

Characterization of IL-22 and antimicrobial peptide production in mice protected against pulmonary *Cryptococcus neoformans* infection

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Cryptococcus neoformans is a significant cause of fungal meningitis in patients with impaired T cell-mediated immunity (CMI). Experimental pulmonary infection with a *C. neoformans* strain engineered to produce IFN- γ , H99 γ , results in the induction of Th1-type CMI, resolution of the acute infection, and protection against challenge with WT *Cryptococcus*. Given that individuals with suppressed CMI are highly susceptible to pulmonary *C. neoformans* infection, we sought to determine whether antimicrobial peptides were produced in mice inoculated with H99 γ . Thus, we measured levels of antimicrobial peptides lipocalin-2, S100A8, S100A9, calprotectin (S100A8/A9 heterodimer), serum amyloid A-3 (SAA3), and their putative receptors Toll-like receptor 4 (TLR4) and the receptor for advanced glycation end products (RAGE) in mice during primary and recall responses against *C. neoformans* infection. Results showed increased levels of IL-17A and IL-22, cytokines known to modulate antimicrobial peptide production. We also observed increased levels of lipocalin-2, S100A8, S100A9 and SAA3 as well as TLR4⁺ and RAGE⁺ macrophages and dendritic cells in mice inoculated with H99 γ compared with WT H99. Similar results were observed in the lungs of H99 γ -immunized, compared with heat-killed *C. neoformans*-immunized, mice following challenge with WT yeast. However, IL-22-deficient mice inoculated with H99 γ demonstrated antimicrobial peptide production and no change in survival rates compared with WT mice. These studies demonstrate that protection against cryptococcosis is associated with increased production of antimicrobial peptides in the lungs of protected mice that are not solely in response to IL-17A and IL-22 production and may be coincidental rather than functional.

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INTRODUCTION

Cryptococcus neoformans, the aetiological agent of cryptococcosis, is an opportunistic fungal pathogen that typically affects individuals with impaired T-cell function (i.e. individuals with AIDS and lymphoid malignancies, and recipients of immunosuppressive therapies) (Levitz, 1991; Mitchell & Perfect, 1995; Shoham & Levitz, 2005; Singh *et al.*, 1997, 2008). Previous studies have shown that protective immunity against this organism is dependent

upon the induction of Th1-type cytokine responses (Blasi *et al.*, 1994; Buchanan & Doyle, 2000; Chuck & Sande, 1989; Hill & Harmsen, 1991; Huffnagle *et al.*, 1991; Mody *et al.*, 1990; Shoham & Levitz, 2005; Singh *et al.*, 2008; Wormley *et al.*, 2007; Wozniak *et al.*, 2009). Pulmonary infection in BALB/c mice using a *C. neoformans* strain engineered to produce murine IFN- γ (H99 γ) results in induction of Th1-type cytokines, IL-17A, and significantly reduced fungal burden. Mice immunized with H99 γ are completely protected against a subsequent pulmonary challenge with an otherwise lethal WT *C. neoformans* strain (Wormley *et al.*, 2007; Wozniak *et al.*, 2009). IL-17A and IL-22 function cooperatively to induce the production of antimicrobial peptides and acute phase proteins such as

Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; HK *C.n.*, heat-killed *Cryptococcus neoformans*; RAGE, receptor for advanced glycation end products; SAA3, serum amyloid A-3; TLR4, Toll-like receptor 4;

β -defensins, S100A8, S100A9 and lipocalin-2 in bronchial epithelial cells (Aujla *et al.*, 2008; Gessner *et al.*, 2012; Kolls *et al.*, 2008; Liang *et al.*, 2006; Wolk *et al.*, 2011). Studies have shown the importance of IL-17A and IL-22 clinically to mediate clearance in patients with chronic mucocutaneous candidiasis (Eyerich *et al.*, 2008) and experimentally to protect mice against disseminated candidiasis (Huang *et al.*, 2004), pulmonary aspergillosis (Gessner *et al.*, 2012) and pulmonary *Klebsiella pneumoniae* infection (Aujla *et al.*, 2008).

S100A8 and S100A9, which can collectively form a heterodimer called calprotectin (Lagasse & Clerc, 1988; Odink *et al.*, 1987; Zwadlo *et al.*, 1988), are involved in neutrophil recruitment and are known to be produced by epithelial cells following induction by IL-22 (Aujla *et al.*, 2008; Kolls *et al.*, 2008; Liang *et al.*, 2006). S100 proteins are calcium-binding cytosolic proteins that can be expressed by multiple cell types, including alveolar epithelial cells, endothelial cells, monocytes, macrophages, neutrophils and dendritic cells (DCs) (Ehrchen *et al.*, 2009; Foell *et al.*, 2007; Ramirez-Ortiz *et al.*, 2011). Importantly, calprotectin may have direct antifungal and fungistatic activity against fungi, including *C. neoformans* (Mambula *et al.*, 2000; McCormick *et al.*, 2010; Urban *et al.*, 2009).

Lipocalin-2, an acute phase protein that is a component of neutrophil granules, is involved in iron sequestration and is a biomarker for inflammation and sepsis (Bachman *et al.*, 2009; Borregaard & Cowland, 2006; Li & Chan, 2011). Secretion of lipocalin-2 involves signalling via Toll-like receptor 4 (TLR4) and subsequent NF- κ B binding to consensus sequences upstream of the lipocalin-2 promoter (Flo *et al.*, 2004; Li & Chan, 2011), and its production is regulated via IL-1 β , IL-17A and other cytokines (Chan *et al.*, 2009; Kolls *et al.*, 2008; Li & Chan, 2011). Although lipocalin-2 is increased in the lungs of mice during infection with *Aspergillus fumigatus*, lipocalin-2-deficient mice do not demonstrate impaired fungal clearance (Gessner *et al.*, 2012), and lipocalin-2 does not bind to fungal siderophores (Flo *et al.*, 2004). Thus, lipocalin-2 may only serve as a biomarker for inflammatory immune responses against fungi and may not have any direct fungicidal effects, although its role against *C. neoformans* remains to be determined.

Serum amyloid A-3 (SAA3) is a member of the SAA family of acute phase proteins and has been long considered a biomarker for inflammatory diseases (Uhlir & Whitehead, 1999). SAA3 is induced via a TLR4/NF- κ B-dependent pathway, and SAA3 in turn induces chemotactic proteins,

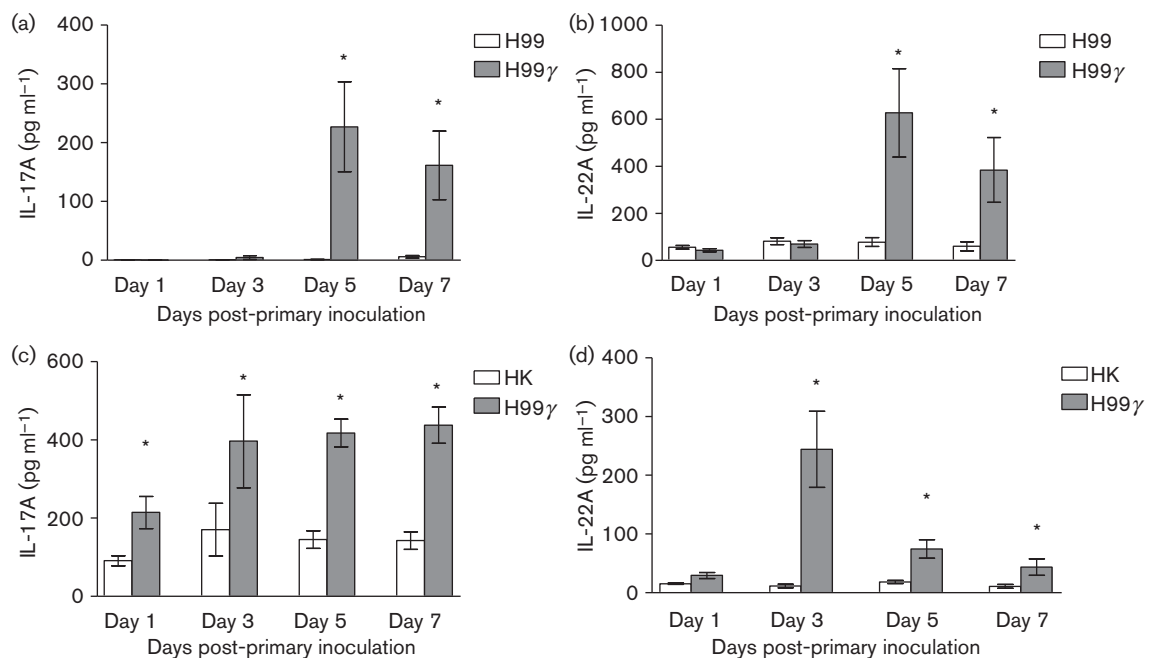


Fig. 1. Pulmonary levels of IL-17A and IL-22 following inoculation with *C. neoformans* strain H99 γ or H99 and during secondary challenge of protectively and non-protectively immunized mice. BALB/c mice were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 or H99 γ (a, b). Alternatively, BALB/c mice received an initial immunization with HK *C.n.* yeasts or *C. neoformans* strain H99 γ and were allowed 60 days to resolve the infection. Mice then received a second intranasal challenge with 1×10^4 c.f.u. of *C. neoformans* strain H99 (c, d). Lung homogenates were prepared from lungs excised on days 1, 3, 5 and 7 post-inoculation and assayed for IL-17A and IL-22 production by ELISA. Data are cumulative of three experiments utilizing four mice per group per time point for each experiment. Asterisks indicate significant differences (* $P < 0.05$).

pro-inflammatory cytokines (including IL-1 β) and nitric oxide from neutrophils, macrophages and epithelial cells (Ather *et al.*, 2011; Hiratsuka *et al.*, 2008). SAA3 instillation into the lungs elicits robust pro-inflammatory cytokine production and phagocyte recruitment into the lungs (Ather *et al.*, 2011). Our previous data show significant IL-1 β increases in the lungs of H99 γ -immunized mice that are protected against experimental pulmonary *C. neoformans* infection (Wozniak *et al.*, 2009), which could be induced by mediators such as SAA3. Therefore, SAA3 may serve as more than just a 'biomarker' during the protective immune response to *C. neoformans* infection.

Receptors for these antimicrobial peptides include the receptor for advanced glycation end products (RAGE) and TLR4 (Ehrchen *et al.*, 2009; Foell *et al.*, 2007; Herold *et al.*, 2007). RAGE is expressed on endothelial cells as well as macrophages, neutrophils, DCs, T cells, B cells, alveolar type II cells and alveolar epithelial cells (Fehrenbach *et al.*, 1998; Katsuoka *et al.*, 1997). S100A8, S100A9 and SAA3 are TLR4 agonists and induce the activation of MyD88-dependent pathways as well as ERK1/2, p38, mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and protein kinase C (PKC). Stimulation of TLR4 induces the release of proinflammatory cytokines, chemokines and reactive oxygen species necessary for the activation of potent host defences (Ehrchen *et al.*, 2009; Hiratsuka *et al.*, 2008).

The studies described herein were designed to examine the effect of high levels of IL-17A and IL-22 on the presence of antimicrobial peptides during protective cryptococcal infection and the recruitment of TLR4⁺ and RAGE⁺ effector cells in these mice. We hypothesized that IL-17A and IL-22 in protected mice would lead to increased production of antimicrobial peptides and increased recruitment of RAGE⁺ and TLR4⁺ antigen-presenting cells (APCs), and reveal a role for those cells and mediators in protection against pulmonary cryptococcal infection.

METHODS

Mice. Female BALB/c (H-2^d) mice (National Cancer Institute/Charles River Laboratories) with a mean weight of 20–25 g and IL-22 knockout (IL-22^{-/-}) mice and their controls, '129 mice' (Mutant Mouse Resource Center), which were subsequently bred in our facility, were used throughout these studies. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Mice were housed at The University of Texas at San Antonio Small Animal Laboratory Vivarium. These animal experiments were approved by The University of Texas at San Antonio Institutional Animal Care and Use Committee (IACUC), and mice were handled according to IACUC guidelines. All efforts were made to minimize animal suffering.

Strains and media. *C. neoformans* strains H99 (serotype A, Mat α) and H99 γ (an INF- γ -producing *C. neoformans* strain derived from H99) were recovered from 15% glycerol stocks stored at -80 °C prior to use in the experiments described herein. The strains were maintained on yeast-extract-peptone-glucose (YPD) media. Yeast

cells were grown for 16–18 h at 30 °C with shaking in YPD broth, collected by centrifugation and washed three times with sterile PBS, and viable cells were quantified using trypan blue dye exclusion in a haemocytometer.

Pulmonary inoculations. Pulmonary immunizations or inoculations were initiated by nasal inhalation as previously described (Wormley *et al.*, 2007; Wozniak & Levitz, 2009). Mice were anaesthetized with 2% isoflurane using a rodent anaesthesia device (Eagle Eye Anaesthesia) and then given a yeast inoculum of 1×10^4 c.f.u. of *C. neoformans* strain H99 or H99 γ in 50 μ l of sterile PBS pipetted directly into the nares. The inocula used were verified by quantitative culture on YPD agar. The mice were fed *ad libitum* and were monitored by inspection twice daily. Mice were killed at specific time points post-inoculation by CO₂ inhalation followed by cervical dislocation, and lung tissues were excised using an aseptic technique. Tissues were homogenized in 1 ml of sterile PBS, followed by culture of 10-fold dilutions of each tissue on YPD agar supplemented with chloramphenicol (Mediatech). Colony-forming units were enumerated following incubation at 30 °C for 48 h. For secondary challenge studies, mice were immunized with either 1×10^4 c.f.u. of *C. neoformans* strain H99 γ or heat-killed *C. neoformans* (HK *C.n.*) in 50 μ l of sterile PBS and were allowed 60 days to resolve the infection. Subsequently, the immunized mice received a second experimental pulmonary challenge with 1×10^4 c.f.u. of *C. neoformans* strain H99 in 50 μ l of sterile PBS and were killed at specific time points post-inoculation. Alternatively, mice intended for survival analysis were monitored by inspection twice daily and killed if they appeared to be in pain or moribund. Mice were killed using CO₂ inhalation, followed by cervical dislocation.

Cytokine and antimicrobial peptide analysis. Cytokine and antimicrobial peptide levels in lung tissues were analysed using ELISAs (R & D Systems or EMD Millipore) performed according to

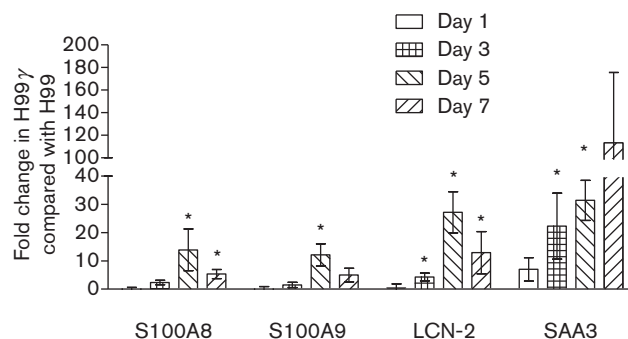


Fig. 2. Antimicrobial peptide gene expression in mice following inoculation with H99 γ compared with H99. BALB/c mice were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 γ or H99 in 50 μ l sterile PBS. At days 1, 3, 5 and 7 post-inoculation, mice were killed and pulmonary RNA was isolated from whole lung tissues and homogenized in Trizol reagent. cDNA was prepared from total RNA and real-time PCR was conducted to measure S100A8, S100A9, lipocalin-2 (LCN-2) and SAA3 expression. Bars represent the fold change in gene expression in pulmonary tissues from H99 γ -infected mice compared with tissues from H99-infected mice. Data shown are cumulative of three independent experiments utilizing individual tissues from four mice per group per time point per experiment and are reported as means \pm SEM (* $P < 0.05$).

the manufacturers' instructions. Briefly, lung tissue was excised and homogenized in ice-cold sterile PBS (1 ml). An aliquot (50 μ l) was taken to quantify the pulmonary fungal burden and an anti-protease buffer solution (1 ml) containing PBS, protease inhibitors (inhibiting cysteine, serine and other metalloproteinases) and 0.05% Triton X-100 were added to the homogenate. Samples were then clarified by centrifugation (800 g) for 5 min. Supernatants from pulmonary homogenates were assayed for the presence of IL-22, IL-17A, SAA3, lipocalin-2, S100A8 and S100A9 by ELISA.

Flow cytometry. Standard methodology was employed for the direct immunofluorescence of pulmonary leukocytes. Briefly, in 96-well U-bottom plates, 100 μ l containing 1×10^6 cells in PBS+2% FBS (FACS buffer) were incubated with 50 μ l Fc Block (BD Biosciences) diluted in FACS buffer for 5 min to block non-specific binding of antibodies to cellular Fc receptors. Subsequently, an optimal concentration of fluorochrome-conjugated antibodies (eBioscience and Molecular Probes) (0.06–0.5 μ g/ 1×10^6 cells in 50 μ l FACS buffer) were added in various combinations to allow for dual or triple staining experiments, and plates were incubated for 30 min at 4 °C. Following incubation, the cells were washed three times with FACS buffer and cells were fixed in 200 μ l of 2% ultrapure formaldehyde (Polysciences) diluted in FACS buffer (fixation buffer). Cells incubated with either FACS buffer alone or single fluorochrome-conjugated antibodies were used to determine positive staining and spillover/compensation calculations. The samples were analysed using BD FACSArray software on a BD FACSArray flow cytometer (BD

Biosciences). Dead cells were excluded on the basis of forward angle and 90° light scatter. For data analyses, 30 000 events (cells) were evaluated from a predominantly leukocytic population identified by backgating from CD45⁺-stained cells. The absolute number of total leukocytes was quantified by multiplying the total number of cells observed by haemocytometer counting by the percentage of CD45⁺ cells determined by flow cytometry. The absolute number of each leukocyte subset (F4/80⁺ macrophages, CD11c⁺/CD11b^{int} DCs) expressing TLR4 or RAGE was determined by multiplying the percentage of each gated population by the total number of CD45⁺ cells.

Antimicrobial gene expression. Total RNA was isolated from lung homogenates using TRIzol reagent (Invitrogen), and then treated with DNase to remove possible traces of contaminating DNA according to the manufacturer's instructions (Qiagen). Total RNA was subsequently recovered using the Qiagen RNeasy kit. cDNA was synthesized from 1 μ g of total RNA using the oligo dT primer and reagents supplied in the SuperScript III RT kit (Invitrogen) according to the manufacturer's instructions. The cDNA was used as a template for real-time PCR analysis using the TaqMan Gene Expression Assay (Applied Biosystems) according to the manufacturer's instructions. All real-time PCRs were performed using the 7300 Real-Time PCR System (Applied Biosystems). For each real-time PCR, a master mix was prepared on ice with TaqMan Gene Expression Assays specific for S100A8, S100A9, SAA3, β -defensins 1 and 2, and LCN-2 (Applied Biosystems). Each reaction was normalized to the endogenous control GAPDH, and relative mRNA expression was derived using the

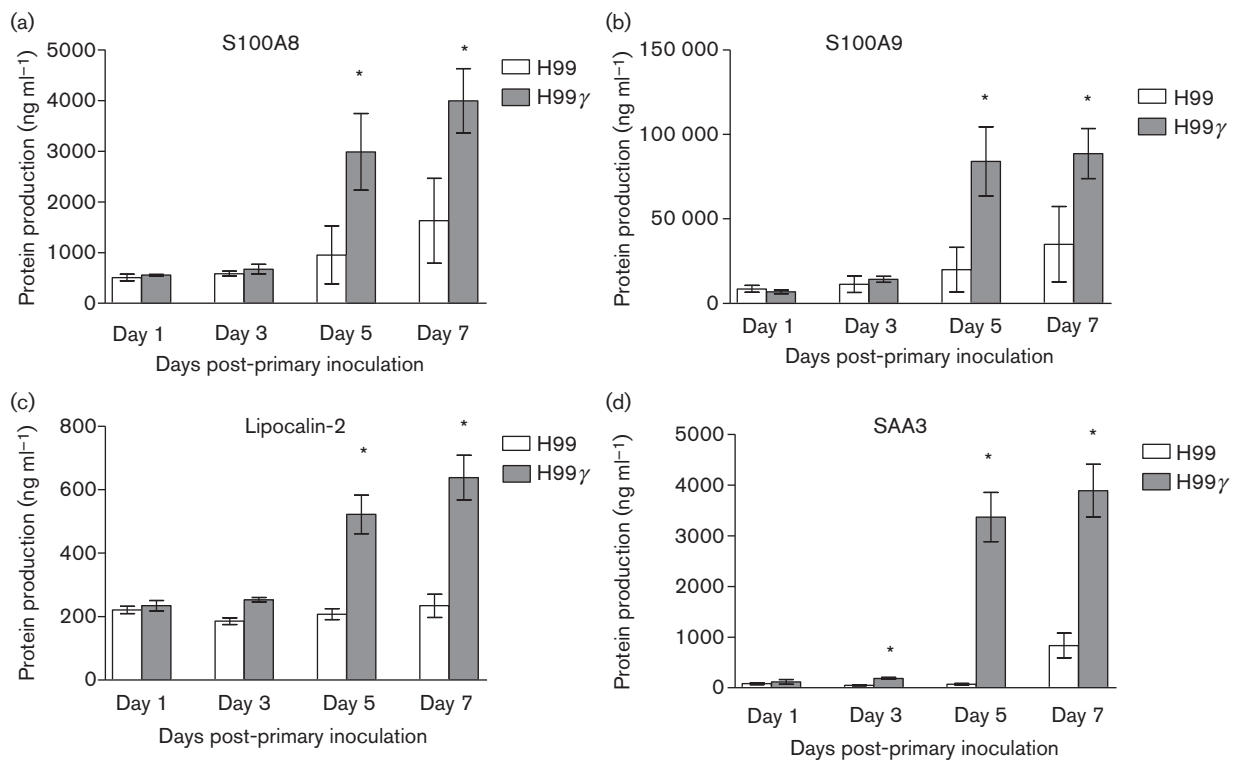


Fig. 3. Pulmonary antimicrobial peptide production following inoculation with *C. neoformans* strain H99 γ or WT *C. neoformans*. BALB/c mice were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 or H99 γ . Lung homogenates were prepared from lungs excised on days 1, 3, 5 and 7 post-inoculation and assayed for S100A8 (a), S100A9 (b), lipocalin-2 (c) and SAA3 (d) production by ELISA. Data are cumulative of three experiments utilizing four mice per group per time point for each experiment. Asterisks indicate significant differences (* $P < 0.05$).

comparative Ct method. The results shown represent the fold increase or decrease in gene expression in BALB/c H99 γ -infected compared with H99-infected mice or IL-22^{-/-} mice compared with WT 129 mice.

In vitro antimicrobial assays. For these experiments, purified S100A8, S100A9, SAA3 or lipocalin-2 (R&D Systems) were incubated with *C. neoformans* strain H99 at a concentration of 500 ng ml⁻¹, the maximum concentration available for these peptides (Hole *et al.*, 2012; Miyakawa *et al.*, 1996). Cryptococci were resuspended in 10 mM phosphate buffer with 2% RPMI medium, pH 5.5 (phosphate buffer). Fungi were added to a 96-well plate in a volume of 50 μ l (2.5 \times 10⁵ ml⁻¹). Phosphate buffer was added to control wells, and 50 μ l of antimicrobial peptide was added to test wells, for a total volume of 100 μ l per well in duplicate wells. Plates were incubated for 24 h at 37 °C, with 5% CO₂. Cryptococci were diluted in sterile PBS and plated on YPD agar to determine c.f.u. numbers. Killing was defined as c.f.u. significantly below the inoculum, and inhibition was defined as c.f.u. significantly below that of growth in phosphate buffer alone (Hole *et al.*, 2012). All peptides were commercially manufactured and are \geq 95% pure.

Statistical analysis. The unpaired Student's *t*-test (two-tailed) was used to analyse fungal burden, pulmonary cell populations and cytokine/antimicrobial peptide data using GraphPad Prism version 5.00 for Windows (GraphPad Prism Software). Survival data were analysed using the log-rank test (GraphPad Software). Significant differences were defined as *P*<0.05.

RESULTS

Protection against *C. neoformans* is associated with increased IL-17A and IL-22 production in lungs

Experimental pulmonary inoculation in mice using a *C. neoformans* strain engineered to express IFN- γ , H99 γ , results in the induction of Th1-type cell-mediated immunity responses, resolution of the acute infection and complete protection against a second pulmonary challenge with a pathogenic *C. neoformans* strain (Wormley *et al.*, 2007; Wozniak *et al.*, 2009). Additionally, we observed significant increases in IL-17A production in the lungs of mice following inoculation with H99 γ and in H99 γ -immunized mice challenged with WT *C. neoformans*, which does not express IFN- γ (Wormley *et al.*, 2007; Wozniak *et al.*, 2009). IL-17A and IL-22 are crucial regulators of antimicrobial peptide production in the lungs (Liang *et al.*, 2006). Thus, we determined the levels of IL-17A and IL-22 present in lung homogenates derived from mice infected with WT *C. neoformans* strain H99 compared with H99 γ on days 1, 3, 5 and 7 post-inoculation. We observed significantly increased levels of IL-17A (Fig. 1a) and IL-22 (Fig. 1b) on days 5 and 7 post-inoculation in the lungs of mice infected with H99 γ

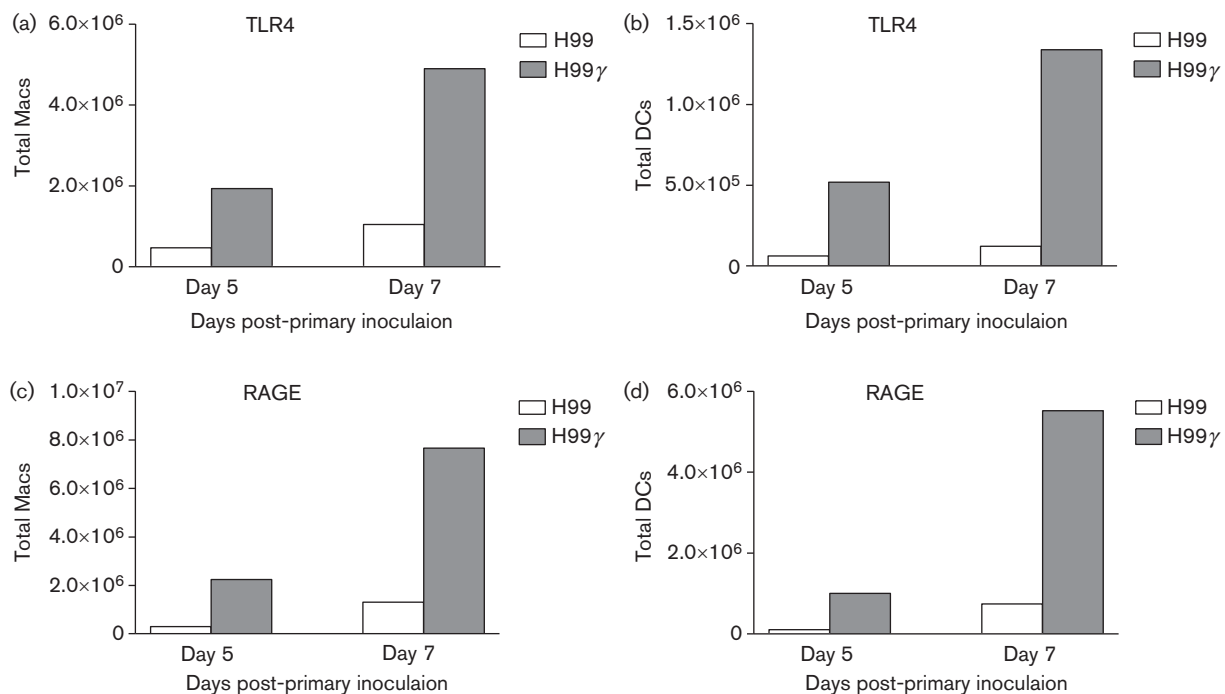


Fig. 4. Antimicrobial peptide receptors on macrophages (Macs) and DCs following inoculation with H99 γ compared with H99. BALB/c mice were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 or H99 γ . Lungs were excised at days 5 and 7 post-inoculation and a single cell suspension was generated using enzymic digestion. The leukocytes were evaluated by flow cytometry for the presence of TLR4 on the surface of macrophages (a) or DCs (b) or RAGE on the surface of macrophages (c) or DCs (d). Flow cytometry data are representative results of two independent experiments using pooled leukocytes from four mice per group per time point for each experiment.

compared with those infected with WT *C. neoformans*. We then determined IL-17A and IL-22 production in mice protectively immunized with *C. neoformans* strain H99 γ or non-protectively immunized with HK *C.n.* and subsequently challenged with WT *C. neoformans*. A significant increase in IL-17A production was observed in lung homogenates prepared from protectively immunized mice (H99 γ immunized) compared with non-protectively immunized mice (HK *C.n.* immunized) on days 1, 3, 5 and 7 post-secondary challenge (Fig. 1c). Also, significantly greater IL-22 production was observed in lung homogenates prepared from protectively immunized mice compared with non-protectively immunized mice on days 3, 5 and 7 post-secondary challenge (Fig. 1d). Thus, IL-17A and IL-22 production is increased during protective anti-cryptococcal immune responses.

Enhanced antimicrobial peptide and acute phase protein production correlates with protective anti-*C. neoformans* immune responses

We next determined if the increased pulmonary IL-17A and IL-22 production observed in mice infected with *C.*

neoformans H99 γ was associated with enhanced antimicrobial peptide and/or acute phase protein production. Mice were intranasally inoculated with *C. neoformans* strain H99 γ or WT *C. neoformans* strain H99. RNA was then extracted from lungs excised from mice in each group at days 1, 3, 5 and 7 post-inoculation to measure the levels of S100A8, S100A9, β -defensins 1 and 2, lipocalin-2, and SAA3 transcripts. Results showed an increased expression of transcripts for S100A8 and S100A9 at days 5 and 7 post-inoculation and for lipocalin-2 and SAA3 at days 3, 5 and 7 post-inoculation within the lungs of mice inoculated with the H99 γ strain compared with H99 (Fig. 2). We observed no difference in protein levels of cathelicidins or gene expression of β -defensins 1 and 2 between each group of mice on all days tested (data not shown).

Subsequently, we determined if increased gene expression of the various antimicrobial peptides and acute phase proteins tested would translate to increased protein production. For this, BALB/c mice were intranasally inoculated with either *C. neoformans* strain H99 γ or WT *C. neoformans*. We then measured S100A8, S100A9, β -defensins 1 and 2, lipocalin-2, and SAA3 production in

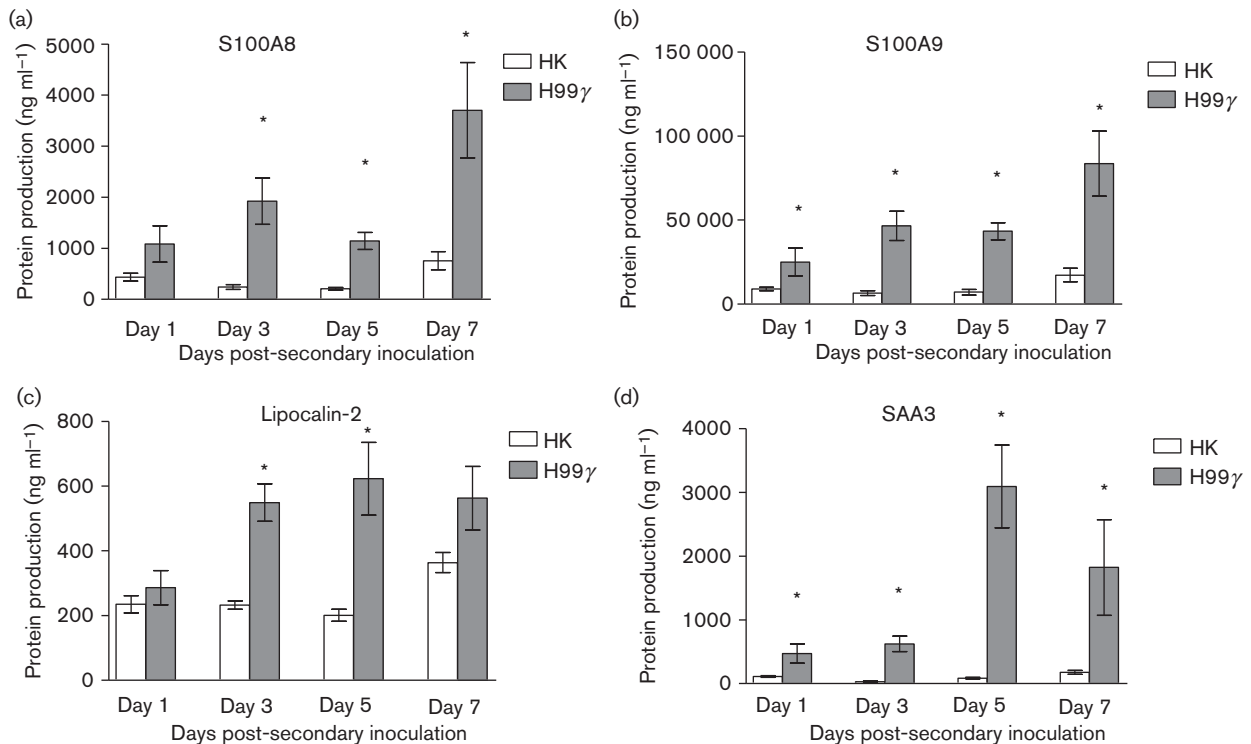


Fig. 5. Pulmonary antimicrobial peptide production during the recall response to *C. neoformans* in protectively and non-protectively immunized mice. BALB/c mice received an initial immunization with HK *C.n.* (HK) or *C. neoformans* strain H99 γ and were allowed 60 days to resolve the infection. Mice then received a second intranasal challenge with 1×10^4 c.f.u. of *C. neoformans* strain H99. Lung homogenates were prepared from lungs excised on days 1, 3, 5 and 7 post-inoculation and assayed for S100A8 (a), S100A9 (b), lipocalin-2 (c) and SAA3 (d) production by ELISA. Data are cumulative of two experiments utilizing four mice per group per time point for each experiment. Asterisks indicate significant differences ($*P < 0.05$).

total lung homogenates derived from each mouse on days 1, 3, 5 and 7 post-inoculation by ELISA. We observed significantly increased levels of SAA3 on days 3, 5 and 7 (Fig. 3d) and S100A8 (Fig. 3a), S100A9 (Fig. 3b) and lipocalin-2 (Fig. 3c) on days 5 and 7 post-inoculation in the lungs of mice inoculated with H99 γ ($P < 0.05$ for each) compared with mice inoculated with WT yeast. However, we observed no difference in β -defensins 1 and 2 expression between each group of mice on all days tested (data not shown).

Experimental pulmonary inoculation with *C. neoformans* strain H99 γ results in the recruitment of TLR4⁺ and RAGE⁺ macrophages and DCs to the lungs during infection

TLR4 and RAGE have been identified as receptors for S100A8/A9 and SAA3 (Ehrchen *et al.*, 2009; Foell *et al.*, 2007; Herold *et al.*, 2007). Thus, we determined the expression of TLR4 and RAGE on the surface of pulmonary macrophages and DCs derived from mice inoculated with WT *C. neoformans* strain H99 or *C. neoformans* strain H99 γ on days 5 and 7 post-inoculation, the time points at which production of antimicrobial peptides and acute phase proteins, respectively, was highest in mice inoculated with

C. neoformans strain H99 γ . Flow cytometric analysis of leukocyte infiltrates demonstrated an increase in the absolute number of TLR4⁺ pulmonary macrophages and DCs (Fig. 4a, b, respectively) and RAGE⁺ pulmonary macrophages and DCs (Fig. 4c, d, respectively).

Protective recall responses to pulmonary *C. neoformans* infection are associated with enhanced antimicrobial peptide and acute phase protein production

We then determined if similar increases in antimicrobial peptide and acute phase protein production in the lungs would be observed during protective recall responses to pulmonary *C. neoformans* infection. BALB/c mice were intranasally immunized with *C. neoformans* strain H99 γ , which induces protective anti-cryptococcal immune responses, or HK *C.n.* strain H99 yeasts, which results in non-protective immune responses, and allowed 60 days to resolve the infection. Subsequently, the mice were challenged with WT *C. neoformans* strain H99, and S100A8, S100A9, lipocalin-2 and SAA3 production in total lung homogenates was determined. We observed significantly increased levels of S100A8 (days 3, 5 and 7), S100A9 (days 1, 3, 5 and 7), lipocalin-2 (days 3 and 5) and SAA3 (days 1,

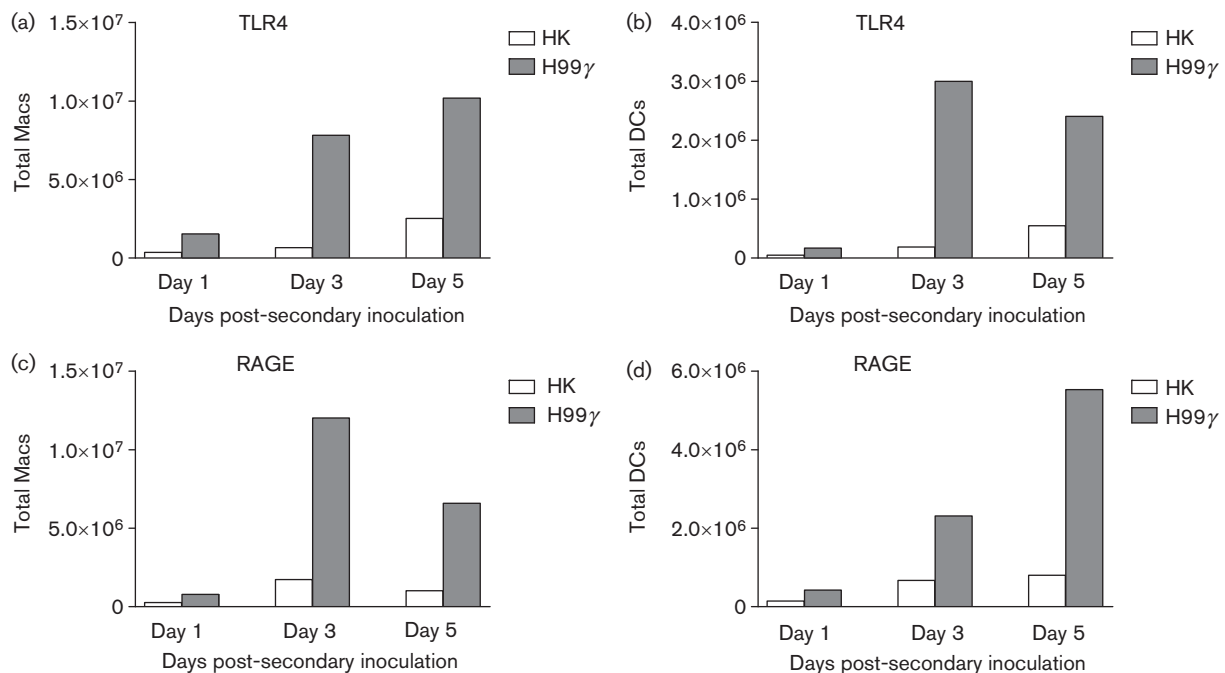


Fig. 6. Antimicrobial peptide receptors on macrophages (Macs) and DCs during secondary challenge in protectively compared with non-protectively immunized mice. BALB/c mice received an initial immunization with HK *C.n.* (HK) or *C. neoformans* strain H99 γ and were allowed 60 days to resolve the infection. Mice then received a second intranasal challenge with 1×10^4 c.f.u. of *C. neoformans* strain H99. Lungs were excised at days 1, 3 and 5 post-challenge and a single cell suspension was generated using enzymic digestion. The leukocytes were then evaluated by flow cytometry for the presence of TLR4 on the surface of macrophages (a) or DCs (b) or RAGE on the surface of macrophages (c) or DCs (d). Flow cytometry data are representative results of two independent experiments using pooled leukocytes from four mice per group per time point for each experiment.

3, 5 and 7) during the recall response to pulmonary challenge with WT *C. neoformans* in mice that were protectively immunized compared with non-protectively immunized mice (Fig. 5). Thus, protection against pulmonary *C. neoformans* infection correlates with increased production of S100A8, S100A9, lipocalin-2 and SAA3.

Protection against pulmonary *C. neoformans* challenge correlates with recruitment of TLR4⁺ and RAGE⁺ pulmonary macrophages and DCs

We next determined the expression of TLR4 and RAGE on pulmonary macrophage and DC infiltrates during the recall response to *C. neoformans* in mice protectively immunized with *C. neoformans* strain H99 γ or non-protectively immunized with HK *C.n.* on days 1, 3 and 5 post-secondary inoculations. Flow cytometric analysis of pulmonary leukocyte infiltrates demonstrated dramatic increases in the absolute number of TLR4⁺ macrophages (Fig. 6a), TLR4⁺ DCs (Fig. 6b), RAGE⁺ macrophages (Fig. 6c) and RAGE⁺ DCs (Fig. 6d) during the recall response to *C. neoformans* in protected mice compared with non-protected mice. These results are similar to the results obtained during primary infection (Fig. 4), and suggest that macrophages and DCs are trafficking to the lungs in response to increased antimicrobial peptide and acute phase protein production in the pulmonary mucosa.

IL-22^{-/-} mice have less antimicrobial peptide and acute phase protein expression compared with WT mice at the protein, but not transcript, level, during experimental pulmonary infection with *C. neoformans* strain H99 γ

Experimental pulmonary inoculation with *C. neoformans* strain H99 γ in mice results in significantly increased production of IL-22 and various antimicrobial peptides and acute phase proteins. We thus determined whether IL-22 was critical to the production of S100A8, S100A9, lipocalin-2 and SAA3 in H99 γ -infected mice. IL-22^{-/-} mice and WT 129 mice were intranasally inoculated with *C. neoformans* strain H99 γ and lungs were excised at day 5 post-inoculation, the time point when the antimicrobial peptide production is at its highest in our model system (data not shown). Gene expression for the various antimicrobial peptides and acute phase proteins was examined. Fig. 7 shows that no significant differences in gene expression of S100A8, S100A9, lipocalin-2, or SAA3 were observed in IL-22^{-/-} mice compared with WT 129 mice. We then performed studies to further examine the importance of IL-22 in modulating protein-level expression of antimicrobial peptides and acute phase proteins in pulmonary tissues of WT 129 mice and IL-22^{-/-} mice following inoculation with *C. neoformans* strain H99 γ at day 5 post-inoculation. Fig. 8 shows that all antimicrobial peptides tested, except S100A9 (Fig. 8b), were significantly reduced in IL-22^{-/-} mice compared with WT mice at day 5 post-inoculation (Fig. 8a, c, d). Interestingly, the overall

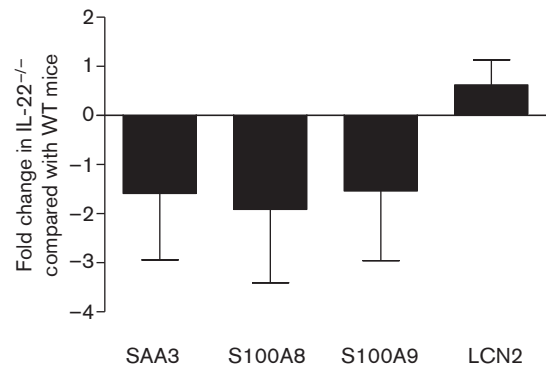


Fig. 7. Antimicrobial peptide gene expression in WT and IL-22^{-/-} mice following inoculation with H99 γ . BALB/c mice were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 γ in 50 μ l sterile PBS. At day 5 post-inoculation, mice were killed and pulmonary RNA was isolated from whole lung tissues homogenized in TRIzol reagent. cDNA was prepared from total RNA and real-time PCR was performed to measure S100A8, S100A9, lipocalin-2 (LCN-2) and SAA3 expression. Bars represent the fold change in gene expression in pulmonary tissues from IL-22^{-/-} mice compared with tissues from WT mice. Data shown are cumulative of three independent experiments utilizing individual tissues from four mice per group per time point per experiment and are reported as means \pm SEM (* $P < 0.05$).

levels of antimicrobial peptides present in the pulmonary homogenates of the WT and IL-22^{-/-} mice were lower than those in previous experiments (Fig. 3); however, these experiments were performed in 129 mice (the appropriate background strain for the IL-22^{-/-} mice), whereas the previous experiments were performed in BALB/c mice, which may explain this difference. In addition to antimicrobial peptides, we assessed pulmonary fungal burden in mice at day 14 post-inoculation, and observed no significant difference between the IL-22^{-/-} and WT mice (Fig. 8e). We then determined whether IL-17A may compensate for the loss of IL-22 in the IL-22^{-/-} mice, as these cytokines cooperatively induce antimicrobial peptide production. IL-17A depletion in IL-22^{-/-} mice showed no further reduction in antimicrobial peptide production at day 5 post-infection (data not shown).

IL-22^{-/-} mice have similar survival rates to WT mice during pulmonary *C. neoformans* strain H99 γ infection

To determine the role of IL-22 on survival during cryptococcal infection with H99 γ , IL-22^{-/-} mice and WT 129 mice were intranasally inoculated with *C. neoformans* strain H99 γ . After 40 days, all mice (100%) survived and were killed (Fig. 9). Fungal burden was determined from brain and lungs of surviving mice, and no cryptococci were detected in either group from either organ tested (data not shown).

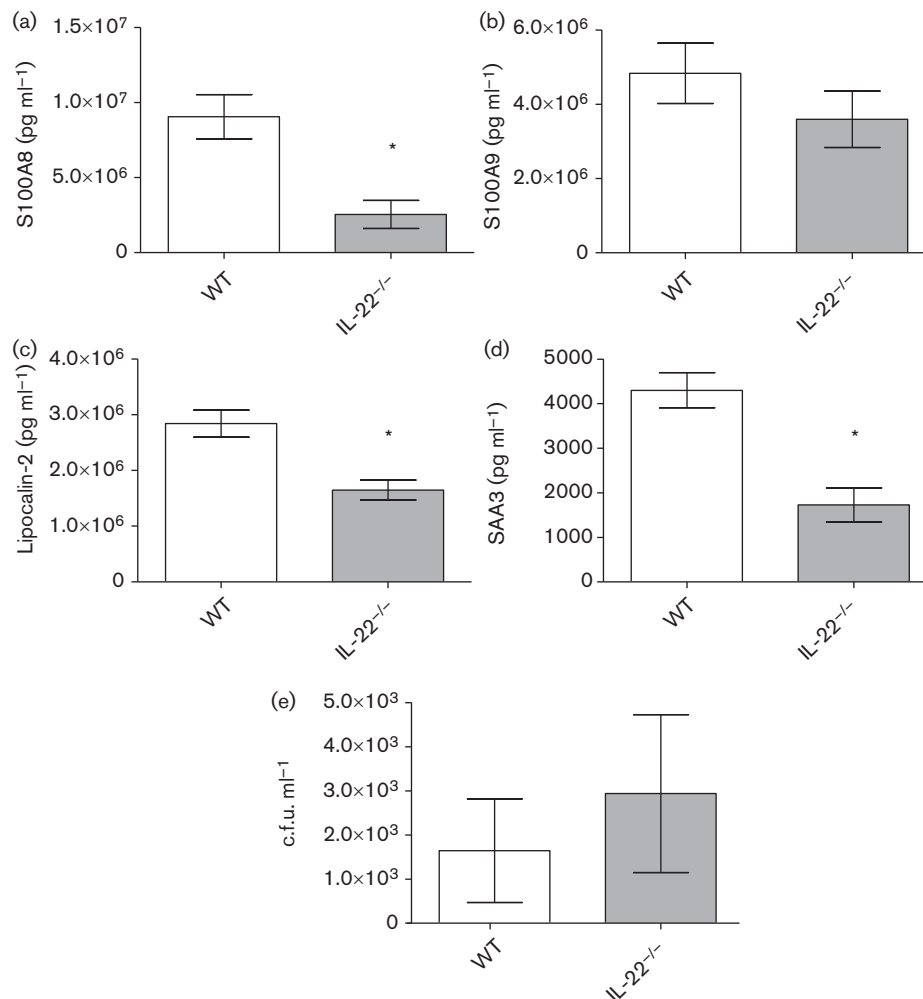


Fig. 8. Pulmonary antimicrobial peptide production and fungal burden in WT and IL-22^{-/-} mice following inoculation with *C. neoformans* strain H99 γ . IL-22^{-/-} mice and WT 129 mice were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 γ yeast cells. For pulmonary antimicrobial peptide analysis, lung homogenates were prepared from lungs excised on day 5 post-inoculation and assayed for S100A8 (a), S100A9 (b), lipocalin-2 (c) and SAA3 (d) production by ELISA. For pulmonary fungal burden analysis (e), mice were killed at day 14 post-inoculation and pulmonary homogenates were plated for quantitative culture on YPD agar. Data are cumulative of three experiments utilizing four to five mice per group (for antimicrobial peptides) or two experiments utilizing four mice per group (for fungal burden analysis) per time point for each experiment. Asterisks indicate significant differences (* $P < 0.05$).

Experimental pulmonary inoculation of IL-22^{-/-} mice with *C. neoformans* strain H99 γ results in normal recruitment of TLR4⁺ macrophages and DCs but reduced recruitment of RAGE⁺ macrophages and DCs to the lungs during infection

We sought to understand why IL-22^{-/-} mice survived infection with *C. neoformans* strain H99 γ by determining the recruitment of TLR4⁺ and RAGE⁺ macrophages and DCs during infection in IL-22^{-/-} mice compared with WT 129 mice. Flow cytometric analysis of pulmonary leukocyte infiltrates on day 7 post-inoculation demonstrated similar

absolute numbers of total TLR4⁺ cells, TLR4⁺ macrophages and TLR4⁺ DCs (Fig. 10a–c) in IL-22^{-/-} mice compared with WT mice. However, a significant decrease in total RAGE⁺ cells was found in IL-22^{-/-} mice compared with WT mice (Fig. 10d), and decreased (albeit non-significant) numbers of RAGE⁺ macrophages and RAGE⁺ DCs were observed in the lungs of knockout mice compared with WT mice ($P = 0.067$ and 0.064 , respectively). These results suggest that although there is some defect in the recruitment of RAGE⁺ cells during H99 γ pulmonary infection in IL-22^{-/-} mice, all putative receptors for antimicrobial binding are not equally affected and may not uniformly rely on IL-22 for expression and/or activity.

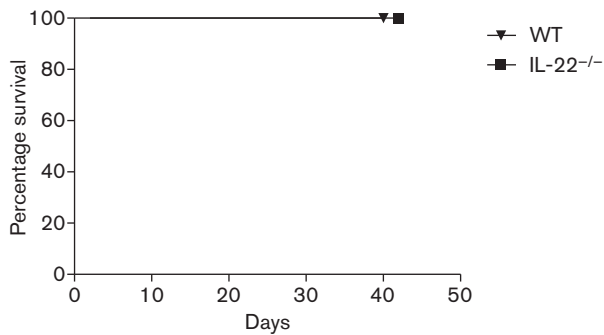


Fig. 9. Role of IL-22 in survival following inoculation with *C. neoformans* strain H99 γ . IL-22^{-/-} mice and WT mice were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 γ . Mice were monitored for 40 days to determine survival during infection with H99 γ . The survival data shown are from one experiment using 10 mice per group.

DISCUSSION

Antimicrobial proteins constitute a phylogenetically conserved and highly effective mechanism of protection against invading organisms at the skin and mucosal surfaces (Kolls *et al.*, 2008). IL-17A and IL-22 are considered critical regulators of antimicrobial protein expression in the lungs. Consequently, we sought to determine the contribution of IL-22, IL-17A, and various antimicrobial peptides and acute phase proteins towards providing protection against pulmonary cryptococcosis. We demonstrated that mice inoculated with *C. neoformans* strain H99 γ produce increased pulmonary levels of IL-22

and IL-17A during infection that correlate with increased transcript expression and protein levels of antimicrobial peptides in both primary and secondary infection models. Furthermore, mice inoculated with H99 γ have increased expression of receptors TLR4 and RAGE, which interact with these proteins on innate immune cells of the lung. IL-22^{-/-} mice infected with H99 γ show no change in antimicrobial gene expression, but do have reduced levels of pulmonary antimicrobial peptides. However, these decreases do not affect fungal burden or survival in this model. Furthermore, IL-22^{-/-} mice show defective recruitment of RAGE⁺ macrophages and DCs to the lung during infection with *C. neoformans* strain H99 γ , but normal recruitment of TLR4⁺ macrophages and DCs. These results led us to conclude that although antimicrobial peptides may be important in the initiation of the immune response, reductions in antimicrobial peptides in IL-22^{-/-} mice are not sufficient to abrogate the protective response. In addition, IL-22 and IL-17A do not appear to be solely responsible for the production of antimicrobial peptides, recruitment of TLR4⁺ macrophages and DCs, and/or protective anti-cryptococcal immune responses.

Several literature examples show that IL-22 and antimicrobial peptides have protective effects during infection, especially in some pulmonary disease models (Aujla *et al.*, 2008; Miyakawa *et al.*, 1996; Pociask *et al.*, 2013). IL-22 has also been shown to be important in influenza infection, where the presence of this cytokine is critical to lung epithelial repair (Pociask *et al.*, 2013). In other pulmonary infections, most notably *K. pneumoniae*, IL-22 is extremely important in the induction of antimicrobial peptides and resolution of infection (Aujla *et al.*, 2008). However, bacteria are generally more sensitive to the antimicrobial

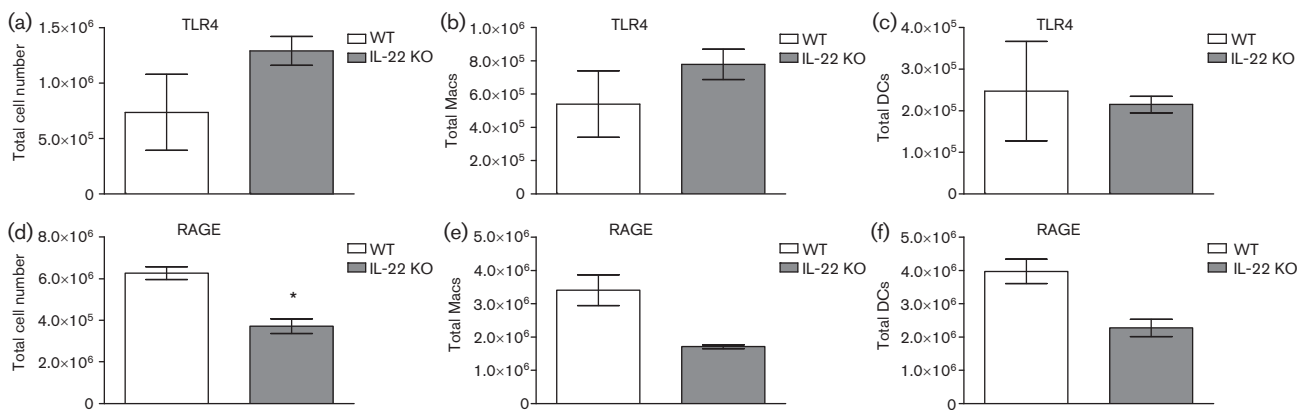


Fig. 10. Antimicrobial peptide receptors on macrophages (Macs) and DCs following inoculation of WT and IL-22^{-/-} mice with H99 γ . Mice of 129 (WT) and IL-22^{-/-} (KO) strains were intranasally inoculated with 1×10^4 c.f.u. of *C. neoformans* strain H99 γ . Lungs were excised at day 7 post-inoculation and a single cell suspension was generated using enzymic digestion. The leukocytes were evaluated by flow cytometry for the presence of TLR4 on the surface of all leukocytes (a), macrophages (b) or DCs (c) or RAGE on the surface of all leukocytes (d), macrophages (e) or DCs (f). Flow cytometry data are cumulative of two independent experiments using pooled leukocytes from four mice per group per time point for each experiment and are reported as means \pm SEM. Asterisks indicate significant differences (* $P < 0.05$).

effects of the peptides themselves – including pore formation and iron sequestration (reviewed by Ganz, 2003; Zasloff, 2002) – whereas fungi have not been shown to be as susceptible to these actions. The inherent resistance of fungi to these antimicrobial peptides may be due to their cell-wall structure, which differs greatly from bacterial cell walls. Alternatively, the antimicrobial peptides may act as messengers during fungal infections rather than as effector molecules. In studies of *Candida albicans* vaginitis, the S100 antimicrobial peptides have been examined, and data have shown that their role is more important in neutrophil recruitment than in direct microbicidal activity against *Candida* (Johnston *et al.*, 2013; Yano *et al.*, 2010, 2012a, b). However, additional studies by other laboratories have shown that IL-22 is critical to protection in a vaginal model of *Candida albicans* infection (De Luca *et al.*, 2010, 2013). Furthermore, our *in vitro* studies did not show anti-fungal activity of any of the purified AMPs against *C. neoformans* (data not shown).

Although we did not observe a change in survival of IL-22^{-/-} mice compared with WT mice during infection with *C. neoformans* strain H99 γ , it is possible that the antimicrobial peptides are active in early cell recruitment and may serve as biomarkers of disease (Li & Chan, 2011; Uhlar & Whitehead, 1999). As reported elsewhere in the literature, antimicrobial peptides can induce deleterious inflammatory responses if not controlled (Jäger *et al.*, 2010), so it is possible that in our system the antimicrobial peptides are required at early time points, but are ‘turned off’ to control the inflammatory response. Interestingly, IL-22^{-/-} mice still produced antimicrobial peptides during H99 γ infection, albeit in lower amounts than that found in WT mice (Fig. 8). Furthermore, IL-22^{-/-} mice had no deficiency in recruitment of TLR4⁺ macrophages and DCs to the pulmonary tissues, but they did have a deficiency in the recruitment of RAGE⁺ macrophages and DCs, suggesting that IL-22 signalling may be involved in the expression of RAGE, a receptor that binds multiple antimicrobial peptides (Fig. 10). Therefore, as in many biological systems, there are probably redundancies in pathways leading to antimicrobial peptide production, and IL-22 is not solely responsible for their production. As IL-22 and IL-17A are known to collaborate to induce antimicrobial peptide production, we examined whether IL-17A depletion in IL-22^{-/-} mice would further reduce the amounts of antimicrobial peptides produced. Depletion of IL-17A in IL-22^{-/-} mice did not affect the pulmonary levels of antimicrobial peptides or result in any change in c.f.u. (data not shown). These data correlate with our earlier findings that protection is not dependent on IL-17A in our model system (Hardison *et al.*, 2010; Wozniak *et al.*, 2011).

The upregulation of the receptors for antimicrobial peptides points to a currently unidentified mechanism of antimicrobial action for APCs. Just the presence of antimicrobial peptides may be involved in cryptococcal killing. However, their interaction with receptors on the

cell surface of APCs may be necessary for initiation of immune responses by these APCs. It is possible that the production of these antimicrobial peptides leads to a faster, more efficient immune response by these APCs but that IL-22 is not solely responsible for their production, again pointing to redundancy in the induction of antimicrobial peptides.

In conclusion, these data show that IL-22 and IL-17A production are associated with antimicrobial peptide production and the expression of receptors for antimicrobial peptides in mice protected against *C. neoformans* infection. In addition, the receptors for antimicrobial peptides are increased on macrophages and DCs infiltrating into the pulmonary tissues during infection. However, survival of mice and recruitment of TLR4⁺ macrophages and DCs during infection with *C. neoformans* strain H99 γ are not significantly affected by the loss of IL-22. Additional studies are in progress to determine what additional factor(s) may be responsible for the induction of antimicrobial peptides and the ultimate resolution of cryptococcal infection.

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