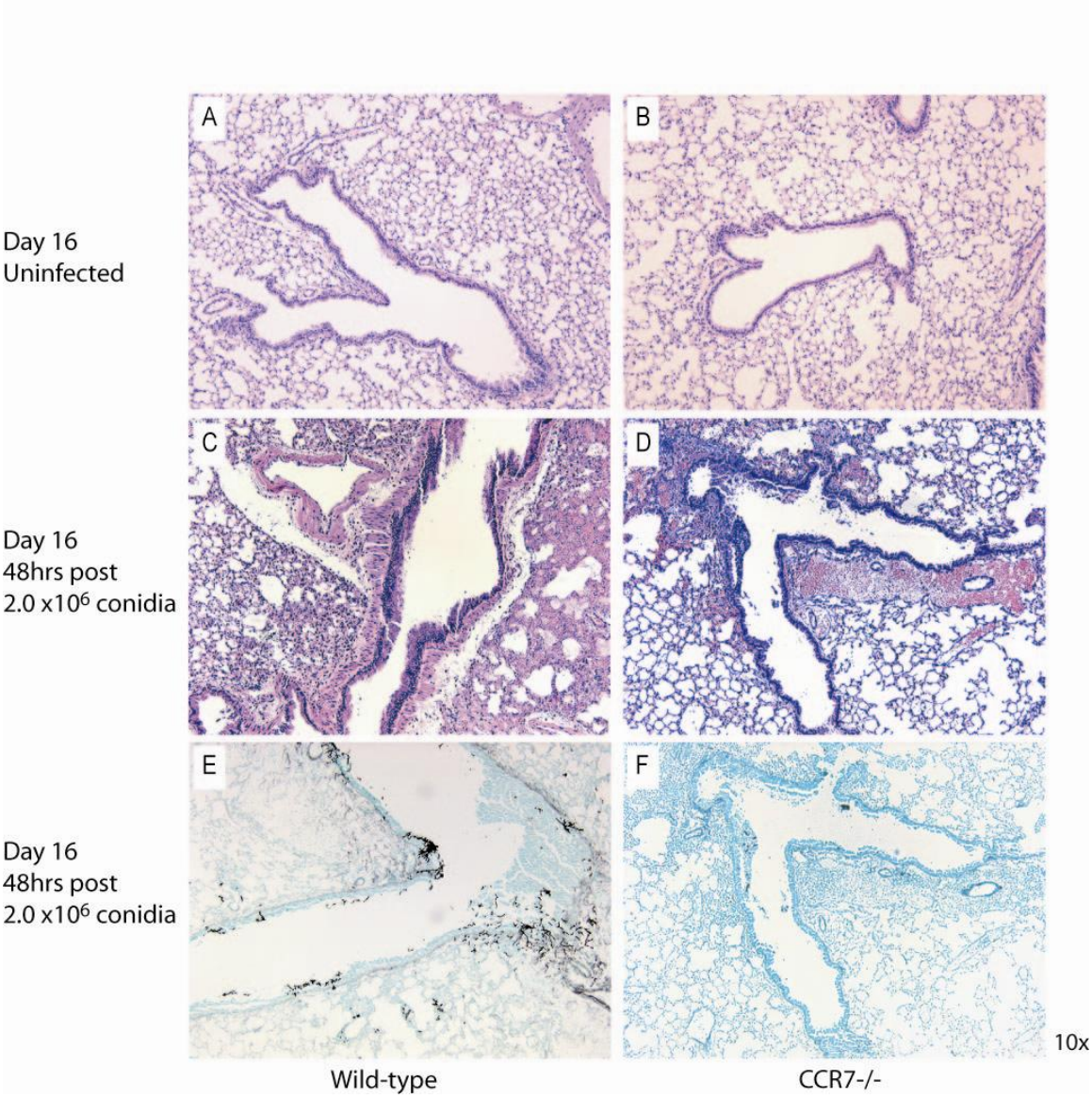

FIGURE 4.2. Histology of WT and CCR7-/- chimeras challenged with *A. fumigatus* following HSCT. WT mice were lethally irradiated and reconstituted with 600 HSCs, 10^4 CMPs and 10^4 GMPs from WT or CCR7-/- mice. Two weeks after transplant mice were given an i.t. injection of 2.0 x10^6 conidia. A-F, Histologic analysis of lung tissue two days after conidia challenge. H & E staining of uninfected lungs (A and B) or 48 hours after conidia challenge (C and D). *E and F*, representative GMS stained sections from wild-type (E) or CCR7-/- (F) chimeras following conidial challenge, fungal elements are stained in black. Original magnification was 10x, for H & E and GMS stained sections.

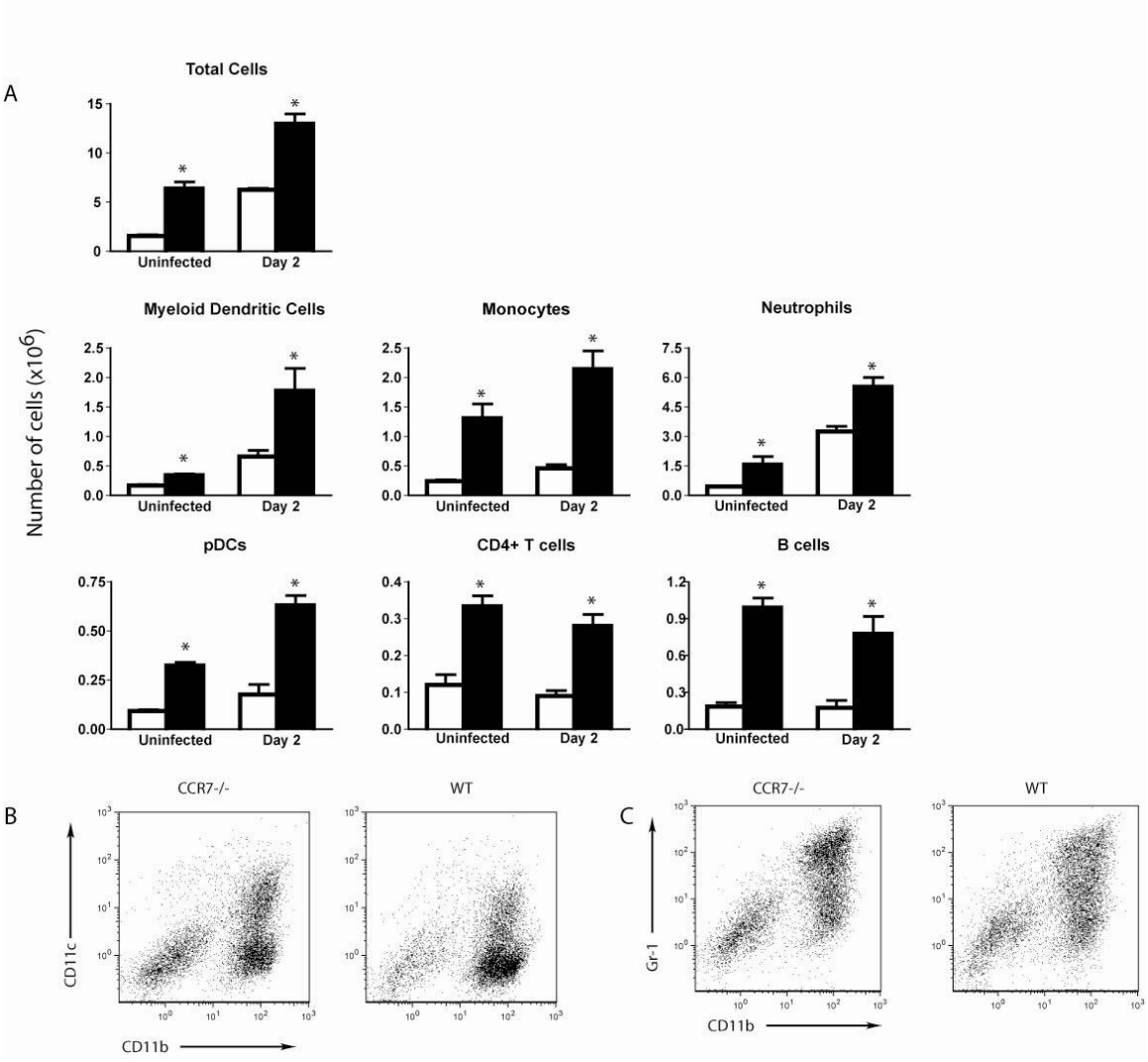

FIGURE 4.3. Leukocyte influx into the lungs prior to, and following, *A. fumigatus* challenge in WT and CCR7-/- chimeras. Fourteen days after HSCT, mice were left unchallenged or were injected with 2.0 x10^6 conidia. Cellular infiltration into the lung was analyzed 48 hours after challenge. A, Myeloid DCs were CD11c high, CD11b high cells. Neutrophils were CD11b high, Gr-1 high, and CD11c-. Monocytes/macrophages were CDllb$^+$, F4/80$^+$, and CD11c$^-$. pDCs were B220$^+$ and CD11c$^+$, CD4+ T cells were CD3$^+$ and CD4$^+$, and B cells were B220$^+$, CD11c$^-$. B and C, Dot plots of DCs and neutrophils respectively. *P <0.05 when comparing WT and CCR7-/- chimeric mice.

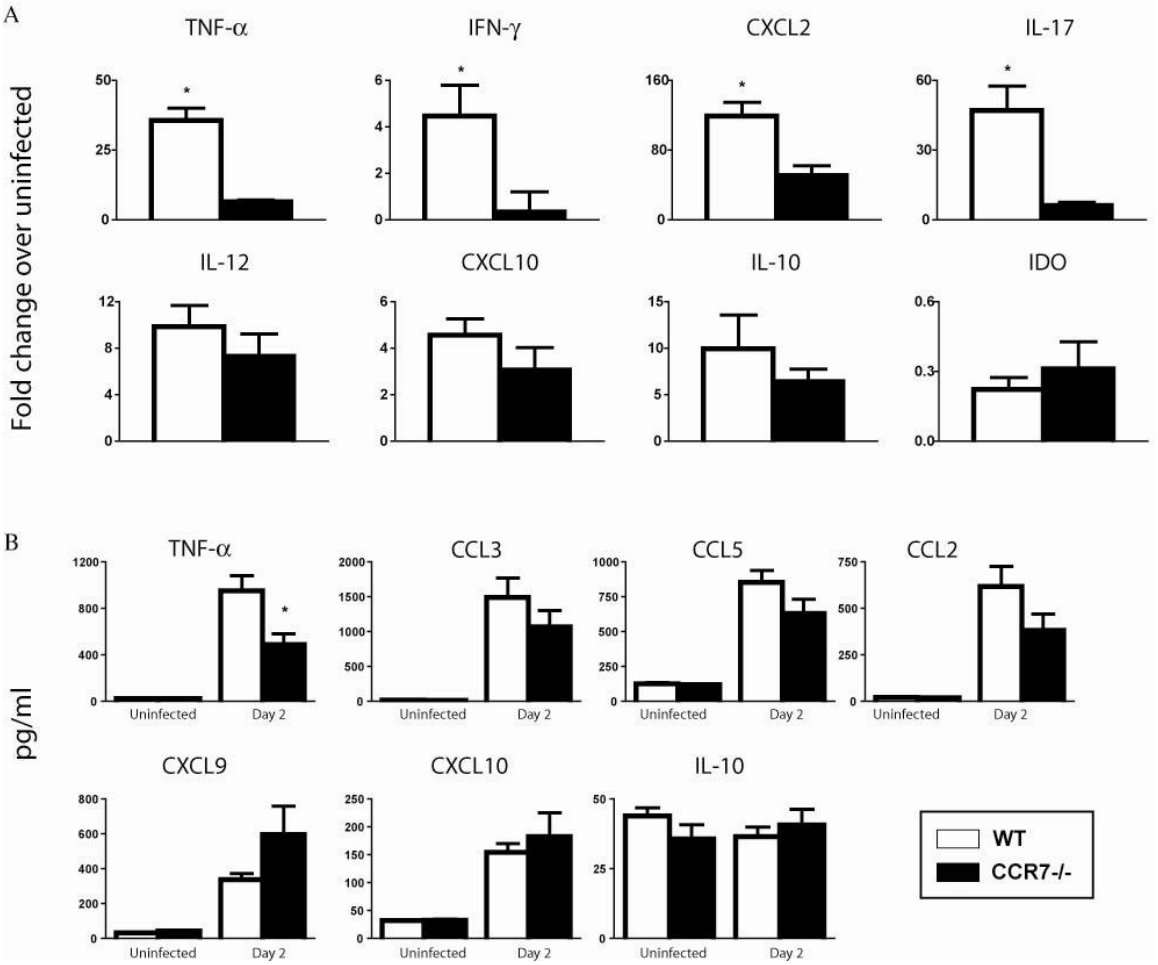

FIGURE 4.4. Whole lung RNA and protein levels 48 hours after conidial challenge in WT and CCR7-/- chimeras following HSCT. A, RNA levels were determined by real-time PCR. Values represent the fold change of infected mice compared with uninfected mice. B, Cytokine and chemokine levels were determined using a Bioplex multiplex assay; all values are represented as pg/ml. **P* <0.05 when comparing WT and CCR7-/- chimeras following conidia challenge.

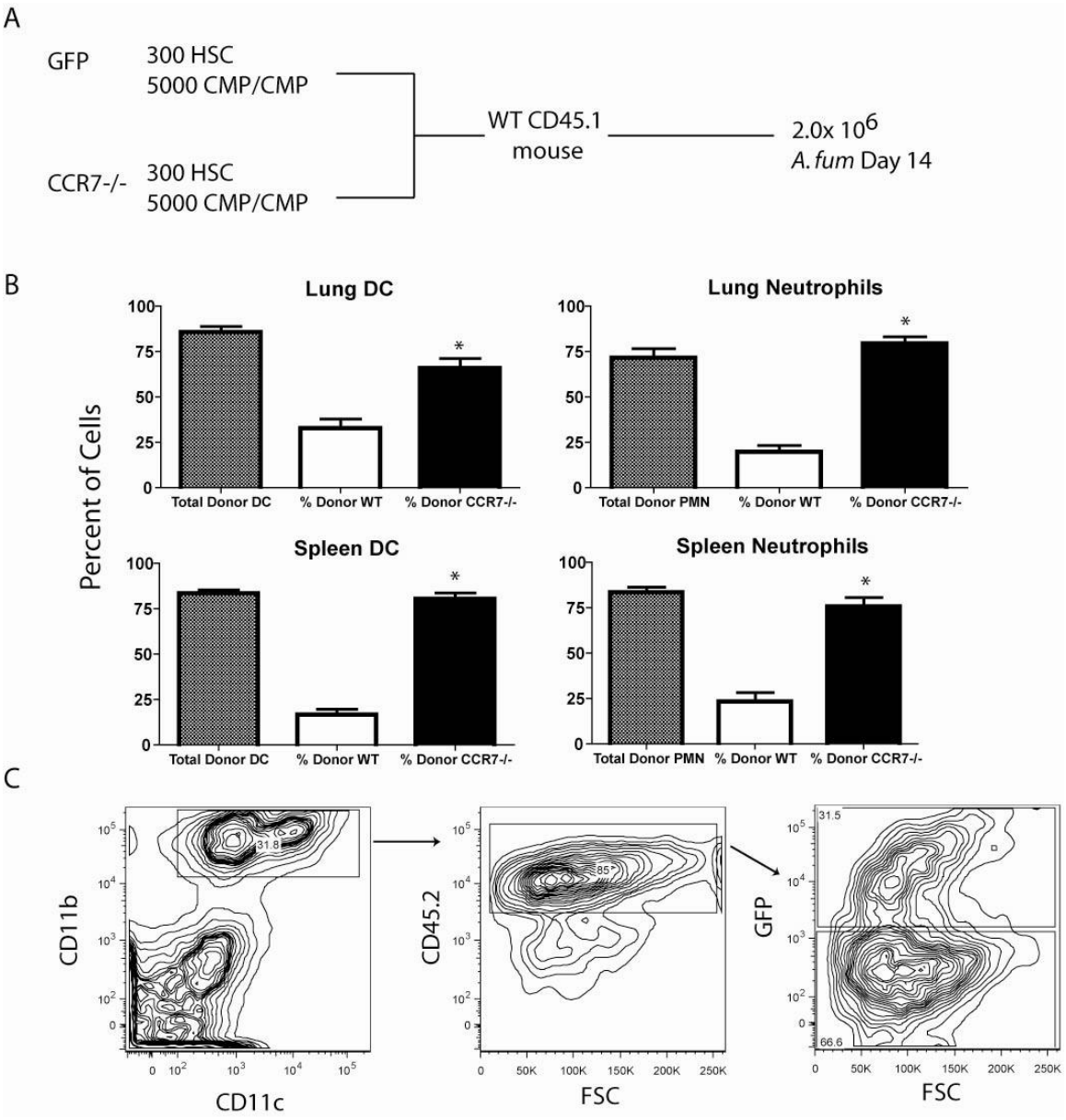

FIGURE 4.5. Competitive reconstitution between WT and CCR7-/- HSCs, CMPs, and GMPs following HSCT. A, Experimental scheme in which WT CD45.1 mice were lethally irradiated and reconstituted with 300 HSCs, 10^4 CMPs, and 10^4 GMPs from CD45.2 WT GFP^+ and CCR7-/- mice. Fourteen days after HSCT, mice were challenged with 2.0 x10^6 conidia and were analyzed by flow cytometry 48 hours later. B, Percent of total, WT and CCR7-/-, donor cells in the lungs of chimeric mice following conidial challenge (gray bars), and the percent of donor cells derived from WT or CCR7-/- mice (white and black bars). C, Contour plots showing donor derived DCs from WT GFP^+ or CCR7-/- mice. *P <0.05 when comparing GFP^+ and CCR7-/- cells following HSCT.

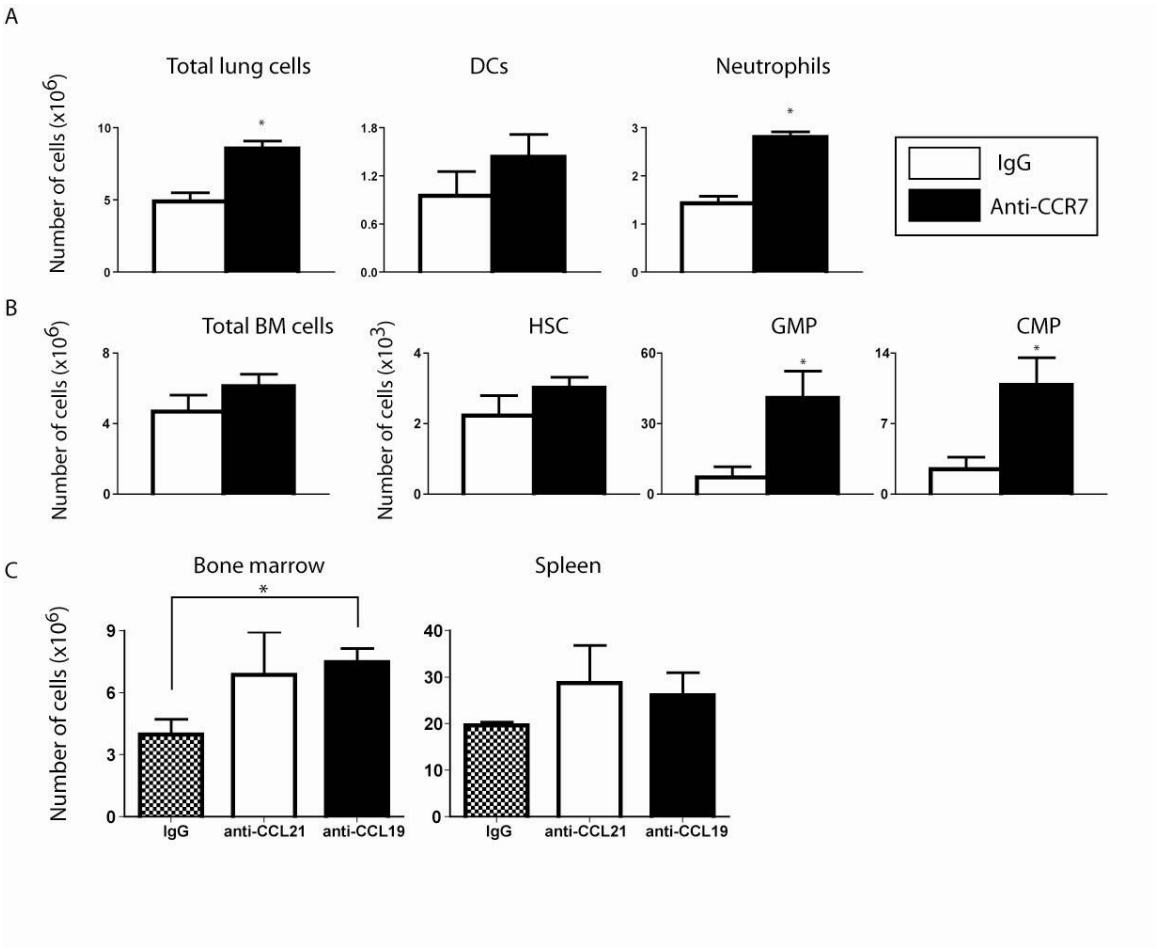

FIGURE 4.6. Effects of antibody treatment on hematopoietic proliferation following HSCT. WT mice were treated with 250μg of anti-CCR7 (A and B), anti-CCL19 or anti-CCL21 (C), or IgG (A, B, and C) every other day, for 14 days following HSCT. A and B, Fourteen days after HSCT and anti-CCR7 treatment, mice were challenged with 2.0 x10^6 conidia. Lungs (A) and BM (B) were analyzed 48 hours after infection. C, The number of cells present in the BM and spleen of uninfected HSCT mice treated with anti-CCL19 or anti-CCL21. *P <0.05 when comparing anti-CCR7 or anti-CCL19 and IgG treated chimeras.

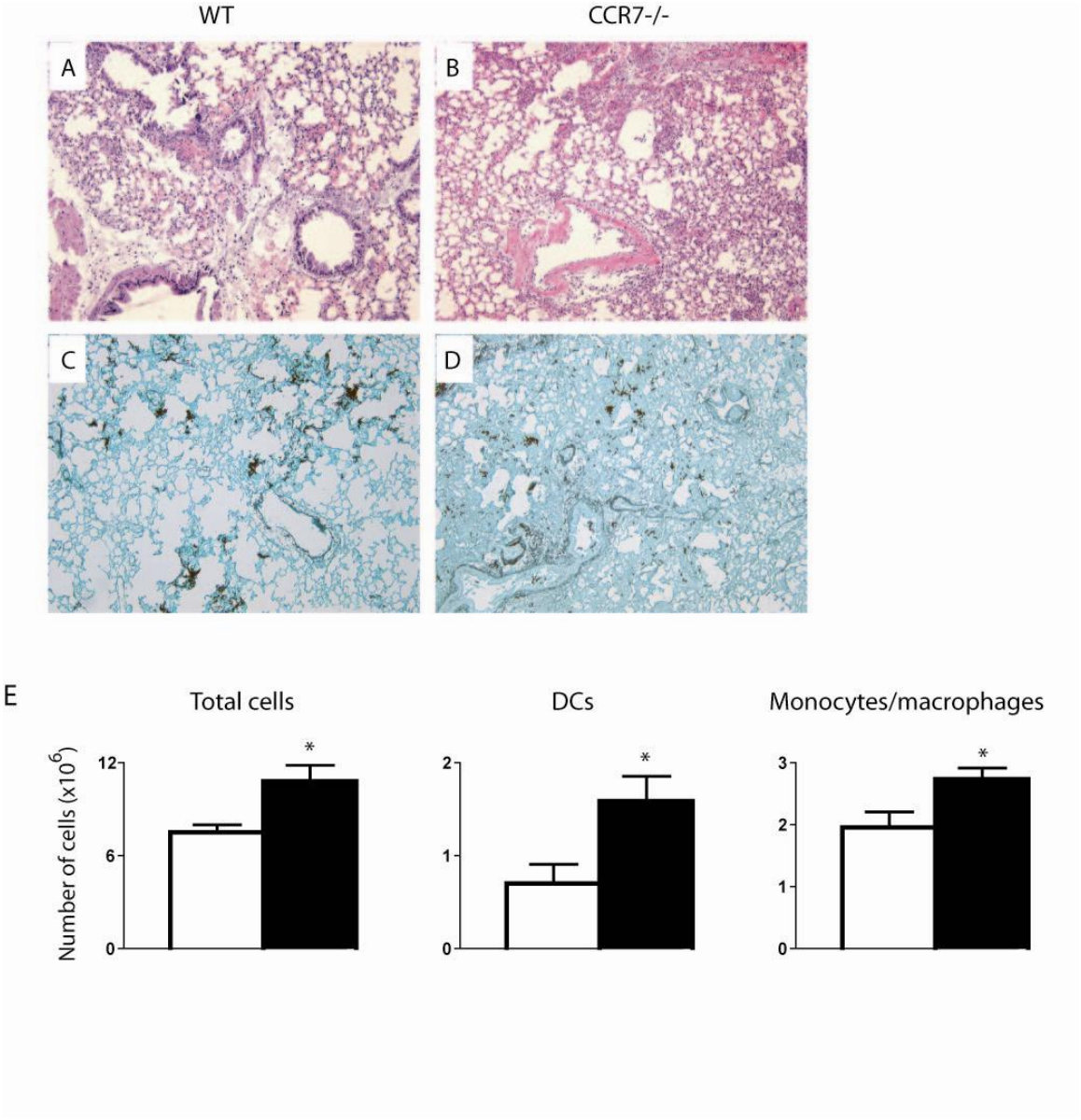

FIGURE 4.7. Effect of anti-Gr-1 treatment during IA following HSCT. WT or CCR7-/- chimeras were treated with anti-Gr-1 13 days after HSCT, and challenged with conidia 24 hours later. *A-D*, Histologic analysis of lung tissue two days after conidia challenge. A and B, H & E staining of lungs 48 hours after conidia challenge. C *and D*, Representative GMS stained sections from WT (C) or CCR7-/- (D) chimeras following conidial challenge. Original magnification was 10x for all images. E, Cellular infiltration into the lungs of WT and CCR7-/- chimeric mice following conidial challenge. *P <0.05 when comparing WT and CCR7-/- chimeras following conidia challenge.

Chapter 5

Conclusion

CCR7 hampers antifungal innate immunity

The development of new therapies to reduce the duration and severity of immunosuppression in patients susceptible to IA, and other pathogens, is a clinically relevant goal. Herein, we have shown that CCR7 plays a modulatory role on the immune response to *A. fumigatus.* The use of anti-Gr-1 to induce transient neutropeina in mice, thereby making them susceptible to IA, has been well established and used by several investigators to study *A. fumigatus* infection (117, 119, 152-154). Using this model, we found that CCR7-/- chimeric mice (WT mice reconstituted with a CCR7-/- hematopoietic system) were far less susceptible to IA than WT chimeras (WT mice reconstituted with a WT hematopoietic system) were. They exhibited increased survival, decreased fungal burden, and a modulated cytokine response. The enhanced protection seen in CCR7-/- chimeric mice appeared to be the result of an increase in myeloid DC numbers in the lung, and this result is consistent with the finding that CCR7 is upregulated on DCs upon pathogenic insult. In this neutropenic mouse model, we found that CCR7 expression on DCs appeared to promote their movement out of the lungs of infected mice, thereby negating their effector role in the lung, and leaving the neutropenic mice susceptible to

IA. Thus, in this model, CCR7 deficiency prevented the trafficking of DCs from the lung to the draining lymph nodes, allowing the DC to continue to provide an effector response at the site of infection (**Fig 5.1**).

We have also shown that CCR7 inhibits hematopoietic stem cell (HSC) and myeloid progenitor cell proliferation in a murine model of IA following hematopoietic stem cell transplantation (HSCT) (**Fig 5.2**). This negative regulatory effect of CCR7 prolonged the period of immunosuppression in mice following HSCT, and left them susceptible to the development of IA. Taken together, our data suggest that targeting CCR7 or its ligands, CCL19 and CCL21, could prove beneficial during IA. Clinical studies are certainly warranted at this time to examine the expression of CCR7 by various effector cell types in patients with IA.

IA following neutrophil depletion by anti-Gr-1

Though our study shows a detrimental role for CCR7 during IA, there are two pitfalls to our experiments that require further discussion: 1) The use of anti-Gr-1 to induce neutropenia in a murine model, and 2) the observation that CCR7-/- mice are prone to multi-organ autoimmunity.

Gr-1 is highly expressed on neutrophils, but has also been show to be expressed on plasmacytoid DCs (pDCs), monocytes, and other immature myeloid cells (209-211). In our studies, we used the monoclonal antibody RB6-8C5 (anti-Gr-1) to deplete neutrophils, but in reality we are likely depleting pDCs as well as immature myeloid cells. Consequently, our results might reflect the effect of depleting these cells rather

than neutrophils. Therefore, an additional model of IA to test our hypothesis is certainly warranted, but unfortunately there are few options in this regard. Administration of cyclophosphamide four days prior to a challenge with *A. fumigatus* has been shown to induce neutropenia in mice, which lasts for 10 days (142). However, this model is also characterized by major effects on regulatory T cells (Tregs). While the role of Tregs is controversial during IA, it is thought that this cell has important regulatory effects on the antifungal immune response (212).

CCR7-/- mice have significant generalized multi-organ autoimmunity, which leads to autoreactive T and B cells, but also to the production of proinflammatory cytokines. The autoimmunity associated with CCR7 deficiency is compounded by a lack of functional Tregs in vivo (172). The failure of Tregs to effectively travel to secondary lymphoid organs leads to the generation of lymphoid aggregates in the lung, gut, and other mucus membranes in the absence of pathogenic stimuli (173, 206). In addition, these mice lack functional secondary lymphoid organs including lymph nodes and Peyer's Patches. We attempted to circumnavigate these problems via the use of bone marrow chimeras, but secondary lymphoid tissue appeared in the lungs of WT mice given CCR7-/- BM. Another approach to eliminate the concerns regarding the use of CCR7-/- knockout mice involves the transient administration of an antibody targeting its ligands. Indeed, we observed in our HSCT model, that the administration of 250μg/mouse of anti-CCR7, CCL19, and CCL21 antibodies every other day had a significant impact on stem cell proliferation and subsequent immune cell functions during IA. Future studies will address the impact of the administration of anti-CCR7, and potentially its ligands, during fungal challenge. We expect that an antibody approach will alter the dynamics of DC

movement from the lung following encounter with *A. fumigatus*, confining them to the lung and thus enhancing their antifungal response.

The impact of CCR7 deficiency on the BM compartment and DC phenotype

One of our more interesting findings arose when we examined the differences between WT and CCR7-/- bone marrow derived DCs (BMDCs). We observed that both uninfected and conidia-challenged cultures of BMDCs were more activated and mature when they originated from CCR7-/- mice. Also, CCR7-/- BMDCs had higher levels of proinflammatory cytokine production, increased CD86 expression, and were better able to stimulate proliferation of allogeneic T cells, following fungal challenge. One explanation for the differences in BMDC phenotype might relate to the autoimmune environment in CCR7-/- mice. Two separate approaches could be taken to look first at how autoimmunity affects the BM: 1) Assess the effects of autoimmunity due to improper thymic education and 2) determine the role of non-functional Treg induced autoimmunity on the BM compartment.

To assess the role of autoimmunity due to improper thymic education during HSC and myeloid progenitor cell development, and consequent DC maturation, CCR7-/- BMDCs could be compared to BMDCs from other autoimmune mice such as autoimmune regulator knockout mice (Aire-/-). The advantage of Aire-/- mice is that they have fully functional Tregs both in vitro and in vivo, thus, phenotypic similarities between Aire-/- and CCR7-/- mice could be attributed to the development of an autoimmune phenotype, and not due to alterations in Treg function (213). Autoimmune

similarities exist between Aire-/- and CCR7-/- mice. Aire induces thymocyte self-tolerance by inducing the expression of peripheral-tissue antigens in thymic epithelial cells, meaning that Aire-/- T cells are not educated for self-tolerance and these mice develop autoimmunity. Similarly, it has been reported that CCR7 is required for proper T cell development in the thymus, as this receptor is required for intrathymic migration (162). Therefore the effects of autoimmunity, as a result of improper thymic education, in the BM of CCR7-/- mice could be elucidated.

To determine whether Tregs regulate the normal development of myeloid hematopoietic cells, mixed congenic chimeras could be made. Mixing CD45.1 WT BM and CD45.2 CCR7-/- BM to create chimeras would allow us to examine a potential role of WT Tregs on hematopoietic myeloid development. Following engraftment, myeloid progenitor cells would be sorted based on their expression of CD45, and in vitro differentiated into BMDCs in conjunction with the same cells from WT and CCR7-/- animals. Provided that functional changes in CCR7-/- HSCs and progenitor cells is not permanent, this approach would allow us to determine whether Tregs play a role in maintaining BM homeostasis, and have any effect on the maturation status of BMDCs.

CCR7-/- HSCs and myeloid progenitors have accelerated homeostatic proliferation following HSCT

We have shown that immune reconstitution following HSCT is accelerated in WT mice receiving CCR7-/- HSCs, CMPs, and GMPs (**Figure 5.2**). This period of enhanced engraftment and reduced immunosuppression proved to be beneficial during infection

with *A. fumigatus*. The numbers of effector cells, including neutrophils, macrophages and monocytes, and DCs, were all elevated in the lungs of CCR7-/- chimeric mice, prior to and during infection. This enhanced cellular response, in combination with the production of appropriate proinflammatory Th1 type cytokines and anti-inflammatory mediators rendered mice far less susceptible to IA than mice with functional CCR7 on hematopoietic cells. In addition, we showed that HSCs and myeloid progenitors from CCR7-/- mice had a significant proliferative advantage over corresponding WT cells. This advantage was seen in both a competitive reconstitution assay and in naïve WT and CCR7-/- mice. Though the mechanism behind this enhanced proliferation remains unclear, immune signaling pathways are likely involved.

The effects of CCR7 deficiency on proliferation and apoptosis signaling pathways

One explanation for the rapid engraftment of CCR7-/- HSC and myeloid progenitor cells might be related to SCF and its receptors. Stem cell factor (SCF) is the ligand for the receptor c-kit, which is found on HSCs, CMPs, and GMPs. This ligand-receptor interaction is responsible for survival, proliferation, and differentiation of these hematopoietic cells (214, 215). Several studies, though not directly, have made a connection between c-kit and CCR7 expression. One study showed that c-kit signaling led to suppressor of cytokine signaling-1 (SOCS1) upregulation, which in turn suppressed SCF-dependent proliferation, but maintained cell survival signals sent via receptor-ligand interactions in a myeloid precursor cell line (216). It has also been shown that SOCS1 can regulate the expression of CCR7 (217). SOCS1- deficient mice have a similar

phenotype to CCR7-/- mice, exhibiting inflammatory cell infiltration into the skin and eyes. In addition, SOCS1-deficiency correlates with a significant reduction in CCR7 expression on T cells, limiting their migratory potential. Taken together, this data suggests that CCR7 and SOCS1 may be intrinsically linked; a deficiency in one may lead to a deficiency in the other. Therefore, should CCR7-/- HSCs, CMPs, or GMPs exhibit a decrease in SOCS1 expression, they might have enhanced proliferation, as SOCS1 is unable to limit the proliferation signal sent from SCF binding to c-kit. To better understand this process and other signaling events that differ between WT and CCR7-/- HSCs, CMPs and myeloid progenitors, future experiments would be directed toward the analysis of RNA and protein to examine the expression pattern of hematopoietic genes in the BM. One small drawback is the small number of HSCs obtained from one mouse, thus BM from 40-60 animals would be required. Luckily, CCR7-/- mice have significantly higher numbers of HSCs, requiring far fewer mice. Protein differences would be analyzed using a Bio-Rad system in which phosphorylated proteins can be differentiated from non-phosphorylated proteins, helping to distinguish between activated and inactive proteins.

In addition to differences in gene and protein expression patterns during cell signaling, a more macroscopic view of differences between WT and CCR7-/- HSCs and myeloid progenitors might be gained by examining proliferation and apoptosis in these cell types. Apoptosis could be measured using the Transferase-mediated dUTP Nick-End Labeling (TUNEL) assay, and by flow cytometry by examining the expression of Annexin V on these hematopoietic cells. Proliferation may be assessed using propidium iodide (PI), or 7-Amino-actinomycin D (7-AAD), to examine the cell cycle. Since PI

binds to DNA, differences in the cell cycle can determined based on the intensity of the PI signal during flow cytometry, as dividing cells contain twice as much DNA as resting cells. Together, these studies would elucidate the role of CCR7 in the proliferative capacity of HSCs and other progenitor cells.

In addition to the above studies, we could additionally explore the effects on progenitor proliferation using the CCR7 ligands, CCL19 and CCL21. A study by Kim et al. demonstrated that recombinant human CCL21 was able to suppress human myeloid progenitor cell proliferation (204). Proliferation of progenitor cells was reduced by as much as 50% with the addition of 30-40 ng/ml of recombinant CCL21. In our studies, we would examine the effects of recombinant murine CCL19 and CCL21 during culture of WT HSCs, CMPs, and GMPs. The minimum media required for the proliferation and differentiation of HSCs in vitro is a-MEM with FCS, BSA, murine SCF, human Thrombopoietin (TPO), murine IL-3, and human erythropoietin (EPO). Using these culture conditions, one HSC per well, in a 96 well plate, would proliferate and form a colony in about 14 days. CCL19, CCL21, or a combination of both could be used to assess the inhibitory effects of these cytokines. In addition, cellular RNA and proteins could be analyzed to determine whether or not signaling differences occur between cells treated with recombinant cytokines. A final study could be conducted in which CCR7-/- HSCs or myeloid progenitor cells were exposed to recombinant CCL19 or CCL21 to determine if any signaling pathways other than CCR7 exist for these cytokines.

The role of common lymphoid progenitors during IA

Our studies have focused on the role of CMPs and GMPs following HSCT and their beneficial role during IA. In addition to myeloid progenitors, which give rise to all cells from the myeloid lineage, a common lymphoid progenitor (CLP) exists, giving rise to all lymphocytes and lymphoid-derived cells (218). We chose not to look at CLPs in our HSCT model, and were thus able to examine the role of CCR7 deficiency in the lung environment devoid of lymphoid aggregates and autoimmunity associated with improper trafficking of CCR7-/- Tregs (173, 206). Although CCR7 deficiency might not be a therapeutic option for CLPs, it would still be very informative to know whether a lack of CCR7 on these cells resulted in increased proliferation following HSCT. Our murine model of HSCT would remain the same, but CLPs could be used in place of CMPs and GMPs. At 14 days post-transplantation, T cell, B cell, plasmacytoid DC, and NK cell numbers would be determined in WT and CCR7-/- chimeras in the lung, spleen, blood, and BM. A potentially interesting result might be similar findings in the lymphoid and myeloid compartments, showing enhanced expansion of CCR7-/- lymphoid cells compared to WT cells. If this was the case, the use of anti-CCL19, anti-CCL21 or anti-CCR7 could be used to examine the subsequent effects on WT CLPs. A short period of antibody treatment following HSCT could be highly beneficial to reconstituting both the innate and adaptive immune systems, which may be useful in the prevention of other opportunistic infections, without the induction of autoimmunity observed in CCR7-/- mice. In our models, the severity of IA was determined by the presence or absence of effector cells of the innate immune system. Unfortunately, HSCT patients are susceptible

to a wide range of pathogens, including many which require an adaptive immune response. To determine the role (beneficial or otherwise) of CCR7 during lymphocyte expansion, a viral model of murine respiratory syncytial virus (RSV) or cytomegalovirus could be used, both of which have been shown to be clinically relevant infections during HSCT (28, 31). A recent study demonstrated that the addition of CLPs during HSCT provided host protection during cytomegalovirus (37). Thus, the use of CCR7-/- CLPs during a model of viral infection may prove beneficial if they behave similarly to CCR7-/- myeloid progenitor cells.

Impact of CCR7 deficiency during allogeneic HSCT, graft-versus-host disease, and anti-tumor responses

GVHD is a serious complication of allogeneic HSCT. During the disease process, donor T cells, introduced for their anti-tumor activity, develop reactivity toward tissues and organs of the host. GVHD is the leading cause of morbidity and mortality following allogeneic HSCT. Significant research has been devoted to diminishing the effects of GVHD, while maintaining the beneficial graft-versus-tumor (GVT) response the transplant was designed for. Based on the need to prevent or reduce GVHD following HSCT while preserving GVT immunity, several experiments could be performed to determine the effects of CCR7 deficiency on hematopoietic cells during these processes.

A common model for GVHD is the use of BALB/c and C57BL/6 (B6) mice during HSCT. This is a good model for allogeneic HSCT, as BALB/c mice express the MHC class II allele $H2^d$ and B6 mice express $H2^b$. In this model, BALB/c mice would

be the recipients and B6 mice would act as the donors, as our CCR7-/- strain of mice are fully backcrossed to B6 mice. BALB/c mice would be lethally irradiated and reconstituted with T cell-depleted BM and 1.0-2.0 x10^6 splenic T cells from B6 or CCR7-/- mice. HSCs, CMPs, and GMPs could be used in place of whole BM, but the addition of T cells is required for the induction of GVHD. Following transplantation, mice would be followed weekly for survival and the development of GVHD. A scoring system developed by Cooke et al. could be used for evaluating GVHD severity (219). Recent data has suggested that CCR7 is required for the proper function of Tregs, and there is clear evidence for the beneficial role of Tregs in the prevention of GVHD (172, 220, 221). Therefore, HSCTs using mixed donor cells may be required to determine the full benefit of CCR7 deficiency on HSCs and myeloid progenitors. Here, BALB/c mice would again be the recipient mice, but donor cells would come from a mixture of CCR7-/- and B6 mice. Transplantation could be performed using the following combination: B6 BM and B6 T cells, B6 BM and CCR7-/- T cells, CCR7-/- BM and CCR7-/- T cells, and CCR7-/- BM and B6 T cells. In addition, WT or CCR7-/- Tregs could be given as supplemental cellular therapy during any donor cell combination. The desired outcome of using CCR7-/- HSCs and myeloid progenitor cells would be to see a reduction in GVHD. However, given the beneficial effect of CCR7 deficiency on myeloid cells during IA following HSCT, GVHD levels similar to those seen in WT mice would also be sufficient.

Should we observe a beneficial combination using CCR7 HSCs and progenitors during HSCT, the next step would be to evaluate the role of CCR7-/- hematopoietic cells during an anti-tumor response. To examine the GVT response, the murine tumor line

P815 would be used, as this tumor is syngeneic to BALB/c mice and allogeneic to B6 mice. HSCT conditions would be identical to those mentioned above, with 100 to 1000 P815 cells being given in combination with the BM and T cell injections. Mice would be observed for morbidity and mortality and tumor analysis could be performed when mice need to be euthanized. Finally, if an appropriate combination of CCR7-/- HSCs, myeloid progenitors, and T cells in conjunction with or without functional Tregs is found, then experiments examining IA following allogeneic HSCT could be performed to determine whether the experimental conditions still provided a protective benefit during fungal infection. These combinations of experiments would provide insight into three significant clinical obstacles following HSCT: GVHD, the GVT response, and the development of opportunistic infections.

To conclude, IA is a serious life threatening disease, primarily found in immunocompromised patients. We have shown that in two distinct murine models involving immunosuppression that the absence of functional CCR7 provides protection against *A. fumigatus* infection. Although many future experiments should be performed to better address the mechanisms of protection, it is clear that there exists a potential for a therapeutic benefit derived from our studies.

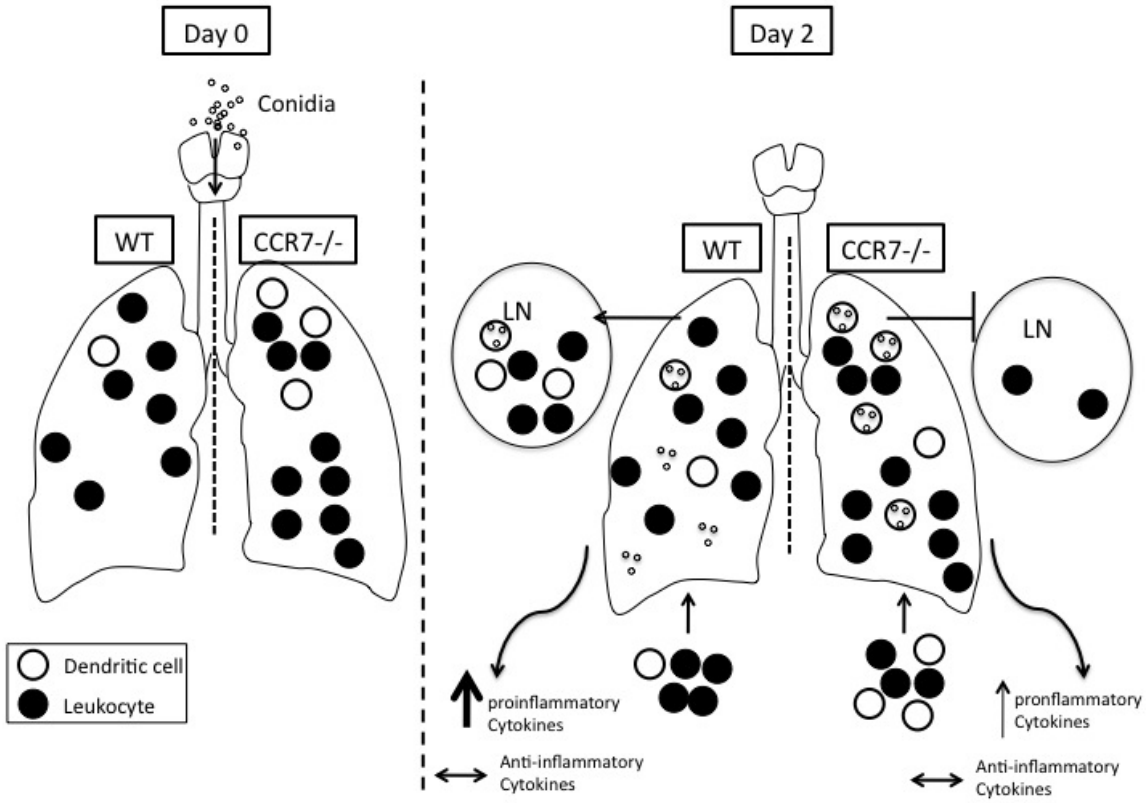

FIGURE 5.1. Working model of enhanced fungal clearance in CCR7-/- mice. Prior to challenge with conidia, the lungs of neutropenic CCR7-/- chimeric mice have significantly more total leukocytes, including twice as many DCs, when compared to the lungs of neutropenic WT chimeras. In contrast to WT chimeras, when CCR7-/- chimeric mice are challenged with *A. fumigatus*, the increased number of lung DCs begins to clear conidia prior to germination and hyphal growth. In addition, CCR7 deficient DCs are unable to respond to the ligands CCL19 and CCL21 and thus do not migrate to the lung draining lymph nodes following A. fumigatus challenge. Consequently, CCR7-/- chimeras have increased fungal clearance as DCs continue to provide an effector response at the site of infection, rather than trafficking to the lymph nodes. The decrease in fungal burden results in a decrease in proinflammatory cytokines and less overall inflammation in the CCR7-/- chimeras, when compared to WT chimeras.

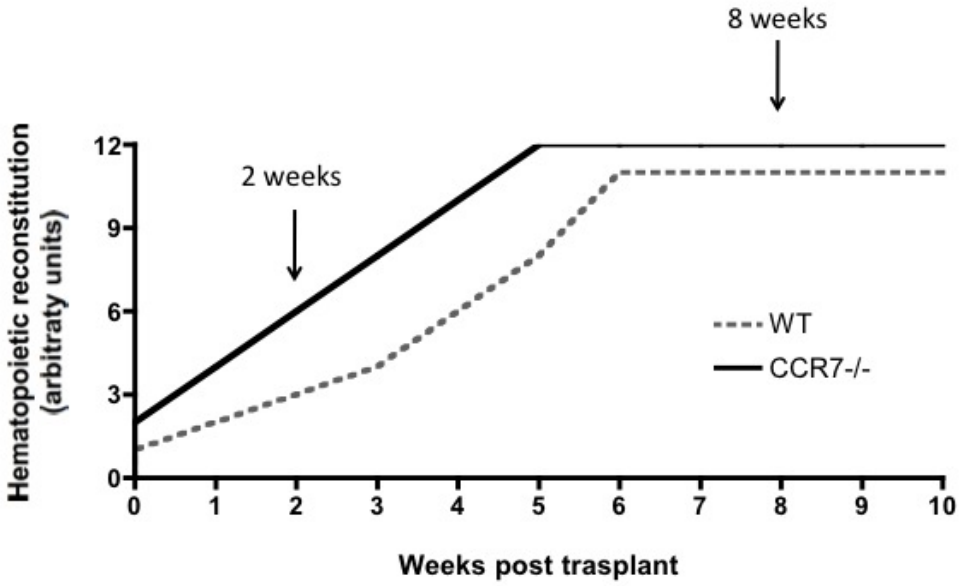

FIGURE 5.2. Model of accelerated hematopoietic reconstitution following HSCT in mice receiving CCR7-/- HSCs and myeloid progenitors. CCR7-/- HSCs, CMPs, and GMPs have a significant proliferative advantage over WT HSCs, CMPs, and GMPs following HSCT into WT mice. At two weeks post HSCT there were more than twice as many cells in the lungs of mice receiving CCR7-/- HSCs and progenitor cells as there were in mice receiving WT HSCs and progenitors. The proliferative advantage of CCR7-/- hematopoietic cells reduced the period of immunosuppression of lethally irradiated mice. As the period of immunosuppression decreases mice are less susceptible to infection with A. fumigatus. By 8 weeks post transplantation WT HSCs and progenitior cells have fully reconstituted the hematopoietic compartment, but absolute numbers are still slightly less in the lung and spleen when compared to CCR7-/- chimeras.

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