Role of sphingosine-1-phosphate (S1P) and S1P receptor 2 in the phagocytosis of Cryptococcus neoformans by alveolar macrophages

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The pathogenic fungus Cryptococcus neoformans is a major cause of morbidity and mortality in immunocompromised individuals. Infection of the human host occurs through inhalation of infectious propagules following environmental exposure. In the lung, C. neoformans can reside in the extracellular environment of the alveolar spaces or, upon phagocytosis, it can survive and grow intracellularly within alveolar macrophages (AMs). In previous studies, we found that sphingosine kinase 1 (SK1) influenced the intracellular residency of C. neoformans within AMs. Therefore, with this study we aimed to examine the role of the SK1 lipid product, sphingosine-1-phosphate (S1P), in the AMs–C. neoformans interaction. It was found that extracellular S1P enhances the phagocytosis of C. neoformans by AMs. Using both genetic and pharmacological approaches we further show that extracellular S1P exerts its effect on the phagocytosis of C. neoformans by AMs through S1P receptor 2 (S1P2). Interestingly, loss of S1P2 caused a dramatic decrease in the mRNA levels of Fcγ receptors I (FcγRI), -II and -III. In conclusion, our data suggest that extracellular S1P increases antibody-mediated phagocytosis through S1P2 by regulating the expression of the phagocytic Fcγ receptors.

INTRODUCTION

Cryptococcus neoformans is a major cause of morbidity and mortality in individuals with an immunocompromised state, particularly among HIV-infected patients, as it is diagnosed in approximately 1 000 000 individuals per year and is responsible for an average of at least 600 000 deaths per year (Park et al., 2009). Upon environmental exposure, infectious C. neoformans propagules are inhaled and enter the host lungs where resident alveolar macrophages (AMs) can internalize the fungal cells to initiate the host immune response. As the central effector function of AMs, phagocytosis of C. neoformans by these phagocytes can lead to the killing of internalized fungal cells, induction of an inflammatory response and the development of a cell-mediated adapted immune response, which is required for host survival. However, C. neoformans is a facultative intracellular pathogen capable of surviving not only in the extracellular environment of the alveolar spaces but also intracellularly within AMs (Feldmesser et al., 2000). Thus, when AMs are unable to kill intracellular C. neoformans, internalization of C. neoformans is detrimental to the host as it provides a protective environment that promotes survival and can exacerbate the dissemination from the lung to other organs (Chrétien et al., 2002; Goldman et al., 2000; Kechichian et al., 2007; Levitz et al., 1999; Luberto et al., 2003; Rittershaus et al., 2006). Therefore, it is important to our understanding of the pathogenesis of C. neoformans to define the host factors modulating the effector functions of AMs, specifically phagocytosis, in order to develop new anticytrococcal therapeutic regiments.

As components of the sphingolipid biosynthesis pathway in mammalian cells, sphingosine kinases 1 (SK1) and 2 (SK2) catalyse the phosphorylation of sphingosine to produce the bioactive lysophospholipid sphingosine-1-phosphate (S1P), which is well documented to regulate numerous

Abbreviations: Ab, antibody; AM, alveolar macrophage; BAL, broncho-alveolar lavage; GXM, glucuronoxylomannan; JTE-013, N-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methylethyl)-1H-pyrazolo[3,4-b]pyridin-6-yl]hydrazinecarboxamide; RT, reverse transcriptase; S1P, sphingosine-1-phosphate; S1PR, S1P receptor; SK, sphingosine kinase.
facets of the immune system (Rivera et al., 2008). The SK1/SIP pathway is particularly important in macrophage function (Weigert et al., 2009) and greatly affects the immune response in the lungs (Garg et al., 2006; Jolly et al., 2002). Previously, we showed that SK1 has a key role in the formation of a granulomatous inflammation in response to pulmonary cryptococcosis (McQuiston et al., 2010). In particular, we found that SK1 is essential for preventing the dissemination of C. neoformans when the host and/or C. neoformans factors promote intracellular parasitism. These previous results indirectly indicate that the product of SK1 activity, SIP, affects the phagocytosis of C. neoformans by AMs.

SIP is an extracellularly secreted sphingolipid that evokes its effects on cells through its binding to a family of five G-protein-coupled receptors (S1P1–S1P5) located on the cell surface. S1P1 is universally expressed on immune cells and is well established to govern the chemotactic effects of extracellular SIP (Rivera et al., 2008). In contrast, the expression profile of the other SIP receptors (S1PRs) is cell-type-dependent, and the effects on the functions of immune cells have not been elucidated. Extracellular SIP has been shown to have numerous effects on macrophages, such as modulating receptor expression (Duong et al., 2004), inducing a proinflammatory state (Hammad et al., 2008) and increasing the killing of internalized bacteria (Allende et al., 2004), inducing a proinflammatory state (Hammad et al., 2008). Previously, we showed that SK1 has a key role in the dissemination of C. neoformans when the host and/or C. neoformans factors promote intracellular parasitism. These previous results indirectly indicate that the product of SK1 activity, SIP, affects the phagocytosis of C. neoformans by AMs.

In this study we examined the effects of extracellular SIP on AMs—C. neoformans interaction and investigated which SIP receptor is involved in this interaction. We show that extracellular SIP increases the phagocytosis of C. neoformans by AMs through S1P2. Using S1P2−/− mice we found that AMs from these mice have decreased expression of Fcγ receptors and, thus, they ingest fewer C. neoformans compared with wild-type AMs. Taken together, these results suggest that the S1P–S1P2 interaction and the consequent Fcγ regulation are important for favouring phagocytosis of C. neoformans.

METHODS

Mouse strains. Five- to seven-week-old wild-type C57BL/6J mice (The Jackson Laboratory), SK1-deficient mice (SK1+/−) and S1P2-deficient mice (S1P2−/−) were used for this research. SK1+/− and S1P2−/− mice were generated previously and colonies were main- tained as described previously (Allende et al., 2004; Kono et al., 2004). All mice were available to us through the Medical University of South Carolina Center of Biomedical Research Excellence (MUSC COBRE) Animal Core Facility, directed by Dr Toshihiko Kawamori, who provided breeding pairs for this study. Travis McQuiston performed all breeding, weaning and genotyping (data not shown). For all experiments, SK1+/− and S1P2−/− mice were age- and sex-matched with SK1+/+/ wild-type mice (C57BL/6J). Both SK1+/− and S1P2−/− mice are isogenic to C57BL/6J mice. Wild-type C57BL/6J mice are also interchangeably called SK1+/+ or S1P2+/+ mice.

Isolation and cell culturing of AMs. AMs were isolated from the lungs of mice using a 1 × sterile PBS bronchoalveolar lavage (BAL). BAL fluid was subjected to centrifugation at 500 g for 10 min. Cell pellets were resuspended in serum-free RPMI supplemented with 0.1% penicillin–streptomycin and cell number was determined by using a haematocytometer. For all co-incubation assays, 1 × 105 cells were plated on the glass portion of a poly-δ-lysine-coated glass-bottomed confocal cell dish (MatTek Corporation). AMs were allowed to adhere for 30 min before the cell dishes were washed three times and fresh media was added. Afterwards, cells were incubated for an additional 90 min prior to experimentation.

C. neoformans strains and growth media. C. neoformans var. grubii serotype A strain H99 is a facultative intracellular pathogen and is universally considered to be a wild-type strain of C. neoformans (WT). C. neoformans cells were grown in yeast extract–peptone–glucose (YPD) medium for 16–18 h at 30 ºC in a shaking cell culture incubator.

Real-time reverse transcriptase (RT)-PCR. mRNA was isolated from AMs using the RNeasy mini kit from Qiagen. cDNA was generated from 0.5 µg RNA using random hexamer primers using the SuperScript III first strand cDNA synthesis system from Invitrogen. Real-time RT-PCR was conducted using a Bio-Rad iCycler to quantify mRNA levels. The standard real-time RT-PCR volume was 25 µl, which comprised 12.5 µl SYBR Green PCR reagents (Bio-Rad), 5 µl cDNA template, 1 µl forward primer (4 µM), 1 µl reverse primer (4 µM) and 5.5 µl water. The sequences of primer pairs for SK isoforms and S1PRs, along with the RT-PCR steps for amplification, were described previously (Argraves et al., 2008; Snider et al., 2009; Xing et al., 2008). All reactions were performed in triplicate. Q-Gene software was used to analyse data, which were then expressed as fold-change mean of normalized expression from control value. As shown, the mean normalized expression is directly proportional to the amount of mRNA of the target gene relative to the amount of mRNA of the reference gene, β-actin. Melt curves were also examined to ensure that the data corresponded to production of the single desired RT-PCR fragment for each target gene. Data represent the average of three separate trials.

In vitro phagocytosis assay. AMs were plated as described above and the desired C. neoformans strain was grown as noted. C. neoformans cultures were subjected to centrifugation at 500 g for 10 min. YPD media was removed and the cell pellet was resuspended in sterile H2O. This was repeated three times. After washing, C. neoformans cells were resuspended in the desired cell media, and the cell number was calculated using a haematocytometer. The volume corresponding to 1 × 106 C. neoformans cells was brought up to a total volume of 1 ml with RPMI containing either 10% mouse serum or 10 µg anti-glucuronoxylomannan (GXM) monoclonal IgG1 antibody 1B87 ml−1 (kindly provided by Arturo Casadevall, Albert Einstein College of Medicine, Bronx, NY, USA) or both 10% mouse serum and antibody. On the day the phagocytosis assay was conducted, mouse blood collected from the hearts of wild-type mice and sera was isolated. C. neoformans suspensions were vortexed vigorously and opsonized with complement, using the freshly isolated mouse serum, and/or with 1B87 antibody for 20 min at 37 ºC. This length of time and serum percentage has been shown by several groups to allow for maximum inactive complement 3b (iC3b) binding and adequate for complement-mediated phagocytosis (Dromer et al., 1989; Kozel & Pfrommer, 1986; Levitz et al., 1997). After opsonization, C. neoformans cell suspensions were centrifuged, washed three times with
serum-free RPMI and finally resuspended to a concentration of \(1 \times 10^6\) \(C.\ neoformans\) cells ml\(^{-1}\). The medium from the confocal dishes containing the AMs was removed and replaced with 100 \(\mu\)l opsonized \(C.\ neoformans\) solution containing \(1 \times 10^5\) \(C.\ neoformans\) cells, thereby making the m.o.i. 1:1. After 2 h co-incubation, the medium was removed, and the plates were washed three times with PBS, fixed in ice-cold methanol and stained with Giemsa stain for analysis by light microscopy using a 100 \(\times\) objective under oil immersion. For each confocal dish, a minimum of 500 macrophages was examined for \(C.\ neoformans\) internalization. As described in the literature, the phagocytic index is the percentage of macrophages with internalized fungal cells multiplied by the average number of internalized fungal cells (Bianco et al., 1975; Taborda & Casadevall, 2002). It is important to note that \(C.\ neoformans\) attachment to AMs was not calculated as part of the phagocytic indices.

**In vitro \(C.\ neoformans\) intracellular growth assay.** The ability of internalized \(C.\ neoformans\) to grow in a co-culture with AMs was examined after 4 h. \(C.\ neoformans\) and AMs were plated and treated as described for the in vitro phagocytosis assay above. To determine the intracellular growth of \(C.\ neoformans\), the medium from co-incubations was removed after 2 h, and plates were washed three times to remove any extracellular \(C.\ neoformans\). Fresh medium was added for an additional 2 h. After a total of 4 h, plates were processed for light microscopy to allow for visualization of daughter cells, also known as ‘buds’. A minimum of 100 internalized \(C.\ neoformans\) per plate was inspected for budding, and intracellular growth was calculated as the percentage of internalized \(C.\ neoformans\) cells with buds in the observed population of internalized fungal cells.

**Statistics.** Data from each experimental group were subjected to an analysis of normality and variance. Statistical significance between the means of two experimental datasets composed of normally distributed values was analysed using Student’s two-tailed \(t\) test. The two-way ANOVA was used when the analysis of the effects of two independent variable concurrently was required. For all statistical tests, \(SD\) with \(P\)-values less than 0.05 were considered significant.

**RESULTS**

**Effect of extracellular S1P on the phagocytosis and intracellular growth of \(C.\ neoformans\)**

The quintessential effector function of AMs is the phagocytosis of microbes, as it is required for subsequent antimicrobial actions of these host phagocytes. Previous studies on *Mycobacterium*–AMs interaction led us to hypothesize that SK1 and S1P may have roles in the antimicrobial actions of AMs against \(C.\ neoformans\). Therefore, to investigate if extracellular S1P affects the actions of AMs against the facultative intracellular pathogen \(C.\ neoformans\), wild-type AMs and wild-type \(C.\ neoformans\) strain H99 were co-incubated in the presence of varying concentrations of S1P, and phagocytosis and intracellular growth of \(C.\ neoformans\) were analysed by light microscopy. The S1P concentrations used in this initial experiment have been demonstrated to induce optimal S1P receptor activation (Hla et al., 2001) and are physiologically relevant to the extracellular environment of the mouse lung (Ammit et al., 2001; Garg et al., 2006).

\(C.\ neoformans\) cells were opsonized with only anti-GXM IgG1 antibody (Ab) 18B7 and the in vitro phagocytosis assay was conducted in serum-free conditions since serum possesses micromolar concentrations of S1P. Treatment with 1 nM and 10 nM S1P were observed to significantly increase the phagocytosis of \(C.\ neoformans\) (Fig. 1a). Greater concentrations of S1P (100 nM and 1 \(\mu\)M) did not significantly increase internalization, possibly due to saturation of the S1P–S1P receptor pathway. S1P increased the phagocytosis of the alveolar-macrophage-derived MH-S cell line and the peritoneal-derived J774.A cell line in nearly an identical pattern to that observed in primary AMs, suggesting the effect of S1P on the phagocytosis of \(C.\ neoformans\) is not exclusive to primary AMs (data not shown). Interestingly, treatment with exogenous S1P did not affect the growth of intracellular \(C.\ neoformans\) after 4 h (Fig. 1b), suggesting that S1P does not induce fungistatic or fungicidal actions of AMs against internalized \(C.\ neoformans\) as occurs in the *Mycobacterium*–macrophage interaction (Garg et al., 2004; Greco et al., 2010).

**Internalization of \(C.\ neoformans\) by AMs occurs through a receptor-mediated phagocytosis**

Although several different types of cell surface receptors recognize various fungal motifs (Levitz, 2002), \(C.\ neoformans\) internalization by host phagocytes requires opsonization. In Fig. 1(a), we found that S1P increased the phagocytosis of IgG1-Ab-opsonized \(C.\ neoformans\). To determine if the S1P-induced increase in phagocytosis is dependent on the host molecule that serves as the opsonin, AMs were co-incubated with \(C.\ neoformans\) cells opsonized with complement, Ab or both complement and Ab with or without S1P. In our hands, AMs have a very low phagocytic index of \(C.\ neoformans\) when opsonized with complement only and, in this case, S1P treatment does not increase phagocytosis (Fig. 2a). On the other hand, S1P increases the internalization of \(C.\ neoformans\) cells when opsonized with Ab only or when opsonized with both complement and Ab (Fig. 2b and c). Notably, \(C.\ neoformans\) were not or very rarely internalized (less than 1%) when they were not opsonized and the addition of S1P did not increase phagocytosis of non-opsonized fungal cells. Together, these results suggest that extracellular S1P increases \(C.\ neoformans\) internalization through an Ab-dependent mechanism.

In a previous study, we showed that AMs isolated from mice lacking SK1 (SK1\(^{-/-}\)) internalize a significantly greater number of \(C.\ neoformans\) cells compared with AMs from wild-type mice SK1/2\(^{+/-}\) (McQuiston et al., 2010), suggesting that a decrease of S1P due to lack of SK1 (please note that S1P is not totally absent in SK1\(^{-/-}\) AMs because of the presence of SK2) enhances phagocytosis of \(C.\ neoformans\) by AMs. In the present study, we found that addition of extracellular S1P increases phagocytosis of \(C.\ neoformans\) by AMs. This apparent contradiction could be explained by considering that extracellular S1P regulates...
phagocytosis differently from intracellular S1P. To address this hypothesis, we sought to determine the effect of extracellular S1P on phagocytosis of *C. neoformans* in conditions in which the intracellular pool of S1P is significantly decreased using AMs from SK1−/− mice. Thus, AMs from SK1−/− or from SK1/2+/+ wild-type mice were co-incubated with *C. neoformans* in the presence of 10 nM S1P, which is the concentration of S1P we found to induce the greatest enhancement in *C. neoformans* phagocytosis. We observed extracellular S1P to increase...
the internalization of *C. neoformans* in both wild-type (SK1+/+) AMs and SK1-deficient AMs and, as previously observed (McQuiston et al., 2010), deficiency in SK1 increases phagocytosis (Fig. 3). Using the two-way ANOVA, we found that both S1P treatment and SK1 deficiency significantly increase phagocytosis (Fig. 3). Intriguingly, the interaction between extracellular S1P and SK1 deficiency is also significant. These data suggest that extracellular S1P modulates the phagocytosis of *C. neoformans* by AMs through an SK1-independent mechanism but extracellular S1P and SK1 affect the actions of each other on *C. neoformans* internalization.

**Identifying the S1PR(s) mediating the effects of extracellular S1P on AMs**

Human and mouse monocytes and macrophages have been shown to express only S1P1 and S1P2 under *in vitro* conditions (Rivera et al., 2008). However, since S1PR expression profiles on monocytes and macrophages are greatly influenced by differentiation, activation and micro-environmental factors, such as extracellular S1P levels (Duong et al., 2004), it should not be assumed that all macrophages possess the same S1PR profile. Therefore, it was imperative to determine the S1P expression repertoire of each macrophage cell type under specific experimental conditions in order to resolve the S1P–S1PR pathway mediating the effects of extracellular S1P. For this, mRNA from WT AMs was isolated and S1P receptor mRNA levels were analysed using real-time RT-PCR (Hornuss et al., 2001), in this study the S1P expression profiles were assessed in freshly isolated AMs incubated in serum-free media in order to preserve the *in vivo* conditions of the alveolar environment, in which serum is not present. Fig. 4 shows that AMs from WT mice express S1P1, S1P2 and S1P4. S1P2 was expressed approximately sixfold more than S1P1, making it the prevalent S1PR expressed by AMs under these conditions. S1P4 was inconsistently expressed between trials, as it was poorly detected.

Since S1P2 was found to be the predominant S1P receptor expressed by AMs, and previous research has implicated S1P2 in regulating effector functions of innate immune cells (Jolly et al., 2004), we hypothesized that extracellular S1P activates S1P2 on the cell surface of AMs to initiate signalling cascades that enhance phagocytosis. To investigate this hypothesis, AMs from WT (S1P2+/+) and S1P2-deficient (S1P2−/−) mice were co-incubated with wild-type *C. neoformans* strain H99 opsonized with anti-GXM 18B7 Ab in the presence of 10 nM S1P for 2 h and analysed for phagocytosis. Untreated S1P2-deficient AMs had significantly decreased phagocytosis of *C. neoformans* compared with untreated S1P2+/+ AMs (39.3 ± 3.8 versus 24.5 ± 2.12, *P* < 0.01) (Fig. 5a). Additionally, in contrast with S1P2+/+, S1P treatment did not increase the internalization of *C. neoformans* by S1P2−/− AMs (51.8 ± 1.8 versus 24.5 ± 7.7, *P* < 0.01) (Fig. 5a). To further corroborate the S1P2 involvement, S1P2+/+ AM–*C. neoformans* co-cultures were treated with the S1P2 antagonist N-(2,6-dichloro-4-pyridinyl)-2-[1,3-dimethyl-4-(1-methyllethy])-1H-pyrazolo[3,4-b]pyridin-6-yl)-hydrazine-carboxamide (JTE-013) and analysed for phagocytosis. JTE-13 is a pyrazopyridine derivative that specifically binds to S1P2 to prevent the extrinsic effects of S1P (Ikeda et al., 2003; Osada et al., 2002). Several concentrations of JTE-013 were used to determine if this S1P2-specific antagonist affects phagocytosis in our model and to determine the concentration with the greatest inhibitory effect (data not shown). We found that 1 μM JTE-013 treatment significantly decreased phagocytosis of *C. neoformans* by S1P2+/+ AMs (Fig. 5b). Importantly, the addition of S1P to AMs at the same time as JTE-013 treatment induced a slight but statistically insignificant increase in phagocytosis compared with AMs treated with JTE-013 alone, thereby demonstrating that antagonist partially inhibits the effects of S1P specifically through S1P2. Furthermore, AMs treated with 1 μM JTE-013 and 10 nM S1P at the same time had a phagocytic index nearly identical to untreated AMs (40.7 ± 3.8 versus 40.8 ± 2.5), (Fig. 5b). Therefore, by using both

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**Fig. 3.** Extracellular S1P increases the phagocytosis of *C. neoformans* by AMs independent of SK1. AMs from C57BL/6J wild-type (SK1+/+) and SK1-deficient (SK1−/−) mice were incubated with *C. neoformans* strain H99 cells at an m.o.i. of 1:1 in the presence of 10 nM S1P to determine if the S1P-induced increase on phagocytosis requires SK1. Co-cultures were stopped after 2 h by removing the media and fixing with ice-cold methanol. Cells were stained with Giesma stain, and internalization was assessed in freshly isolated AMs incubated in serum-free media in order to preserve the *in vivo* conditions of the alveolar environment, in which serum is not present. Fig. 4 shows that AMs from WT mice express S1P1, S1P2 and S1P4. S1P2 was expressed approximately sixfold more than S1P1, making it the prevalent S1PR expressed by AMs under these conditions. S1P4 was inconsistently expressed between trials, as it was poorly detected.

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SIP2-deficient AMs and an SIP2-specific antagonist, these data show that extracellular SIP mediates its effect on the phagocytosis of *C. neoformans* by AMs through SIP2.

On the other hand, AMs also express SIP1 (Fig. 4) and, therefore, it is possible that SIP1 may participate in the modulation of phagocytosis. Studies have shown that SIP1 and SIP2 have opposing functions and even negatively regulate the activation of each other (Jolly et al., 2004; Okamoto et al., 2000). To determine if SIP1 affects the observed increase in phagocytosis following SIP1 treatment, SIP2<sup>+/+</sup> AMs were treated with either the SIP1 antagonist W146 or VPC 23901. A set of control AMs was treated with the specific vehicle solution used to resuspend each antagonist. In contrast with JTE-013 treatment, neither W146 nor VPC 23901 affected the phagocytosis of *C. neoformans* by wild-type AMs (Fig. 5c). Additional concentrations of these antagonists were also examined, and similar results (e.g., no effect on phagocytosis) were obtained (data not shown). The variation in the phagocytic index of control AMs (e.g., AMs treated with vehicle solution alone) is most likely to be due to the differences in the vehicle solutions, as the manufacturer’s directions of each compound required a different concentration of DMSO for reconstitution.

**Effect of extracellular SIP on the Fcγ receptor (FcγR) cell surface expression of AMs**

The data presented here show that SIP2 regulates internalization of Ab-opsonized *C. neoformans* by AMs. The mechanism by which SIP2 affects phagocytosis of *C. neoformans* under these conditions is unknown. Duong et al. (2004) examined the effects of lysophospholipids on human monocyte-derived macrophages and reported that SIP induces mRNA levels and cell-surface expression of FcγRII, thereby implicating SIP in the regulation of phagocytic receptors recognizing Abs (Duong et al., 2004). In light of the data presented here, it was hypothesized that SIP binds to SIP2 to initiate signalling cascades governing FcγR expression on the cell surface of AMs, thereby modulating the phagocytosis of Ab-opsonized *C. neoformans*. Since antibodies specific to each FcγR isoform (e.g., due to high level of homology between FcγII and FcγIII) are not commercially available, we examined the level of FcγRs mRNA in AMs from SIP2<sup>+/+</sup> and SIP2<sup>−/−</sup> mice to address this hypothesis. The deficiency of SIP2 resulted in a dramatically decreased mRNA levels of FcγRI, -II, and -III (Fig. 6). Thus, the data presented here suggest that SIP2 affects internalization of Ab-opsonized *C. neoformans* by modulating the expression of the phagocytic FcγRs.

**Effect of SIP2 on host susceptibility to cryptococcosis**

To determine if SIP2 affects host susceptibility to *C. neoformans* infection, SIP2<sup>+/+</sup> and SIP2<sup>−/−</sup> mice were intranasally challenged with highly virulent wild-type *C. neoformans* strain H99 and monitored for their susceptibility to cryptococcosis. Interestingly, SIP2<sup>−/−</sup> mice survived significantly longer than SIP2<sup>+/+</sup> mice (22.5 ± 2.1 days versus 19.8 ± 1.5 days, P<0.001) (Fig. 7). Along with our data showing that SIP2 modulates the internalization of *C. neoformans* by AMs in vivo, these data demonstrate that the deficiency in SIP2 provides protection against cryptococcosis.

**DISCUSSION**

In this study, we show that extracellular SIP mediates the phagocytosis of the pathogenic fungus *C. neoformans* by AMs through SIP2. Furthermore, we present data suggesting that SIP2 affects Ab-dependent phagocytosis by modulating FcγR expression.

Unlike previously published studies examining the effects of extracellular SIP on the antimicrobial actions of AMs against internalized *Mycobacterium* species (Garg et al., 2004; Sali et al., 2009), treatment with SIP1 did not affect the intracellular growth of *C. neoformans* within AMs. This is not necessarily surprising since *C. neoformans*, in contrast with *Mycobacterium*, does not actively prevent the acidification or maturation of the phagosome (Feldmesser et al., 2000; Levitz et al., 1999), a process that in *Mycobacterium*-infected AMs is indeed controlled by SIP1 (Garg et al., 2004; Kusner, 2005; Malik et al., 2000; Thompson et al., 2005). In addition, *C. neoformans* possesses virulence factors enabling internalized *C. neoformans* cells to survive within this microbicidal intracellular environment (Cox et al., 2001, 2003; Shea et al., 2006; Wright et al., 2007; Zaragoza et al., 2008).

The results presented here show that exogenous (i.e., extracellular) SIP increases the phagocytosis of *C. neoformans* by AMs whereas our previous studies showed that
Fig. 5. S1P2 mediates the phagocytosis of *C. neoformans* by AMs. S1P1/2+/+ and S1P2−/− AMs were incubated with *C. neoformans* strain H99 opsonized with the anti-GXM antibody 18B7 at an m.o.i. of 1:1 with or without 10 nM S1P to determine if S1P increases phagocytosis through S1P2 (a). Student’s *t*-test was conducted to determine significance (*P*<0.01 compared with S1P1/2+/+ AMs without S1P; *P*<0.005 compared with S1P1/2+/+ AMs with S1P). (b) Wild-type (S1P1/2+/+) AMs were co-incubated with 18B7-opsonized *C. neoformans* strain H99 cells in the presence of the S1P2 antagonist JTE-013 (1 μM) with and without S1P (10 nM). Student’s *t*-test was conducted to determine significance (*P*<0.01 compared with S1P1/2+/+ AMs without S1P or JTE-013; *P*<0.01 compared with S1P1/2+/+ AMs with S1P; *P*>0.05, compared with S1P1/2−/− AMs with JTE-013). (c) Wild-type (S1P1/2+/+) AMs were co-incubated with *C. neoformans* strain H99 cells opsonized with the anti-GXM antibody 18B7 in the presence of either vehicle only (+veh), the S1P2 antagonist JTE-013 (1 μM), the S1P1/3 antagonist VPC 23019 (10 μM) or the S1P1 antagonist W146 (10 μM). Note that the vehicle for each antagonist was different. Student’s *t*-test was conducted to determine significance (*P*<0.05 compared with the applicable vehicle only). For all experiments, co-incubations were stopped after 2 h by removing the media and fixing with ice-cold methanol. Phagocytic indices were determined using light microscopy to determine *C. neoformans* internalization. Data are the average from three separate experiments.

Fig. 6. S1P2-deficient AMs have decreased FcγR expression. AMs were isolated and pooled together from four S1P1/2+/+ (wild-type) and S1P2−/− mice. RNA was extracted from these AMs and real-time RT-PCR was conducted to determine whether the presence of S1P2 affects FcγR expression profile. All values were normalized against the expression of a reference gene, β-actin. Data are the average from three separate experiments.
SK1 decreases it (McQuiston et al., 2010). One plausible explanation is that intracellular S1P produced by SK1 mediates phagocytosis through a different signalling pathway than that initiated upon extracellular S1P ligation to S1PRs. This is supported by the observation that SK1 seems not to be required for the action of extracellular S1P since addition of extracellular S1P increases phagocytosis of C. neoformans in AMs in which SK1 is deleted (SK1−/− AMs) (Fig. 3).

The use of appropriate and physiological concentrations of S1P is also very important for studying its effect on biological systems. For instance, in studies examining the extrinsic effects of S1P on AMs (Garg et al., 2004, 2006; Hammad et al., 2008; Hughes et al., 2008; Jiang et al., 2007; Santucci et al., 2007), the concentrations of S1P ranged from 0.5 to 50 μM S1P and/or were conducted in the presence of serum, which has an S1P concentration of 0.4–1.1 μM (Yatomi et al., 1997). With the exception of the blood, in which S1P concentration ranges from 1 to 4 μM, the S1P concentration in all other tissue ranges from 0.5 to 75 pmol (mg tissue)−1 (Allende et al., 2004; Edsall & Spiegel, 1999; Schwab et al., 2005). S1P concentration in the mucosal airway surface, as measured in BAL fluid, from healthy patients has been shown to range from 2 to 4 nM (Ammit et al., 2001). Antigen challenge significantly increases S1P levels in the BAL fluid from asthmatic patients to concentrations ranging between 8 and 14 nM (Ammit et al., 2001). In addition, the S1P concentration in the BAL fluid from Mycobactrium tuberculosis-infected patients has been shown to have a range of 47.5 ± 36.2 nM (Garg et al., 2006). Thus, previous studies conducted in the presence of micromolar concentrations of S1P may not represent how S1P modulates the actions of resident AMs, since, in non-blood tissues, S1P concentrations are in the nanomolar range. The high affinities of the S1PRs, which range from 2 to 63 nM (Kd values) (Hla et al., 2001), suggest that S1P can evoke its effects on cells and in tissue with low S1P content. The research presented here shows that similar S1P concentrations to the ones present in the extracellular spaces of the lung can modulate the phagocytosis of C. neoformans by AMs through S1P2. To our knowledge, this represents the first report showing the ability of extracellular S1P to regulate microbial phagocytosis through an S1PR.

In this research, we show that S1P treatment increases AM phagocytosis of C. neoformans by approximately 30–40 % (Fig. 3). Although this effect on phagocytosis may seem modest, this increase in C. neoformans internalization may have a larger role in the antimicrobial actions of macrophages required to contain cryptococcal infection. C. neoformans cells evoke AMs to secrete chemokines and cytokines to recruit other inflammatory cells, which further enhance the antimicrobial actions of AMs through cytokines and, ultimately, result in the development of an adaptive immune response that controls the cryptococcal infection (Abe et al., 1998; Buchanan & Murphy, 1997; Vecchiarelli et al., 1994; Voelz et al., 2009). Intravenous administration of S1P decreases disease severity and bacterial burden of macrophages during Mycobacterium infections, suggesting that S1P could also modulate the host inflammatory response to C. neoformans (Garg et al., 2004, 2006; Sali et al., 2009). The effects of exogenous S1P on cryptococcosis were not examined in this research but the role of S1P and its respective receptors, particularly S1P1 and S1P2, in cryptococcosis should be examined in future studies.

Research studies addressing S1P and S1P2 suggest that S1P1 and S1P2 may have opposing functions in controlling host immune cells. S1P1 expression is required for immune cell egress from lymphoid tissues (low nanomolar S1P concentrations) into the blood stream (micromolar concentrations) (Chiba, 2005; Singer et al., 2005; Vora et al., 2005). In contrast, binding of extracellular S1P to S1P2 in mast cells results in the inhibition of cell migration through the activation of the small GTPase Rho, which negatively regulates S1P1-induced Rac activity (Okamoto et al., 2000; Yokoo et al., 2004). Furthermore, upregulation of S1P2 expression in mast cells following FcγRI cross-linking via immunoglobulin E inhibits cell migration (Jolly et al., 2004). These conflicting actions are also observed in terms of the inflammatory state of macrophages. Hughes et al. (2008) have shown that S1P binds to S1P1 and prevents the proinflammatory stimulus of lipopolysaccharide on mouse peritoneal macrophages. Extracellular S1P increases the levels of pro-inflammatory molecules COX-2 and PGE2 in the mouse monocyte/macrophage RAW264.7 cell line (Hammad et al., 2008). Similarly, S1P binding to S1P2 increased CAMP levels in PGE2- and isopreteronol-stimulated RAW264.7 cells (Jiang et al.,...
SIP increased internalization of *C. neoformans* cells by AMs only when the fungal cells were opsonized with Ab, specifically an IgG1 molecule targeting capsular GXM. We hypothesized that the binding of extracellular SIP to SIP2 of AMs may augment *C. neoformans* internalization by modulating the cell-surface expression of FcγRs on AMs. Macrophages express three FcγRs (FcγRI, -II and -III) capable of binding IgG1 molecules to illicit an effector function. The activating FcγRI is a high-affinity receptor for IgG1 (Kd 1 x 10^-9) that can bind to monomeric IgG1 and IgG1 immune complexes while FcγRII and -III are considered low-affinity receptors for IgG1 and, therefore, can only bind to IgG1 immune complexes (Daean, 1997; Nimmerjahn et al., 2005). This hypothesis of SIP augmenting phagocytosis through modulation of FcγR expression was also suggested in the previous work by Duong et al. (2004), in which they showed, using a GeneChip assay, that SIP treatment affects expression levels of FcγRII isoforms in human differentiated macrophages. The mechanism by which extracellular SIP regulates the Fcγ receptor expression is not known and our results suggest that this regulation occurs through SIP2.

Human FcγRIIa is an activatory isoform homologous to mouse FcγRII while human FcγRIIb is an inhibitory isoform homologous to mouse FcγRII. Since the relative expression levels of the activatory FcγRI and -III compared with the inhibitory FcγRII determine if phagocytosis occurs (Ravetch & Bolland, 2001), SIP2 could promote phagocytosis by triggering signalling cascades that either increase the expression of FcγRI and/or FcγRII or decrease the expression of FcγRII. It is important to note that, although FcγRII and FcγRIII are both considered low-affinity IgG1 receptors compared with the high affinity of FcγRI for IgG1 (Kd 1 x 10^-9), the inhibitory FcγRII has a tenfold greater affinity for IgG1 immune complexes than activatory FcγRIII (Kd 3.01 x 10^-7 versus Kd 3.2 x 10^-6) (Nimmerjahn et al., 2005). Therefore, modest alteration in expression of the inhibitory FcγRII compared with the activatory FcγRIII could result in a much more pronounced change in the activation threshold and, subsequently, effector cell responses of AMs. Additional investigations are needed to elucidate the molecular mechanism by which SIP2 affects FcγR expression.

It is important to note that even if the effect of SIP1 on phagocytosis is dependent on the ligation of IgG-opsonized *C. neoformans* with FcγRs, it is possible that other receptors are also affected by SIP1 treatment and contribute to the SIP1-induced increase in *C. neoformans* internalization. Complement receptors 3 (CR3) and 4 (CR4) can internalize Ab-opsonized *C. neoformans* in a complement-independent mechanism (Taborda & Casadevall, 2002). Both antibodies against CR3 and CR4 were shown to significantly decrease phagocytosis of IgG1-opsonized *C. neoformans* by macrophages (Taborda & Casadevall, 2002). The effect of SIP1 on the phagocytosis of *C. neoformans* opsonized only with IgG1 was examined in this research since IgG1 serves as an excellent opson for macrophage phagocytosis (Mukherjee et al., 1995, 1996; Netski & Kozel, 2002), shows therapeutic potential in mouse models of *C. neoformans* (Feldmesser & Casadevall, 1997; Mukherjee et al., 1994; Shapiro et al., 2002) and is produced during cryptococcosis in humans (Abadi & Pirofski, 1999; Deshaw & Pirofski, 1995). However, similar to IgG, IgM that is innately produced by naive mature B cells (prior to activation and immunoglobulin class switching) is also recognized by CR3 and CR4 in a complement-independent mechanism (Taborda & Casadevall, 2002). Interestingly, though, hyper-IgM syndrome increases susceptibility to cryptococcosis (Escarccega-Barbosa et al., 2002; Jo et al., 2002). Thus, other Ig classes, particularly IgM, may be an important link between the innate and adaptive immune system during cryptococcosis, affecting disease outcome. Several cell-surface receptors expressed by macrophages, including CD14, TLR2, TLR4 and CD18, that recognize *C. neoformans* capsular GXM do not work independently to internalize *C. neoformans* but rather modulate the phagocytosis and other effector functions as co-receptors and/or by initiating various signalling cascades (Levitz, 2002; Monari et al., 2005; Yauch et al., 2005). Thus, these receptors could act in concert with FcγRs or independently to affect *C. neoformans* internalization by AMs.

The intravenous administration of SIP1 to *Mycobacterium*-challenged mice during the acute phase of infection decreases *Mycobacterium* organ burden, improves lung histopathology and reduces dissemination and disease progression (Garg et al., 2004; Sali et al., 2009). However, how a systemic treatment with this bioactive molecule enhances the localized host immune response in the lungs is unknown. The studies on *Mycobacterium* presuppose SIPR involvement in virulence but that assumption was not investigated. When we tested the susceptibility of SIP2-/- mice to *C. neoformans* we found, surprisingly, that they survived longer compared with SIP2+/+ wild-type mice (Fig. 7). Since SIP2 is expressed in many different immune cells, it is very difficult to conclude that the specific role of SIP2 in virulence is due to its regulation of phagocytosis of *C. neoformans* by AMs. For instance, activation of SIP2 increases the vascular permeability of lung endothelial cells while antagonist inhibition using JTE-013 improved barrier integrity (Sanchez et al., 2007). Therefore, the deficiency in SIP2 may decrease the ability of *C. neoformans* cells to pass...
through the endothelial lining of the lungs to enter the bloodstream and cause disseminated disease. Thus, even though less phagocytosis by AMs occurs in the lungs of S1P2−/− mice, this host may be less permissive to fungal dissemination in the lung tissue than S1P2+/+ mice.

In conclusion, our studies suggest that extracellular S1P mediates phagocytosis of Ab-opsonized C. neoformans by AMs through an S1P2-dependent mechanism.

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