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Epithelial Cell-Derived S100 Calcium-Binding Proteins as Key Mediators in the Hallmark Acute Neutrophil Response during *Candida* Vaginitis[▽]

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Vulvovaginal candidiasis (VVC), caused by *Candida* species, is a significant problem in women of childbearing age. Similar to clinical observations, a robust vaginal polymorphonuclear neutrophil (PMN) migration occurs in a subset of mice without affecting vaginal fungal burden. We hypothesize that the vaginal PMN infiltrate and accompanying inflammation are not protective but instead are responsible for the symptoms of infection. The purpose of this study was to identify the signal(s) associated with the PMN response in the established mouse model. Vaginal lavage fluid from inoculated mice were categorized base on PMN counts, evaluated for PMN chemotactic activity and analyzed by SDS-PAGE and mass spectrometry (MS) for unique protein identification. The lavage fluid from inoculated mice with high, but not low, PMN levels showed increased chemotactic activity. Likewise, SDS-PAGE of lavage fluid with high PMN levels showed distinct protein patterns. MS revealed that bands at 6 and 14 kDa matched the PMN chemotactic calcium-binding proteins (CBPs), S100A8 and S100A9, respectively. The presence of the CBPs in lavage fluid was confirmed by Western blots and enzyme-linked immunosorbent assay. Vaginal tissues and epithelial cells from inoculated mice with high PMN levels stained more intensely and exhibited increased mRNA transcripts for both proteins compared to those in mice with low PMN levels. Subsequent antibody neutralization showed significant abrogation of the chemotactic activity when the lavage fluid was treated with anti-S100A8, but not anti-S100A9, antibodies. These results reveal that the PMN chemotactic CBP S100A8 and S100A9 are produced by vaginal epithelial cells following interaction with *Candida* and that S100A8 is a strong candidate responsible for the robust PMN migration during experimental VVC.

Vulvovaginal candidiasis (VVC), caused by *Candida* species, is an opportunistic fungal infection that affects ca. 75% of healthy women of childbearing age (34). Menarchal women are predisposed to VVC through several exogenous factors, most of which involve elevated hormone levels (e.g., pregnancy, use of high estrogen oral contraception or hormone replacement therapies, and the luteal phase of the menstrual cycle) (34). Frequent antibiotic usage and uncontrolled diabetes mellitus are also known to be linked to increased susceptibility to VVC. Recurrent vulvovaginal candidiasis (RVVC), defined as three or more episodes of VVC per year, affects a separate 5 to 10% of menarchal women. Most cases of RVVC are primary RVVC, where idiopathic infection occurs with no predisposing factors, whereas secondary RVVC (repeated occurrence of acute VVC) could occur as a result of being unable to avoid certain predisposing factors (34).

Although VVC and RVVC have historically been attributed to a putative local immune deficiency, several studies using a mouse model of *Candida* vaginitis and many cross-sectional clinical studies evaluating women with primary RVVC have shown that protection is not mediated by local or systemic adaptive immunity (17, 18). In fact, several forms of immunoregulation seem to be in place to inhibit such responses (11, 19, 26). Instead, innate immunity by epithelial cells appears to provide some level of protection through noninflammatory processes involving direct contact with *Candida* (3, 29, 39). To further investigate factors associated with susceptibility to infection, a human live challenge was established where healthy women were given an intravaginal inoculation of *C. albicans* and followed for the natural history of infection (10). These studies showed that protection indeed occurred in the absence of any inflammatory responses, whereas symptomatic infection was associated with a heavy vaginal cellular infiltrate consisting almost entirely of polymorphonuclear neutrophils (PMNs) (10). Furthermore, vaginal lavage fluid from women with symptomatic infection promoted PMN migration *in vitro*, while lavage fluid from asymptomatically colonized women did not (10). These results suggested that susceptibility to infection may be associated with the secretion of PMN chemotactic factors following the interaction of *Candida* with the vaginal epithelium and are responsible for the robust PMN response.

An experimental mouse model of *Candida* vaginitis has historically been used to study mucosal host defense mechanisms against *C. albicans* (18). Although this model has its limitations (i.e., requirement for a state of pseudoestrus for establishing infection, lack of *Candida* as normal flora at mucosal sites, and a neutral vaginal pH unlike the acidic pH in humans), it parallels many aspects of the clinical disease, including the presence of PMNs in the vagina following inoculation (5, 15, 22). However, no protective role of PMNs was ever demonstrated;

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their presence was somewhat erratic and was never correlated to a reduction in vaginal fungal burden. However, based on the vaginal PMN data from the clinical live challenge study (10), it appears that the presence of PMNs during experimental vaginal infection may have been significant. Hence, the purpose of the present study was to reevaluate the relationship between the PMN migration and infection status in the established mouse model of *Candida* vaginitis and to characterize the associated chemotactic factors that are responsible for the PMN recruitment to the vagina.

MATERIALS AND METHODS

Mice. Female CBA/J mice, 6 to 10 weeks of age, were purchased from Charles River at the National Cancer Institute (NCI), Frederick, MD, and used throughout the studies. For evaluation of different mouse strains in infection, age-matched female C3H/HeN and SJL mice (NCI), BALB/c, C57BL/6, and DBA/2 mice (Jackson Laboratories, Bar Harbor, ME) were used. All animals were housed and handled according to institutionally recommended guidelines. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the LSU Health Sciences Center.

Vaginal *Candida* infection. Vaginal infection was conducted as previously described (16, 18). Briefly, mice were injected subcutaneously with 0.1 mg of β-estradiol (Sigma Chemical Co., St. Louis, MO) in 100 μl of sesame oil (Sigma) 72 h prior to inoculation and then weekly throughout the study period. Intravaginal inoculation was performed by introducing 20 μl of phosphate-buffered saline (PBS) containing 5×10^4 *C. albicans* 3153A blastoconidia from a stationary-phase culture (i.e., 12 to 18 h of culture at 25°C in Phytone-peptone broth with 0.1% glucose) into the vaginal lumen. Control animals were estrogen treated (estrogenized) and inoculated with PBS alone. Separate groups of 5 to 10 mice were sacrificed at 4, 7, and 10 days postinoculation. Vaginal lavages were conducted using 100 μl of sterile PBS with repeated aspiration and agitation. Vaginal fungal burden was quantified by culturing the lavage fluid at 1:10 serial dilutions on Sabouraud-dextrose agar plates (BD Diagnostics, Sparks, MD) supplemented with gentamicin (Invitrogen, Carlsbad, CA). CFU were enumerated after incubation at 35°C for 48 h and expressed as CFU/100 μl of lavage fluid. Supernatants of the lavage fluid were filtered through 0.45-μm-pore-size filter units and stored at −70°C until use.

Quantification of vaginal PMNs. Smears containing 10 μl of vaginal lavage fluid collected from each inoculated and uninoculated mouse were stained using the Papanicolaous technique (pap smear). When present, vaginal leukocytes were identified to be predominantly PMNs by the characteristic trinuclear lobes. PMN counts were taken from five nonadjacent high-power fields (×400) per animal by light microscopy and averaged. As another measure of PMN numbers, absolute numbers of trilobed leukocytes present in 100-μl lavage samples were enumerated by wet-mount preparations. As further confirmation of PMN quantification, cellular fractions of lavage fluids were analyzed by flow cytometry using standard direct immunofluorescence methods. Briefly, cells were incubated with a combination of allophycocyanin-labeled monoclonal anti-mouse Gr-1 antibodies (1.25 μg/ml; R&D Systems, Minneapolis, MN) and phycoerythrin-labeled monoclonal anti-mouse CD45 antibodies (0.3 μg/ml; eBioscience, San Diego, CA) for 30 min at 4°C. Cells were incubated with either buffer alone or fluorochrome-conjugated isotype control (rat IgG2b) to determine background staining. After incubation, the cells were washed and fixed in Poly-Lem fixative (Polysciences, Warrington, PA). Flow cytometric data were collected on a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ) and analyzed using FlowJo (version 7.5.5; TreeStar, Ashland, OR).

Longitudinal evaluation of infection. Quantitative counts of vaginal CFU and PMNs in lavage fluid from 15 mice were evaluated successively at 8h and on days 5, 10, 15, 20, 25, and 30 postinoculation. The mice were anesthetized with isoflurane for each lavage. Lavage samples from estrogenized uninoculated mice were also collected at each time point as controls. Vaginal fungal burden and PMNs were quantified by plate count and pap smear, respectively.

***Candida* vaginal adherence assay.** Mouse vaginae from estrogenized inoculated mice ($n = 4$) were excised at 8, 16, and 24 h postinoculation and individually washed with a continuous vertical flow of 5 ml of sterile PBS. The resulting wash fluid was collected into a 15-ml tube, serially diluted, and cultured on Sabouraud-dextrose agar plates. The washed vaginae were weighed and homogenized in PBS supplemented with 0.1% Triton X-100. The resulting tissue homogenates were serially diluted and cultured on Sabouraud-dextrose agar plates. CFU were enumerated as described above and expressed as CFU/gram of vagina. In addition, sections of inoculated vaginae pre- and postwashing were placed on glass slides and incubated with calcofluor white stain (Sigma) in the dark for 15 min at room temperature to stain *Candida*. Stained *Candida* cells were visualized by confocal microscopy. The percent *Candida* adherence was calculated as follows: % adherence = [CFU for tissue homogenate/(CFU for wash fluid + CFU for tissue homogenate)] × 100.

PMN chemotaxis assay. Mouse PMNs were obtained from peritoneal exudate cells harvested 12 h after intraperitoneal injection of 10% casein in PBS. PMNs were isolated by the standard techniques of Ficoll-Paque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation, followed by hypotonic lysis of erythrocytes. PMN enrichment was confirmed by flow cytometry with the final enrichment ranging from 85 to 95% Gr-1^+ cells. A transwell system (Corning, New York, NY) was used to determine PMN chemotaxis levels of vaginal lavage fluid from inoculated mice. Recombinant mouse MIP-2 (100 ng/ml, a PMN chemoattractant) and RANTES (100 ng/ml, a monocyte chemoattractant) (R&D Systems) were used as positive and negative assay controls, respectively. *C. albicans* culture filtrate antigen (CaCF) and the supernatant of a 24-h *C. albicans* blastoconidia culture (CA sup) were included as controls for the presence of *C. albicans* antigen in the lavage fluid, and lavage vehicle (PBS) served as the true negative control. Chemotaxis buffer (110 μl) consisting of RPMI 1640 containing 2 nM L-glutamine, 25 mM HEPES (Invitrogen), and 1% bovine serum albumin (BSA; Sigma) was added to the upper chambers of the transwell plate, and the controls described above or lavage fluid at 1:3 dilutions were added to the bottom chambers. All controls and lavage samples were tested in duplicate. After 5 min of equilibration, 50 μl of chemotaxis buffer containing 5×10^4 PMNs was added to the upper chambers, and the plate was incubated for 1 h at 37° in 5% CO_2. After incubation, total PMNs that migrated into the bottom chambers were harvested and enumerated microscopically using a hemocytometer. The results were expressed as the fold increase over PMN migration induced by lavage fluid from estrogenized uninoculated mice.

Proteomic analyses. (i) SDS-PAGE. The total protein concentrations of vaginal lavage fluid were determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL). Portions (10 μg) of total proteins in lavage fluid were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, the lavage samples were mixed 1:1 with Laemmli sample buffer (Bio-Rad, Hercules, CA) supplemented with β-mercaptoethanol and heated for 5 min at 95°C prior to electrophoresis in a 15% Tris-HCl polyacrylamide gels (Bio-Rad). Separated proteins were stained with Coomassie blue (Bio-Rad) and visualized on a MultiImage Light Cabinet (Alpha Innotech, San Leandro, CA).

(ii) MS. Protein bands of interest identified by SDS-PAGE were excised and prepared for matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analyses. The peak list of proteins was used to query all entries of the NCBI-nr protein database using the Mascot search program (Matrix Sciences, London, United Kingdom). Protein integrity scores were provided for each protein assessed. The protein integrity score is defined as $-10 \log(P)$, where P is the probability that the observed match is a random event. If the integrity score is higher than 75 (i.e., $P < 0.05$), the match is considered as nonrandom and significant for protein identification.

(iii) Western blotting. Protein in vaginal lavage fluid or vaginal epithelial cell lysates from estrogenized inoculated or uninoculated mice were separated in 18% Tris-HCl polyacrylamide gels (Bio-Rad) and transferred to 0.2-μm-pore-size nitrocellulose membranes. Blots were blocked with 3% nonfat milk (Bio-Rad) and incubated with primary antibodies specific for S100A8 or S100A9 (goat polyclonal, 0.5 μg/ml) or β-actin (sheep polyclonal, 1 μg/ml) (R&D Systems) overnight at 4°C. The blots were then washed and incubated with biotinylated anti-goat or anti-sheep secondary antibodies (IgG, 0.1 μg/ml) for 1 h. After washing, the bands were labeled with streptavidin-horseradish peroxidase (HRP) conjugate and visualized with amplified opti-4CN substrate in accordance with the manufacturer's instructions (Bio-Rad). Recombinant mouse S100A8 and S100A9 (10 ng; R&D Systems) were included as references for the molecular weight and antibody specificity.

(iv) ELISA. Concentrations of S100A8 and S100A9 in vaginal lavage fluids from estrogenized inoculated and uninoculated mice were determined by a standard enzyme-linked immunosorbent assay (ELISA). EIA/RIA plates (Costar, Corning, NY) were coated with monoclonal rat anti-mouse S100A8 or S100A9 antibodies (2 μg/ml; R&D Systems). After overnight incubation at 4°C, the nonspecific protein-binding sites were blocked with 1% BSA in PBS for 1 h at 37°C. The plates were washed three times with ELISA wash buffer (0.5% Tween 20 [Sigma] in PBS), and lavage fluid supernatants (at dilutions ranging from 1:10 to $1:10^5$) and standards (serially diluted recombinant mouse S100A8 or S100A9; R&D Systems) were added in triplicate, followed by incubation for 2 h at 37°C. After washing, the plates were incubated with primary antibodies

(1 μg/ml; polyclonal goat anti-mouse S100A8 and S100A9) for 1 h at 37°C, washed, and incubated with the secondary antibody (biotinylated anti-goat IgG, 0.05 μg/ml) for 1 h at 37°C. After washing, the plates were incubated with streptavidin-HRP (Bio-Rad) for 30 min at room temperature, washed, and reacted with one-step ultra tetramethylbenzidine (TMB; Thermo). The reaction was stopped with sulfuric acid (2 N) when it reached the optimal color intensity. The absorbance was read at 450 nm on a Multiskan Ascent microplate photometer (Labsystems, Helsinki, Finland). The results were expressed as nanograms per 100 μl of lavage fluid.

The ELISA was validated by several means. First, the detectable concentrations for each protein were set within the linear range of the standard curve and extrapolated. At least three dilutions of each sample were tested, and the values within the detectable range of the standard curve were used to determine the final concentrations of the target proteins in the samples. Second, the assay was performed using isotype controls for either capture (monoclonal), primary (polyclonal), or biotinylated secondary antibodies and showed no background absorbance. Third, no cross-reactivity was observed between the anti-S100A8 and anti-S100A9 antibodies to the respective recombinant proteins. Finally, low levels of cross-reactivity were observed between antibodies to S100A8 and S100A9 in lavage samples with high PMN levels. This was presumably due to the presence of the S100A8/S100A9 heterodimeric complex, which appeared quite low (the range was 0.2 to 17.4% of the total S100A8 and S100A9 concentrations).

Immunocytochemistry and immunohistochemistry. For immunocytochemical analysis, cellular fractions of vaginal lavage fluid from estrogenized inoculated and uninoculated mice were cytospun onto glass slides using a Cytospin 4 cytocentrifuge (Thermo) at 1,000 rpm for 5 min. The slides were fixed in ice-cold acetone for 5 min and stored at −20°C until use. For immunohistochemical analysis, mouse vaginae from estrogenized inoculated or uninoculated mice were excised, placed in Tissue-Tek cryomolds (Miles Corp., Elkhart, ID) containing optimum cutting temperature (OCT) medium (Sakura Finetek USA, Torrance, CA) and stored at −70°C. Frozen tissue was sectioned (6 μm) and collected on glass slides. The slides were fixed in ice-cold acetone for 5 min and stored at −20°C until use. Upon hydration of cytospin preparations and tissue sections, all steps were performed using the cell and tissue staining kit HRP-3-amino-9-ethylcarbazole (AEC; R&D Systems). Briefly, cells or tissues were blocked with peroxidase, goat serum, avidin, and biotin blocking buffers and then incubated with monoclonal rat anti-mouse S100A8 or S100A9 antibody (10 μg/ml; R&D Systems), monoclonal mouse anti-human AE1/AE3 antibody (epithelial cytokeratin markers, 5 μg/ml; MP Biomedicals, Solon, OH), or isotype controls (rat IgG2a, rat IgG2b, and mouse IgG1) overnight at 4°C. The slides were then washed and incubated with biotinylated anti-rat IgG antibodies (R&D Systems) or anti-mouse IgG $F(ab')_2$ fragments (Thermo) for 1 h at room temperature. The slides were then washed and incubated with streptavidin-HRP for 30 min. Finally, the slides were washed and reacted with AEC chromogen substrate, counterstained with CAT hematoxylin (Biocare Medical, Concord, CA), and preserved in aqueous mounting medium (R&D Systems). For quantitative analyses of immunocytochemical data, the numbers of positively stained epithelial cells were enumerated in five nonadjacent fields per sample at ×100 magnification and averaged.

Gene expression analysis. To harvest vaginal epithelial cells, mouse vaginae from estrogenized inoculated or uninoculated mice were excised and placed in a 48-well plate containing 0.5 ml/well of Dispase (1.7 U/ml; Invitrogen) and then incubated on a shaking platform overnight at 4°C. The epithelial sheets were harvested from the intact vaginal tissue, minced with a scalpel, and washed in PBS. The pellet was resuspended in 1 ml of 10× trypsin-EDTA (Invitrogen) and incubated at 37°C for 10 min. The suspension was them sheared by using a syringe with 21-gauge needle, washed in PBS, and enumerated by trypan blue dye exclusion. Total RNA from vaginal epithelial cells was collected by using the RNeasy minikit (Qiagen, Valencia, CA). Synthesis of cDNA from 10 ng of total RNA was completed by using 20 U of SuperScript III reverse transcriptase with 5 mM dTT (Invitrogen), GeneAmp 10× PCR buffer II with 5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 1 mM deoxynucleoside triphosphates (GE Healthcare), and 20 U of RNasin RNase inhibitor (Promega, Madison, WI) in a total volume of 10 μl and primed with random hexamers in a 96-well thermal cycler (25°C for 10 min, 48°C for 30 min, and 95°C for 5 min). Real-time PCR was performed by using TaqMan gene expression assays predesigned for mouse *S100A8* and *S100A9* (assay IDs Mm01220132_g1 and Mm00656925_m1, respectively; Applied Biosystems) and Brilliant II QPCR Master Mix (Stratagene, La Jolla, CA) in a total reaction volume of 25 μl. The PCR products were detected in consecutive 44 cycles (95°C for 15 s and 60°C for 1 min) in a CFX96 real-time PCR cycler (Bio-Rad). Signals were normalized to β-actin RNA content. Normalized data were used to quantify relative expression levels of *S100A8* and *S100A9* mRNA using the $\Delta\Delta C_T$ method. The results are expressed as the fold increase over expression in cells from estrogenized uninoculated mice.

Antibody neutralization study. Polyclonal goat antibodies to mouse S100A8, S100A9, or isotype goat IgG (10 μg/ml; R&D Systems) were added separately or in combination to pooled vaginal lavage fluid from inoculated mice with a high PMN infiltrate that had shown significantly increased PMN migration activity. The mixtures were incubated for 30 min at room temperature and then evaluated for PMN migration in the chemotaxis assay described above. The results were expressed as the percentage of control (numbers of migrated PMNs by lavage fluid treated with the isotype IgG).

Statistics. The unpaired Student *t* test was used to analyze the data. Significant differences were defined at a confidence level where P was <0.05 and evaluated using Prism Software (GraphPad, San Diego, CA).

RESULTS

Vaginal PMN response post-*Candida* inoculation. Based on the documented vaginal presence of PMNs in the animal model (15) after vaginal inoculation with *Candida*, our first objective was to classify and/or stratify the migration by PMNs into the vagina. For this, we compiled data for vaginal fungal burden and quantification of PMNs in lavages from estrogenized uninoculated and inoculated mice on days 4, 7, and 10 postinoculation from a total of 22 experiments with 5 to 10 mice per group using the pap smear technique to identify cell types. The average numbers of PMNs/field from five fields at ×400 magnification are illustrated per mouse (Fig. 1A). Visual breakpoints for the PMN numbers were identified from the complete data set of PMN counts and stratified into high, intermediate, and low categories. Using this strategy, ≥50 PMNs/field could be classified as "high" PMN numbers, whereas <25 PMNs/field could be classified as "low" PMN numbers. Intermediate was then defined as 25 to 49 PMNs/field. PMNs from uninoculated mice were predominantly classified as low. The second objective was to verify and confirm these categories by other means. Comparing the cumulative stratified data by pap smear (Fig. 1B), similar results were observed by wet-mount microscopy for absolute quantification per volume (100 μl) based on trilobed nuclei (Fig. 1C) and flow cytometry for the neutrophil marker Gr-1-positive cells (Fig. 1D). In all cases, a significant difference between high versus low PMN categories at days 4 and 7 postinoculation was verified ($P < 0.0001$ and $P < 0.0001$ by pap smear, $P < 0.0001$ and $P < 0.0001$ by wet-mount, and $P = 0.0446$ and $P = 0.0234$ by flow cytometry, respectively). Additional flow cytometric analysis restricted to the leukocytes alone showed that PMNs constituted the predominant cell type, ranging from 64 to 97%. Finally, all inoculated mice from the PMN experiments described above were positive for vaginal *Candida* burden (predominantly hyphae confirmed by wet-mount) and exhibited equal variability in vaginal CFU ($P > 0.05$). Uninoculated mice remained negative for yeast or hyphae throughout the study period (Fig. 1E).

Reevaluations of the animal model. To determine whether earlier or later time points postinoculation in CBA/J mice were more or less consistent for PMN infiltration, vaginal fungal burden and PMN counts were evaluated in lavage fluids collected 8 h, and 1, 2, and 5 days postinoculation and at 5-day intervals thereafter for a period of 30 days. The results showed that the PMN infiltration was initiated as early as day 2 postinoculation and occurred similarly throughout the 30-day period. Although vaginal fungal bur-

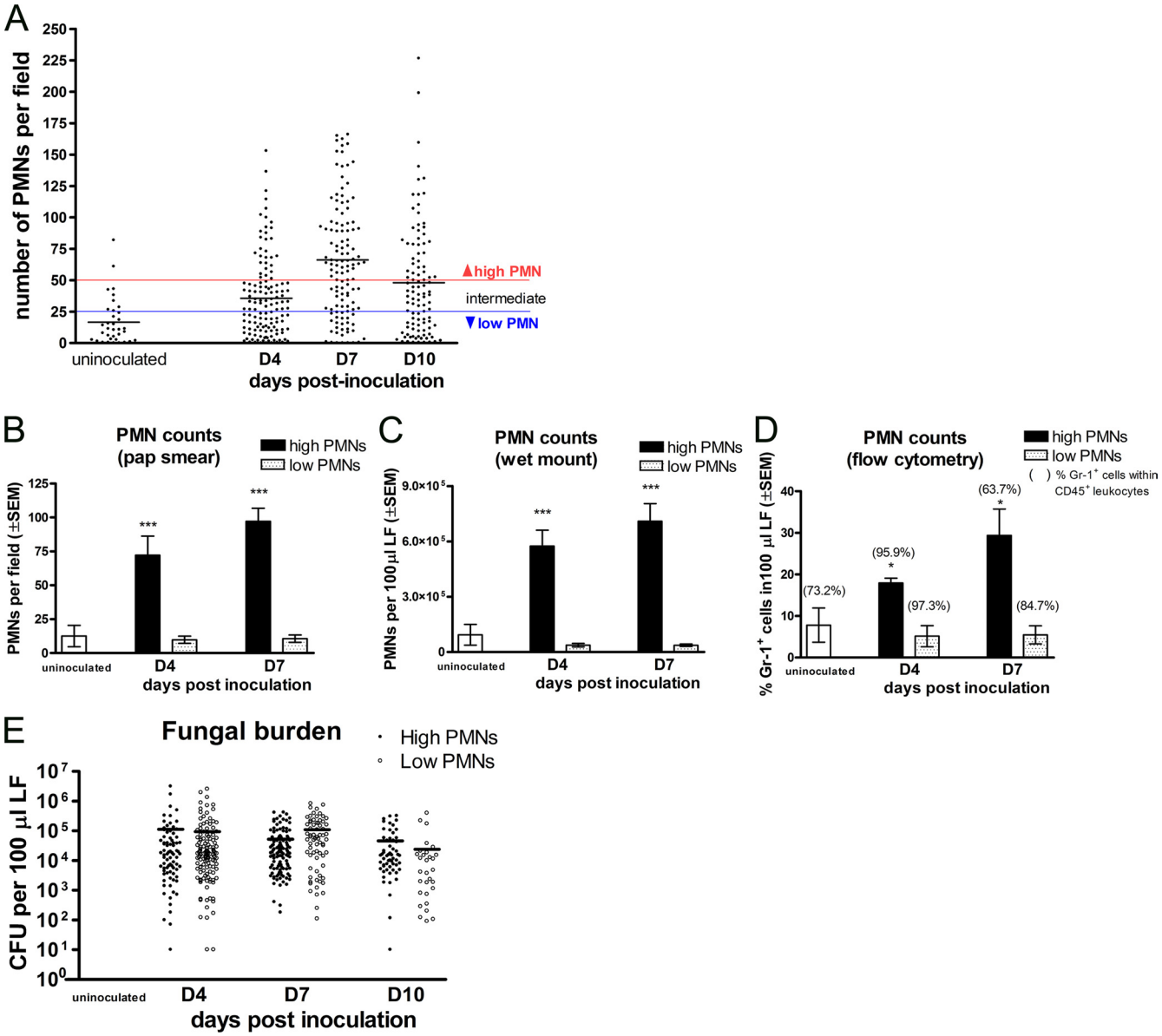

FIG. 1. Classification of vaginal lavage samples based on PMN infiltration levels in response to *Candida* inoculation. (A) Stratification of lavage fluid into high, intermediate and low PMN groups. PMNs were quantified in lavage fluids from estrogenized inoculated and uninoculated mice on days 4, 7, and 10 postinoculation. Each point represents an individual mouse, and the horizontal bar indicates the geometric means. Lavage fluids were classified into high (≥50 PMNs/field), intermediate (26 to 49 PMNs/field), and low (≤25 PMNs/field) groups according to the number of PMNs per field at ×400 magnification. The results include 22 separate experiments with at 5 to 10 mice per group. (B to D) Statistical analysis of high-level versus low-level PMN groups. PMNs were identified by nuclear morphology (pap smear), leukocyte size (wet mount), and Gr-1$^+$ cells (flow cytometry) and enumerated. The % Gr-1$^+$ PMNs within the CD45$^+$ leukocyte population was also assessed by flow cytometry and is indicated in parentheses. (E) Quantification of the vaginal *Candida* burden. CFU/100 µl of lavage fluids from above mice was assessed. *, $P < 0.05$; ***, $P < 0.0001$. LF, lavage fluid. SEM, standard error of the mean.

den was detected at all time points, the PMN levels remained variable among the infected animals, as seen in the original 10-day study (data not shown). Hence, no particular earlier or later time point served as more or less consistent for PMN infiltration. No major signs of distress/discomfort (i.e., weight loss and isolation) were observed during the 30-day period. Likewise, symptoms of an infected state (i.e., itching, soreness, and redness) were not able to be defined qualitatively or quantitatively.

Although several studies have previously shown no differences in susceptibility to experimental vaginitis between different haplotypic strains of mice (4, 12), we conducted a study evaluating vaginal fungal burden and PMN infiltration in C3H/HeN, SJL, BALB/c, C57BL/6, and DBA/2 mice in parallel with CBA/J mice. All strains showed similar vaginal fungal burden and PMN infiltration patterns in the vaginal lavage fluids on days 4 and 7 postinoculation (data not shown). Hence, both fungal burden and PMN infiltration displayed similar results

regardless of mouse strain. Therefore, the remainder of the study was conducted using the original conditions (CBA/J mice evaluated over a 10-day period after inoculation).

***Candida* vaginal adherence during the first 24 h postinoculation.** Recognizing that there is equal variability in vaginal fungal burden days postinoculation despite a differential PMN response, a pilot study was undertaken to evaluate the level of variability in early (<24 h) *Candida* adherence postinoculation that may contribute to differential signaling and ultimately the differential PMN response. For this, we quantified nonadherent and tissue-associated *Candida* in estrogenized mice during the first 24 h after vaginal inoculation. As illustrated in Fig. 2A, *Candida* CFU were successfully detected and enumerated from wash fluid (nonadherent *Candida*) and vaginal tissue homogenates (tissue-associated *Candida*). In addition, firm association between *Candida* and a vaginal epithelium was evidenced by visualization of adherent *Candida* by confocal microscopy after removal of nonadherent *Candida* (Fig. 2B). When further analyzed for a percentage of adherent *Candida* over total fungal burden on each vagina evaluated, the results showed a substantial variation in *Candida* adherence between animals that was maximal at 16 h postinoculation (Fig. 2C).

Chemotactic ability of lavage fluid from mice with high or low PMN levels. Based on the overall concept that PMNs were being signaled to migrate into the vaginal cavity, lavage fluid classified as containing high or low PMN levels from the criteria defined above (Fig. 1) were tested for chemotactic activity. For this, filter-sterilized pooled lavage fluids from estrogenized inoculated mice were evaluated in a PMN chemotactic assay. The results showed that lavage fluid from inoculated mice with high numbers of PMNs on days 4, 7, and 10 postinoculation stimulated substantial PMN chemotaxis compared to that from uninoculated mice ($P = 0.01$, 0.04, and 0.005, respectively), while fluid from inoculated mice with low PMN levels had minimal chemotactic activity. When comparing results for each day postinoculation between high and low PMN groups, fluids containing high PMN levels had significantly greater chemotactic activity than their low PMN counterparts on day 4 and 10 postinoculation ($P = 0.03$ and $P = 0.02$, respectively). A similar trend was observed on day 7 but was not statistically significant (Fig. 3).

Identification of PMN chemotactic factors in lavage fluid. (i) SDS-PAGE. Equal amounts of total protein in lavage fluid from inoculated mice were evaluated by SDS-PAGE for the presence of unique bands in mice with high PMN levels compared to mice with low PMN levels or no PMNs or uninoculated mice. The results showed numerous bands in all lavage fluids. However, in contrast to lavage fluids from uninoculated mice and inoculated mice with low PMN infiltrate, there were two low-molecular-mass proteins at ~6 and ~14 kDa with remarkably increased band intensity in lavage fluid from mice with high PMN levels (Fig. 4).

(ii) Proteomic analysis. Proteomic analysis was used to identify the proteins in the bands of interest from lavage fluid of mice with high and low PMN levels 4 and 7 days postinoculation. The results are reported as the total number of proteins present in each band by protein integrity score (Table 1) in three separate experiments. Proteomic analysis by MS revealed that the 14-kDa band from the high PMN lavage fluid had high homology/match to S100A9 calcium-binding protein (CBP),

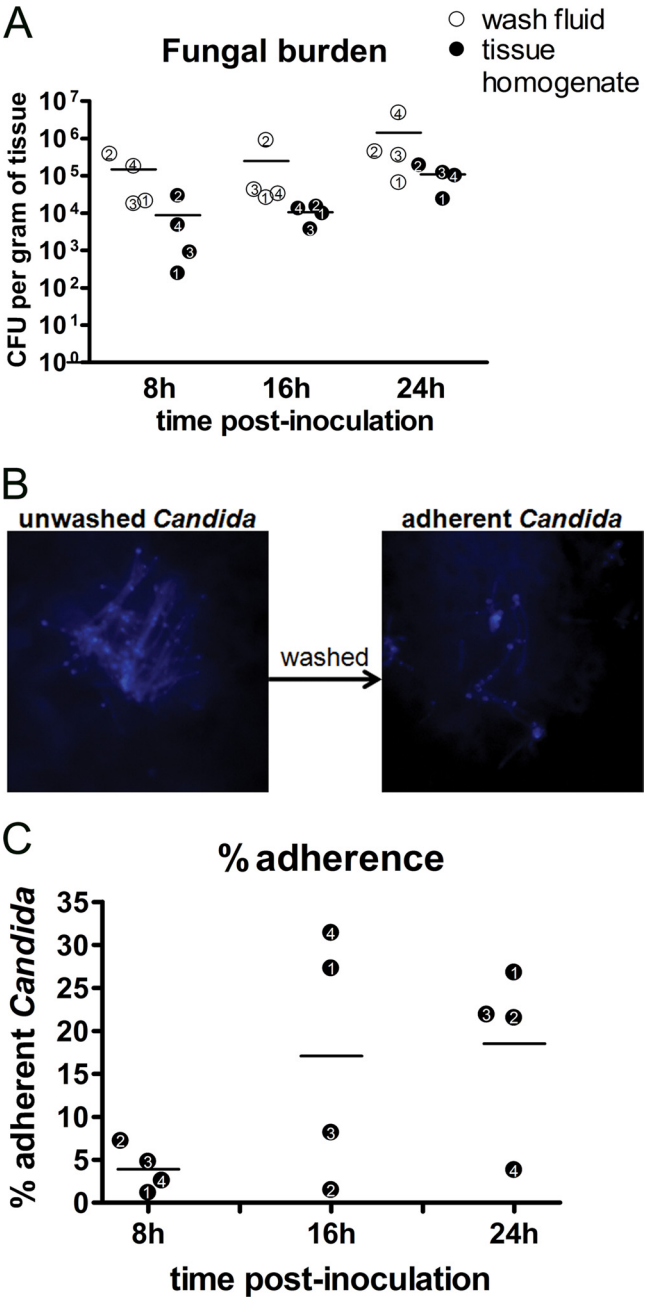

FIG. 2. Variable levels of *Candida* adherence on vaginal epithelia during the first 24 h postinoculation. (A) Quantification of tissue-associated and nonadherent vaginal *Candida* burden over a 24-h period postinoculation. Vaginae from four inoculated mice were removed at each specific time interval, washed with 5 ml of sterile PBS to remove nonadherent *Candida*, and homogenized. Nonadherent and tissue-associated fungal burdens were assessed from wash fluid and tissue homogenates, respectively, and expressed as CFU/g of vaginae. Numbers enclosed in each data point (1 to 4) represent individual mice at each time point postinoculation. (B) Total (unwashed) and tissue-associated (adherent) *Candida* on a vaginal epithelium. Vaginae from above mice pre- and postwashing were incubated with calcofluor white and examined by fluorescent confocal microscopy at ×400 magnification. Blue staining indicates *Candida* present on a vaginal epithelium (C) Vaginal *Candida* adherence between animals over a 24-h period. CFU obtained from above experiments were further analyzed for a percentage of adherent *Candida* for each animal. Numbers enclosed in each data point (1 to 4) represent individual mice corresponding to those in panel A.

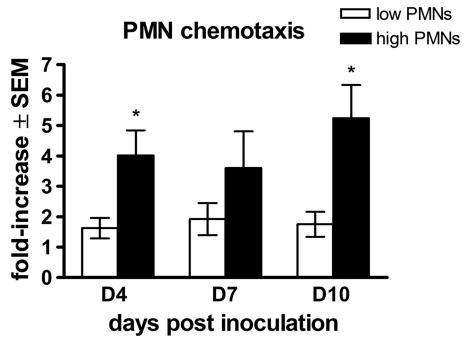

FIG. 3. PMN chemotactic ability of lavage fluid from inoculated mice with high and low PMN levels. Pooled lavage fluid from estrogenized uninoculated or inoculated mice 4, 7, and 10 days postinoculation were evaluated in the PMN chemotaxis assay. The number of PMNs migrating to the bottom chamber were quantified. Chemotactic ability of lavage fluid from high or low PMN groups were normalized to that by lavage fluid from estrogenized uninoculated mice and expressed as the fold increase in migrated PMNs. Figure represents cumulative results from four repeats. *, $P < 0.05$. SEM, standard error of the mean.

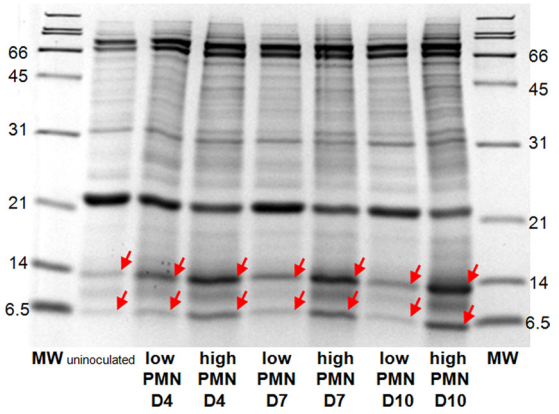

FIG. 4. SDS-PAGE analysis of lavage fluids from inoculated mice. Proteins in pooled lavage fluids from estrogenized uninoculated or inoculated mice 4, 7, and 10 days postinoculation with high or low PMN levels were separated by electrophoresis and visualized with Coomassie blue. Arrows indicate bands exhibiting differential intensities between high- and low-PMN-level groups and uninoculated groups, which were further analyzed for protein identification by MS. MW denotes molecular mass markers. Masses are indicated in kilodaltons on both sides of the image. Image shows a representative result of four repeat experiments.

whereas the 6-kDa band had high homology/match to S100A8 CBP. The integrity scores of the high PMN lavage fluids (mean 132, range 64 to 230, where >75 is a confident match) revealed these proteins to be highest in concentration and purity of all proteins in each band. The same bands analyzed from low PMN lavage fluids suggested the presence of the same proteins, but the integrity scores were lower (mean, 77; range, 45 to 159). Similar results were observed in lavage fluids taken at day 10 postinoculation (data not shown).

Presence of S100A8 and S100A9 in vaginal lavage fluids. Western blotting with antibodies against mouse S100A8 and S100A9 were performed to examine the presence of the proteins in vaginal lavage fluid of inoculated mice with high and low PMN levels. The results in Fig. 5A show that increase levels of both proteins were detected in pooled lavage fluid from mice with high PMN levels (days 4 to 10 postinoculation) compared to pooled fluid from those with low PMN levels. The presence of the proteins was also seen in lavage fluid from uninoculated mice at a low constitutive level. In addition, evaluation by ELISA (Fig. 5B and C) revealed that the concentrations of both S100A8 and S100A9 were significantly increased in the lavage fluid with high PMN levels compared to fluid with low PMN levels ($P = 0.001$ and $P = 0.003$, respectively). The concentrations of S100A8 were ~2.5 μg/100 μl of lavage fluid, whereas concentrations of S100A9 were ~75 μg/100 μl of lavage fluid. Similar to the Western blot results, fluid from uninoculated mice had extremely low concentrations of each protein.

Production of S100A8 and S100A9 by vaginal epithelial cells. To investigate epithelial cells as a source of S100A8 and S100A9 present in lavage fluid, vaginal tissue sections from inoculated mice with high PMN or low PMN levels were tested for the presence of the CBPs by immunohistochemistry. The results in Fig. 6A show that increased levels of both proteins were present on or within vaginal epithelia of inoculated mice with high PMN levels compared to those with low PMN levels or uninoculated mice. These results also revealed that the cells positively stained for the CBPs were localized at the apical layer of the epithelia. Positive staining for a pan-epithelial cell marker (AE1/AE3) confirmed that epithelial tissue and cells were being examined. In addition, examination of cellular fractions collected from vaginal lavages by immunocytochemistry showed that vaginal epithelial cells from inoculated mice with high PMN levels stained more intensely (Fig. 6B) and were significantly elevated (Fig. 6C and D) in the number of S100A8- and S100A9-positive cells compared to cells from inoculated mice with low PMN levels ($P = 0.02$ and $P = 0.003$, respectively, on day 7). In all cases, uninoculated mice showed negligible staining for S100A8 and S100A9.

Confirmation of the S100A8 and S100A9 cellular source during infection. To determine whether vaginal epithelial cells are indeed a source of S100A8 and S100A9 during infection, *S100A8* and *S100A9* mRNA transcripts in vaginal epithelial cells from inoculated mice with high PMN levels or low PMN levels were quantified by real-time PCR. The results showed that expression of both *S100A8* and *S100A9* mRNA transcripts was increased in vaginal epithelial cells from inoculated mice with high PMN levels compared to those with low PMN levels (Fig. 7A and B) and correlated with increased protein expression by vaginal epithelial cell lysates collected from the same animals (Fig. 7C).

Role of S100A8 and S100A9 in PMN chemotaxis. Based on the results displaying the production of S100A8 and S100A9 proteins and secretion into the vaginal lumen by vaginal epithelial cells during infection accompanied by a robust PMN infiltrate, we next conducted an antibody neutralization study in which vaginal lavage fluid was pretreated with either anti-mouse S100A8 or S100A9 antibodies and subsequently tested in the PMN migration assay. The results in Fig. 8 show that the PMN chemotactic activity of the lavage fluid was significantly reduced after neutralization of S100A8 (34.63 ± 9.530% of control isotype IgG), whereas treatment with anti-S100A9 antibody exhibited minimal effects on the activity (79.30 ±

TABLE 1. Protein integrity scores from excised bands at 14 and 6 kDa[a]

Day and band size (kDa)	Low PMN		High PMN	
	Protein	Integrity score	Protein	Integrity score
Day 4, 14 kDa	S100A9	52	**S100A9**	**197**
	Immunoglobulin heavy-chain variable region	41, 33	**Keratin complex 2, gene 6a**	**108, 77**
	HLA-B*2705 α2 domain	40	**Epidermal keratin subunit II**	**99,** 68
	Tigger transposable element derived 4	40	**Keratin complex 2, gene 6b**	**84,** 68
	THO complex 5 isoform 3	39	**Cytokeratin 6B**	**84,** 68
	Human leukocyte antigen B	39	**Keratin 6L**	**83**
	Anti-U5-116kDa hypothetical frame-1 protein	39	Centromere protein E isoform 5	67
	KIAA1729	39	Centromere protein E isoform 1	66
	Novel protein	38	Unnamed protein product	65
	Catenin delta-2	37	Centromere protein E isoform 4	60
Day 4, 6 kDa	Hypothetical protein LOC767961	48	**CP-10**	**110, 111**
	Unnamed protein product	46	**S100A8**	**109, 109**
	Immunoglobulin heavy-chain variable region	41	MRP8	65
	Testicular luteinizing hormone beta subunit	39	Butyrophilin	42
	6-Phosphogluconate dehydrogenase	38	Dopamine receptor protein	41
	Lcmt1 protein	36	Immunoglobulin heavy-chain variable region	41, 40, 30
	Unnamed protein product	36	Insulin-like growth factor 1	40
	Glutamic-pyruvate transaminase	35	Proteasome 26S subunit, ATPase 3	39
	MHC class I	33	Immunoglobulin heavy-chain VHDJ region	38
	Unknown (protein for MGC:13829)	32	Unnamed protein product	30
Day 7, 14 kDa	**S100A9**	**159, 137, 126**	**S100A9**	**230, 152, 110**
	MYEF2 protein	54	Cytokeratin 8	66
	Hematopoietic stem/progenitor cells 176	46	Keratin complex 2, gene 6b	64
	Unnamed protein product	45, 40	Novel protein	64
	Anti-U5-116KDA hypothetical frame-1 protein	44	hCG1821267	59
	CCDC18 protein	44	boIA-like 3	55
	hCG1820607	44	mFLJ00186	54
	Unnamed protein product	42	Hypothetical protein	52
	MHC class I antigen	42	Calprotectin larger component, MRP14	52
	Sarcoma antigen NY-SAR-41	42	MHC class I antigen	44
Day 7, 6 kDa	CP-10	63, 56, 46	**CP-10**	**179, 86**
	S100A8	62, 54, 45	**S100A8**	**178, 84**, 64
	Selenium-binding protein	51	**MRP8**	**95**, 57
	TBC1 domain family, member 15	50	Protein-serine/threonine kinase	62
	MRP8	48	MHC class I antigen	50
	Unnamed protein product	40	Unnamed protein product	50
	Myosin light chain 1	38	GMP reductase 2, isoform 2	48
	Myo5c protein	37	GMP dehydrogenase	48
	Cytochrome P-450PBc2	37	Chain A, crystal structure of human guanosine	48
	IgH antibody heavy chain VDJ region	36	Zcchc14	45

[a] Integrity scores set in boldface indicate a high probability match for a specific protein (≥75). MHC, major histocompatibility complex.

8.893% of control). Comparing the levels of neutralization between each antibody treatment revealed a statistical significance ($P = 0.03$).

DISCUSSION

The vaginal presence of PMNs in the experimental mouse model of *Candida* vaginitis provided a means to further dissect components of the new clinical concept that an acute PMN response was associated with symptomatic *Candida* vaginitis (10). The documented erratic numbers of PMNs in the mouse model (5, 15) first prompted a series of studies to formally classify the PMN response in CBA/J mice. Accordingly, vaginal PMN levels in lavage samples from inoculated mice were categorized as high, intermediate, and low based on PMN counts by microscopic quantification of pap smear preparations from a substantial number of experiments. Although arbitrary, criteria used for the classification was equivalent to the clinical indications of inflammation during vaginitis caused by various pathogens (1, 6, 27). It is important to note as part of the present study that longitudinal studies up to 30 days in CBA/J and several other haplotypic strains of mice showed a similar differential PMN response to that using CBA/J mice over a 10-day period. In addition, previous studies that manipulated estrogen concentrations, inocula, and strains of *Candida* in the model also showed the same differential PMN infiltration patterns (13, 14, 18). Taking this information into account, the PMN classification was further validated by alternate methods, including quantification of viable leukocyte numbers by wet-mount and detection by flow cytometry using the neutrophil marker Gr-1. In addition, results from the flow cytometric analyses

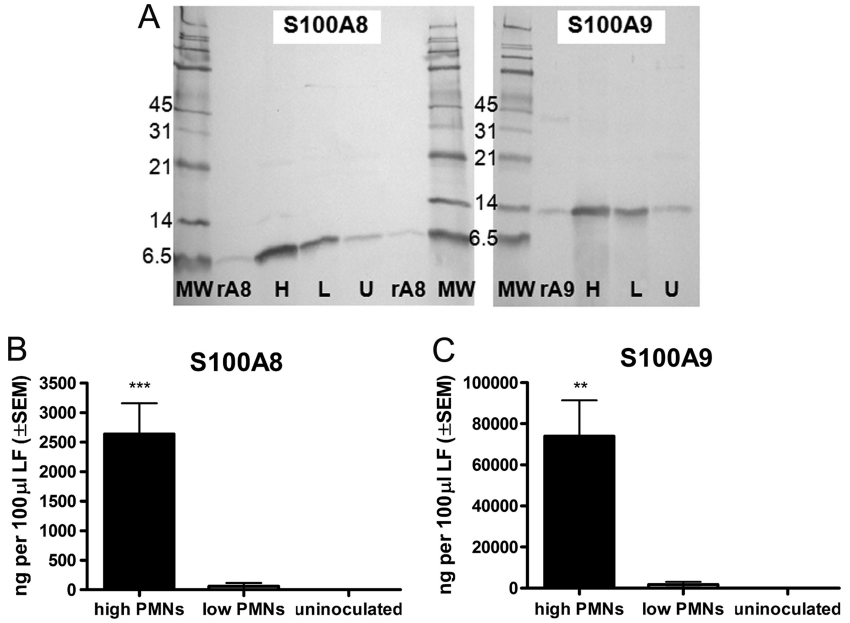

FIG. 5. S100A8 and S100A9 are present *in vivo* postinoculation. (A) Western blot. Proteins in lavage fluids from estrogenized inoculated mice with high PMN levels (H) or low PMN levels (L) or estrogenized uninoculated mice (U) were separated and visualized by Western blotting with anti-S100A8 (left panel) or anti-S100A9 (right panel) antibodies. Recombinant mouse S100A8 (rA8) and S100A9 (rA9) were included as positive controls. MW denotes molecular mass markers. Masses are indicated in kilodaltons on the right side of the MW lanes. The figure shows a representative image of three repeat experiments testing lavage samples collected on day 4 postinoculation. (B and C) ELISA. Diluted lavage fluid from estrogenized inoculated mice with high PMN levels or low PMN levels or uninoculated mice were evaluated for S100A8 and S100A9 concentrations by ELISA. The results are cumulative data of three repeat experiments testing lavage samples collected on day 4 postinoculation. **, $P < 0.005$. ***, $P < 0.001$. LF, lavage fluid. SEM, standard error of the mean.

revealed that the vaginal leukocytes were predominantly PMNs, which is consistent with previous findings (33). With the classification confirmed by several means, we used the high-level and low-level PMN groups to define symptomatic and asymptomatic conditions of *Candida* vaginitis, respectively, and these two groups were used exclusively for the remainder of the studies. Unfortunately, attempts to further classify the groups by the presence of clinical signs and symptoms of *Candida* vaginitis (redness, swelling, irritation, itching, and scratching) failed to produce quantitative or qualitative data to correlate with the PMN infiltration. This is not uncommon in animal studies. Hence, the PMN classification was used as the sole criteria to define a symptomatic condition.

An important question that arises relative to the PMN classification is how an inbred strain of mice would exhibit differential PMN infiltration patterns in response to the same inocula. A reasonable hypothesis from clinical studies is that the sensitivity to *Candida* by vaginal epithelial cells and subsequent PMN response is ultimately dependent on the number of *Candida* interacting with vaginal epithelial cells (10). Indeed, a pilot study in the animal model revealed that the number of *Candida* that actually gain firm adherence to vaginal epithelia differed substantially during the initial 24-h postinoculation. Hence, the differential patterns of vaginal PMN infiltration may be explained by the differential levels of early *Candida* adherence and subsequent signaling within the vaginal epithelial cells. An alternative possibility is that *Candida* directly stimulates PMN migration. However, clinical studies show no direct correlate between symptoms/PMN infiltration and vaginal fungal burden (2, 9).

Based on our previous reports that lavage fluids from women with symptomatic infection accompanied by a robust PMN infiltrate stimulated PMN chemotaxis (10), and the concept of *Candida* triggering epithelial cells to promote PMN recruitment, we were encouraged that vaginal lavage fluid from inoculated mice with high, but not low, PMN levels also stimulated substantial PMN migration. These results prompted a series of studies to identify the source and nature of the chemotactic activity.

Proteomic analysis of the more intense 6- and 14-kDa bands visualized by SDS-PAGE in lavage fluid from mice with high PMN levels revealed a significant match to CBP S100A8 and S100A9, respectively. Both proteins are cytosolic and secretory proteins expressed by PMNs, monocytes, and epithelial cells upon activation (21, 23, 25, 30, 32). Other names for these proteins include CP-10, MRP8, and MRP14 (7, 24, 28, 40). Murine models of infection have shown secreted S100A8 and S100A9 to be potent chemoattractants of PMNs (7, 8, 24). The S100A8/A9 heterodimer, calprotectin, has been shown to have antimicrobial properties (35, 37, 40), while also serving as a potent chemoattractant of PMNs (32, 38). In fact, both proteins have been suggested as biomarkers for monitoring inflammatory disease activity (20). Although numerous proteins were identified to be potentially present at the molecular sizes examined (Table 1), these proteins were not considered as candidate chemotactic factors due to their poor integrity scores (i.e., low probabilities of protein match).

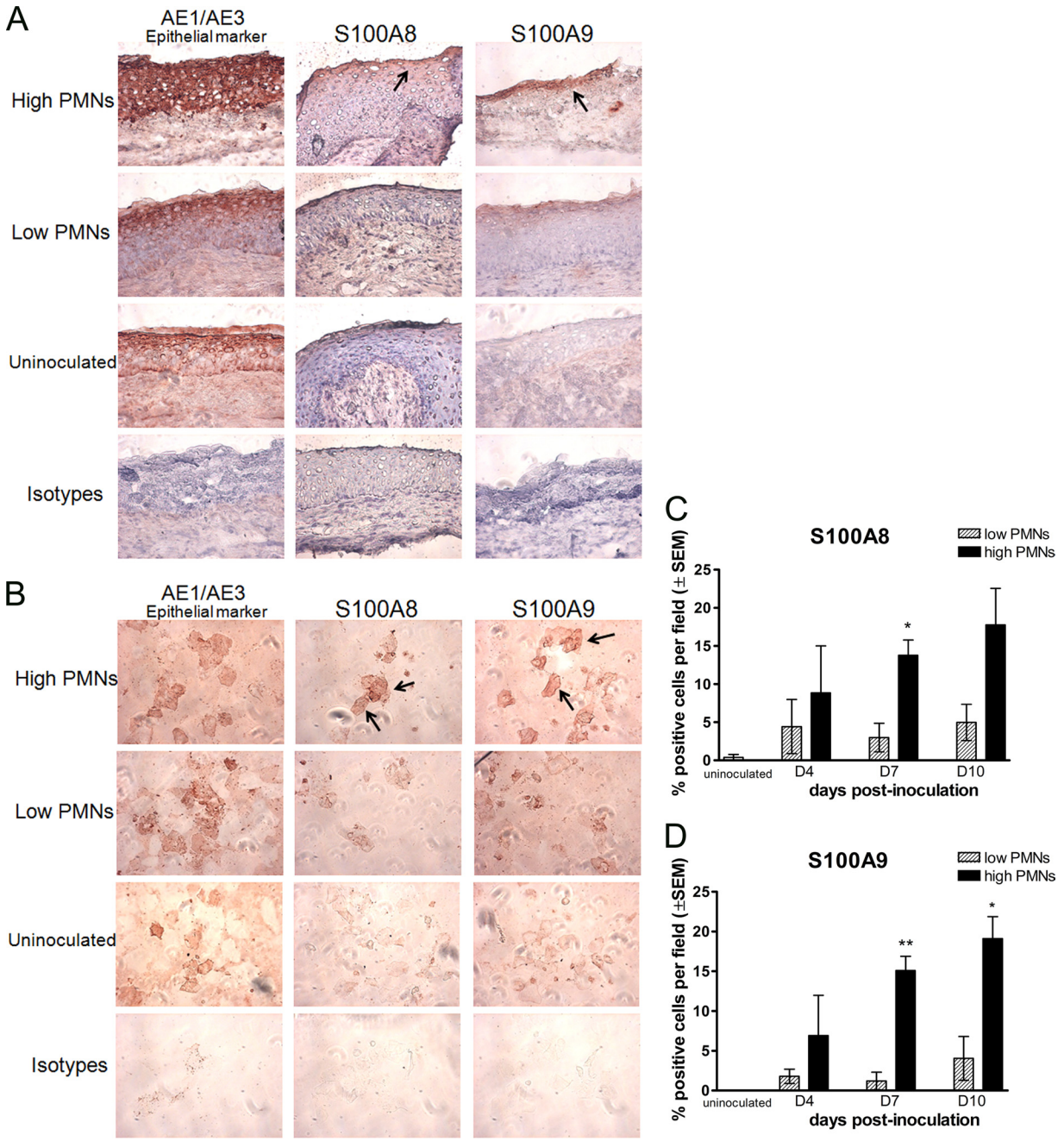

FIG. 6. Presence of S100A8 and S100A9 on vaginal epithelial cells after interaction with *Candida.* (A and B) Vaginal tissue sections (A) and cytospin preparations of cellular fractions in pooled lavage fluid from estrogenized uninoculated or inoculated mice with high PMN levels or low PMN levels (B) were stained with anti-S100A8, anti-S100A9, anti-AE1/AE3 (pan-epithelial cell marker), or isotype control antibodies. Images are shown at ×400 magnification. Arrows represent epithelial cells positively stained for S100A8 or S100A9. Images show a representative result of three repeat experiments testing specimens collected on day 7 postinoculation. (C and D) The numbers of positively stained epithelial cells for S100A8 and S100A9 were counted in five nonadjacent fields per slide at ×100 magnification and averaged. The results are cumulative data of three experiments. *, $P < 0.05$; **, $P < 0.01$. SEM, standard error of the mean.

Western blots of lavage fluids confirmed the qualitative presence of S100A8 and S100A9 consummate with the PMN classification. Further quantitative evaluation by ELISA confirmed the Western blot results and revealed concentrations of S100A8 and S100A9 to be in low microgram amounts. Interestingly, PMN migration was detected as early as 48 h postinoculation and was supported by the presence of both S100A8 and S100A9 in the lavage fluid at that time (data not shown).

Taking into account the fact that the chemotactic S100 proteins are produced by various innate immune cells, it was important to confirm vaginal epithelial cells as a cellular source

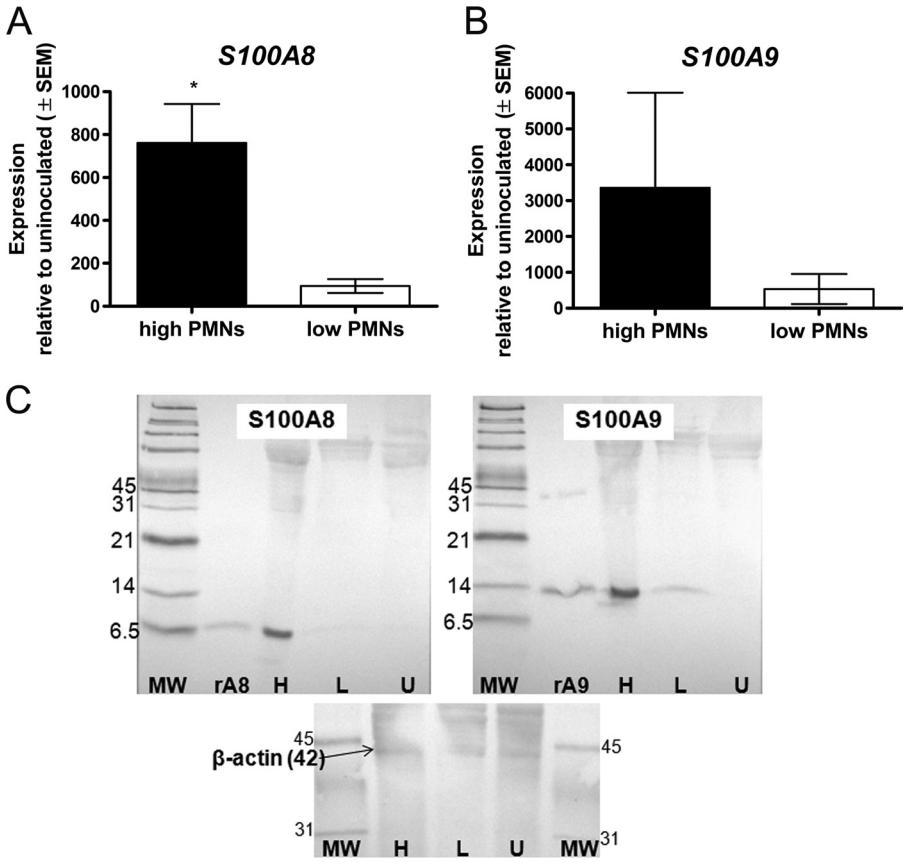

FIG. 7. Vaginal epithelial cells are a source of S100A8 and S100A9 during infection. (A and B) *S100A8* and *S100A9* mRNA expression by vaginal epithelial cells. Total RNA was extracted from vaginal epithelial cells from estrogenized uninoculated or inoculated mice with high PMN levels or low PMN levels and reverse transcribed into cDNA. *S100A8* and *S100A9* mRNA expression was quantified and normalized to β-*actin* mRNA expression. The results are expressed as the fold increase over expression in cells from estrogenized uninoculated mice. The results are cumulative data of 3 experiments testing vaginal epithelial cells collected on day 4 postinoculation. *, $P = 0.0024$. SEM, standard error of the mean. (C) S100A8 and S100A9 protein production by vaginal epithelial cells. Proteins in vaginal epithelial cell lysates from estrogenized inoculated mice with high PMN levels (H) or low PMN levels (L) or estrogenized uninoculated mice (U) were separated and visualized by Western blotting with anti-S100A8 (left panel), anti-S100A9 (right panel) or β-actin (bottom panel) antibodies. Recombinant mouse S100A8 (rA8) and S100A9 (rA9) were included as positive controls. MW denotes molecular mass markers. Masses are indicated in kilodaltons on the right side of the MW lanes. Images show a representative result of three repeat experiments testing vaginal epithelial cells collected on day 4 postinoculation.

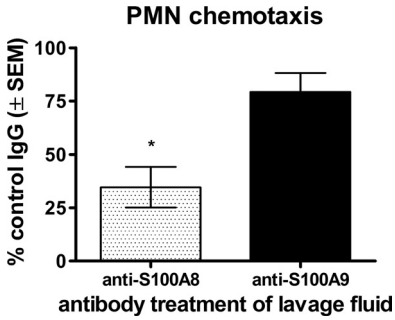

FIG. 8. Role of mouse S100A8 and S100A9 in PMN chemotaxis. Pooled lavage fluid with positive chemotactic activity was incubated with anti-S100A8 or anti-S100A9 antibodies and tested in the PMN chemotaxis assay. The numbers of PMNs migrating to the bottom chamber were quantified by microscopic counts. The results are expressed as the percentage of control compared to the PMN migration by the same fluid incubated with the isotype control IgG antibody. Figure shows cumulative data from three repeats. *, $P < 0.05$. SEM, standard error of the mean.

of the proteins during infection. Direct evidence for the production of the S100 proteins by epithelial cells was shown by immunostaining of vaginal tissue sections and epithelial cells collected from lavage fluid of inoculated mice. Furthermore, the localized positive staining at the apical surface of vaginal epithelia where *Candida* first adheres supports the hypothesis that vaginal epithelial cells are triggered to produce the chemotactic proteins upon interaction with *Candida*. Finally, expression of *S100A8* and *S100A9* mRNA transcripts by vaginal epithelial cells was detected and positively correlated with protein expression in epithelial cells from mice with high and low PMN levels. These results confirm that epithelial cells are a source of S100A8 and S100A9. We recognize that PMNs likely also contribute to the S100 proteins in the lavage fluids. However, based on the clear verification that vaginal epithelial cells produce S100A8 and S100A9, we propose that the initial production is by vaginal epithelial cells that mediate the PMN migration. Once in the vagina, the PMN contribution comes as part of a positive-feedback mechanism (31) to recruit additional PMNs to the vagina to amplify the response.

As further confirmation of the functional role for the S100 proteins in PMN migration, antibody neutralization of S100A8, but not S100A9, reduced PMN chemotactic activity of vaginal lavage fluid. Thus, our results suggest that S100A8 plays a key role in the robust PMN migration under susceptible conditions of vaginitis in the mouse model. We recognize, however, that other mediators may also contribute to the PMN migration since antibody neutralization of S100A8 in lavage fluid did not fully eliminate all PMN migration. We also recognize that S100A9 may contribute to some level of PMN chemotaxis since it reduced the PMN migration by ~20%, although a combination of the two antibodies together did not show any further reduction in PMN migration than anti-S100A8 antibodies alone (data not shown).

Clinical studies have suggested that S100A9, but not S100A8, serves as a PMN chemoattractant (28). We expect that the S100 proteins will be involved in the symptomatic PMN response in clinical vaginitis; a comprehensive evaluation of common inflammatory cytokines and chemokines (granulocyte colony-stimulating factor, tumor necrosis factor alpha, interleukin-1 [IL-1], IL-6, IL-8, and IL-17) in lavage fluids from women with or without symptomatic infection failed to identify candidates for the PMN chemotactic factor. In addition, preliminary proteomic analysis of lavage fluid from women with symptomatic infection and high numbers of vaginal PMNs have revealed the presence of the S100 proteins (P. L. Fidel, unpublished data). It will be interesting to determine whether S100A8 or S100A9 alone or the heterodimer is chemotactic during symptomatic *Candida* vaginitis in women.

Despite their well-characterized antimicrobial properties (35–37, 40), it is interesting that neither PMNs nor the CBPs exhibit protective roles against *Candida* in the vagina. In fact, only a small amount of S100A8 and S100A9 was found to be in the heterodimeric antimicrobial form (calprotectin). It is plausible, too, that PMNs do not function well in the vaginal microenvironment as opposed to the bloodstream or other mucosal sites.

In the present study, we further dissected the vaginal PMN response in the mouse model of *Candida* vaginitis and identified CBP S100A8 as a strong candidate responsible for the robust PMN response. We hypothesize that vaginal epithelial cells of susceptible hosts have a high sensitivity to *Candida* and respond to smaller numbers of *Candida* by the secretion of S100 CBPs as a danger signal, resulting in a robust PMN migration and associated inflammation responsible for the symptoms of infection. On the other hand, epithelial cells of resistant hosts have a low sensitivity to *Candida* and therefore are not triggered to secrete the chemotactic CBPs, allowing the condition of asymptomatic colonization. Accordingly, while the animal model might not fully mimic the epithelial cell sensitivity hypothesis, the model is adequate, based on the differential PMN migration, to dissect the mechanisms of the epithelial cell response leading to the PMN infiltration. Studies to identify pattern recognition receptors involved in *Candida*-epithelial cell interaction and to elucidate biological roles of S100A8 and S100A9 in vaginitis *in vivo* will continue to shed light on this hypothesis.

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