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Effect of Lung Surfactant Collectins on Bronchoalveolar Macrophage Interaction with *Blastomyces dermatitidis*: Inhibition of Tumor Necrosis Factor Alpha Production by Surfactant Protein D

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Alveolar surfactant modulates the antimicrobial function of bronchoalveolar macrophages (BAM). Little is known about the effect of surfactant-associated proteins in bronchoalveolar lavage fluid (BALF) on the interaction of BAM and *Blastomyces dermatitidis*. We investigated BALF enhancement or inhibition of TNF-α production by BAM stimulated by *B. dermatitidis*. BAM from CD-1 mice were stimulated with *B. dermatitidis* without or with normal BALF, surfactant protein A-deficient (SP-A$^{-/-}$) or surfactant protein D-deficient (SP-D$^{-/-}$) BALF, or a mixture of SP-A$^{-/-}$ and SP-D$^{-/-}$ BALF. An enzyme-linked immunosorbent assay was used to measure tumor necrosis factor alpha (TNF-α) in culture supernatants. BALFs were standardized in protein concentration. BAM plus *B. dermatitidis* (BAM-*B. dermatitidis*) TNF-α production was inhibited ≥47% by BALF or SP-A$^{-/-}$ BALF (at 290 or 580 μg of protein/ml, $P < 0.05$ to 0.01); in contrast, SP-D$^{-/-}$ BALF did not significantly inhibit TNF-α production. If SP-A$^{-/-}$ BALF was mixed in equal amounts with SP-D$^{-/-}$ BALF, TNF-α production by BAM-*B. dermatitidis* was inhibited ($P < 0.01$). Finally, pure SP-D added to SP-D$^{-/-}$ BALF inhibited TNF-α production by BAM-*B. dermatitidis* ($P < 0.01$). *B. dermatitidis* incubated with BALF and washed, plus BAM, stimulated 63% less production of TNF-α than did unwashed *B. dermatitidis* ($P < 0.05$). SP-D was detected by anti-SP-D antibody on BALF-treated unwashed *B. dermatitidis* in an immunofluorescence assay (IFA). The BALF depleted by a coating of *B. dermatitidis* lost the ability to inhibit TNF-α production ($P < 0.05$). 1,3-β-Glucan was a good stimulator of BAM for TNF-α production and was detected on *B. dermatitidis* by IFA. β-Glucan incubated with BALF inhibited the binding of SP-D in BALF to *B. dermatitidis* as demonstrated by IFA. Our data suggest that SP-D in BALF binds β-glucan on *B. dermatitidis*, blocking BAM access to β-glucan, thereby inhibiting TNF-α production. Thus, whereas BALF constituents commonly mediate antimicrobial activity, *B. dermatitidis* may utilize BALF constituents, such as SP-D, to blunt the host defensive reaction; this effect could reduce inflammation and tissue destruction but could also promote disease.

Blastomycosis is a pulmonary mycotic disease contracted by inhalation of airborne conidia or mycelial fragments of the dimorphic fungus *Blastomyces dermatitidis*, which promptly converts to its parasitic form, yeasts (2, 23, 39). The disease can become chronic and can disseminate, with granuloma formation and suppuration. Complete understanding of the epidemiology of blastomycosis is hindered by a lack of reporting (23, 25). The lung is an important interface between the host and an environment that contains a plethora of potentially harmful microorganisms. The innate immune defense of the lung includes the bronchoalveolar macrophages (BAM) and pulmonary surfactant.

Under ordinary conditions BAM are a part of the defense against inhaled microorganisms. The bactericidal activity of BAM from humans, rats, or mice is well documented. In contrast, BAM fungicidal activity is limited for such pulmonary pathogens as *Histoplasma capsulatum* (24), *B. dermatitidis* (42), *Coccidioides immitis* (5), and *Aspergillus fumigatus* mycelia (37). A weak fungicidal activity of BAM for *B. dermatitidis* might relate to only a modest respiratory burst triggered by *B. dermatitidis* (8, 10).

Pulmonary surfactant is a complex mixture of lipids, phospholipids, and proteins (36). Two of the surfactant-associated proteins, SP-A and SP-D, are involved in innate immunity and lung homeostasis and belong to a family known as collectins. One important biological role of collectins (20) is to bind to targets such as bacteria (26, 27), viruses (17–19, 32), and fungi (13, 29–31, 35). The collectins bind by recognizing patterns of carbohydrate distribution and, by binding and/or enhancement of phagocytosis, increase the clearance of the target by BAM. Little is known regarding the interaction of *B. dermatitidis* with surfactant protein (SP-A and SP-D) in the pulmonary compartment. The goal of the present study was to investigate the role of SP-A and SP-D in the interaction between BAM and *B. dermatitidis*, especially with regard to tumor necrosis factor alpha (TNF-α) production, since TNF-α is an important orchestrator of

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lung innate and adaptive immune responses, and this role is especially prominent with fungi (11, 15, 34).

MATERIALS AND METHODS

Animals. Male CD-1 mice, 8 to 12 weeks old, were obtained from the breeding colony of Charles River Laboratory, Hollister, CA. Male C3H/HeN SP-A negative (SP-$A^{-/-}$) mice and male NIH Swiss Black SP-D-negative (SP-$D^{-/-}$) mice, 8 to 10 weeks old, were bred and raised in the University of Cincinnati Laboratory Animal Facility. These mice were housed in microisolator cages in rooms with HEPA-filtered laminar airflow and received autoclaved food and bedding. All animal studies conformed to National Institutes of Health, University of Cincinnati, and Veterans Affairs Medical Centers guidelines and were approved by the Institutional Animal Care and Use Committee of the California Institute for Medical Research.

Reagents and media. The tissue culture medium RPMI 1640 with L-glutamine, 10% (vol/vol) heat-inactivated fetal bovine serum, and penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively) was obtained from Gibco Laboratories, Grand Island, NY, and the mixture is referred to as complete tissue culture medium (CTCM). Rabbit anti-mouse SP-D was obtained from Chemicon International Laboratories (Temecula, CA), and fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) and goat anti-mouse IgG (both goat antisera heavy- and light-chain only) from Zymed Lab (San Francisco, CA). β-1,3-Glucan was prepared from *Saccharomyces cerevisiae* by autoclaving cell walls in 19 mM citrate buffer to separate mannans (supernatant) and then extracting the insoluble β-1,3-glucan by hot 3% NaOH extraction under nitrogen (4), and the purity was verified by infrared spectroscopy (Sigma Chemical Co., St. Louis, MO). Monoclonal anti-1,3-β-glucan mouse IgG (kappa light-chain isotype) was obtained from Biosupplies Australia Pty., Ltd. (Parkville, Victoria, Australia).

Fungi. *B. dermatitidis* ATCC 26199, an isolate that is virulent in mice (40), was used in all experiments. *B. dermatitidis* yeasts are so large (>10 μm in diameter) that a single macrophage can only sometimes ingest one and with great difficulty; moreover, the organisms often clump in units of two to three cells, making ingestion even more problematic. Microscopic observations show that *B. dermatitidis* units are commonly only surrounded by macrophages rather than being ingested (9). Viable *B. dermatitidis* was prepared from cultures grown for 72 h on blood agar plates at 37°C, and growth was suspended in 3 ml of saline. The suspension was pelleted by centrifugation at 400 × *g* for 10 min, and the pellet suspended in 1 ml of saline and counted with a hemacytometer. A suspension of 5×10^6 viable *B. dermatitidis*/ml of saline was made.

Heat-killed (HK) *B. dermatitidis* was prepared from cultures grown for 72 h on blood agar plates at 37°C and suspended in 5 ml of saline. The suspension was heated at 65°C for 30 min with mixing every 10 min and pelleted by centrifugation at 400 × *g* for 10 min, the pellet was suspended in 5 ml of saline, and the HK *B. dermatitidis* was counted by using a hemacytometer. The suspension had 53×10^6 HK *B. dermatitidis*/ml. It was diluted to 10 ml to yield 5×10^6 HK *B. dermatitidis*/ml, and portions were stored at −80°C. Viability testing assured the absence of viable *B. dermatitidis*.

BAM. The lungs of CD-1 mice were lavaged with Dulbecco phosphate-buffered saline without calcium or magnesium but containing 10% fetal bovine serum and 0.1% EDTA as previously described (41). Cells obtained by repeated 1-ml lavages (total of 10 ml/mouse) were pelleted by centrifugation (400 × *g*, 10 min). Pelleted cells from 10 mice were pooled, washed once in CTCM, and counted with a hemacytometer. A yield of $(8.5 \pm 2.1) \times 10^5$ cells/mouse, highly enriched for macrophages (97.5% ± 3.2%), was obtained by this method. BAM monolayers were formed by incubating 0.1 ml of lavaged cells (10^6/ml of CTCM) per microtest plate well (A/2, Costar 3696; Corning, Inc., Corning, NY) for 2 h at 37°C in 5% CO_2–95% air humidified atmosphere. After incubation, nonadherent cells were aspirated. A total of 90% of the incubated cells adhered, and the monolayer contained approximately 9×10^4 macrophages per well.

Electrophoresis and immunoblotting. Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tris-glycine and 1-mm precast gels in an X-Cell Sure-Lock chamber (Novex; Invitrogen, Carlsbad, CA) with reagents and according to instructions supplied by the vendor. For the reduction of samples, NuPAGE sample reducing agent (Invitrogen) was added so that it was 10% in the sample.

SDS-PAGE gel proteins were blotted to polyvinylidene difluoride membranes by using an X-Cell II blot module (Novex) using reagents and according to instructions provided by the vendor. Blotted polyvinylidene difluoride membranes were air dried and stored at 4°C until needed.

Western immunoblotting of membranes was done with reagents and according to instructions supplied with the SuperSignal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL). Blocked membranes were probed for 1 h with the primary antibody, rabbit anti-mouse SP-D (1:100), in buffer. After a washing step, the membranes were treated with goat anti-rabbit IgG-horseradish peroxidase conjugate (1:1,000) in buffer for 1 h. After the membranes were washed, the substrate (H_2O_2 plus luminol [Invitrogen, Carlsbad, CA]) was added for 5 min. After the substrate was drained, the membrane was exposed to X-ray film (CL-X Posure, 5 by 7 in.; Pierce), the film was developed, and the images were digitized (Bio-Image, Ann Arbor, MI).

BALF. The lungs of CD-1, SP-$A^{-/-}$, and SP-$D^{-/-}$ mice were lavaged with 1 to 3 ml of PBS with penicillin-streptomycin per mouse, and the lavage fluid was centrifuged. Subsequently, CD-1 (wild-type) BALF will be referred to as normal bronchoalveolar lavage fluid (BALF) or, when not specified, it is this BALF that is meant. Pools of BALF from four to five SP-$A^{-/-}$ or SP-$D^{-/-}$ mice or from ten CD-1 mice were prepared. Cell-free BALF was lyophilized, suspended in 1 ml of distilled water, and dialyzed (Spectra/Por-1 membrane; Spectrum Laboratories, Inc., Rancho Dominguez, CA; exclusion, 6,000 to 8,000 molecular weight) against 45 ml of saline. The protein concentrations of BALF were measured (BCA protein assay reagent; Pierce).

Pure SP-D (purified recombinant rat SP-D dodecamers [116.3 μg/ml]), with Tris-buffered saline and 2 mM EDTA, was studied (17). This protein was purified from conditional medium of stably transfected CHO-K1 cells by gel filtration chromatography on maltosyl agarose, followed by agarose gel filtration chromatography. The reduced product appeared as a single protein band on SDS-PAGE. Endotoxin levels were determined by chromogenic assay and was 1.9 pg/μg of protein.

Treatment of macrophages and challenges. BAM were incubated (37°C, 5% CO_2 + 95% air) with BALF, prepared as described above and diluted in CTCM, from SP-$A^{-/-}$, SP-$D^{-/-}$, or SP-$A^{-/-}$ BALF with SP-$D^{-/-}$ BALF in a 1:1 ratio and challenged with 0.01 ml of viable *B. dermatitidis* or HK *B. dermatitidis* (the effector/target ratio thus being 2:1). Culture supernatants were collected after 24 h, and the TNF-α levels in supernatants were measured by enzyme-linked immunosorbent assay (ELISA; Pierce/Endogen, Woburn, MA). The assay produces a linear dose response for the TNF-α concentration and absorbance at 450 nm, and a standard curve verifies this in each run.

Absorption of BALF and coating of *B. dermatitidis*. Absorption of BALF and the coating of *B. dermatitidis* was done in 1-ml microcentrifuge tubes. Heat-killed *B. dermatitidis* (6×10^8 in 0.5 ml of saline) was pelleted by centrifugation (10,000 rpm, 1 min), the saline was removed, and *B. dermatitidis* was suspended in 0.5 ml of BALF (1,290 μg of protein/ml). *B. dermatitidis* plus BALF (*B. dermatitidis*-BALF) was incubated, with mixing every 10 min, at 4°C for 1 h. After centrifugation, the supernatant (once-absorbed BALF) was removed, and pelleted *B. dermatitidis* was suspended in 0.5 ml of saline and counted. Coated *B. dermatitidis* was suspended to 5×10^6/ml of saline, and 0.01-ml portions were used to stimulate BAM.

Once-absorbed BALF (0.5 ml) was incubated with 6×10^8 *B. dermatitidis* for 1 h as described above and centrifuged. The supernatant BALF (twice-absorbed BALF) was collected and tested at 290 μg of protein/ml.

Staining procedure. CD-1 BALF (290 μg/ml BALF) in CTCM, SP-$D^{-/-}$ BALF (290 μg/ml BALF) in CTCM, and pure SP-D (20 μg/ml) with SP-$D^{-/-}$ BALF (290 μg/ml BALF) in CTCM (0.1-ml volumes) were incubated with 0.01 ml of viable *B. dermatitidis* (5×10^6/ml) for 1 h at room temperature and centrifuged at 6,000 rpm/min for 1 min. The pellet was washed with 0.1 ml of CTCM and incubated for 30 min with rabbit anti-mouse SP-D diluted 1:10 in CTCM. The suspension was centrifuged, and the pellet was suspended for 30 min in fluoresceinated goat anti-rabbit IgG conjugate diluted 1:10 in CTCM. The suspension was recentrifuged, and the pellet was washed and resuspended in 0.01 ml of CTCM. The pellet was studied with a fluorescence microscope.

For photographic recording, the same procedure with BALF was used except that HK *B. dermatitidis* (because of biohazard considerations in the photography laboratory) and goat anti-rabbit antibody conjugated to phycoerythrin were substituted for the corresponding cells and antibody described above.

A total of 0.01 ml of viable *B. dermatitidis* (5×10^6/ml) was incubated with 0.1 ml of anti-β-glucan mouse IgG diluted 1:10 in CTCM for 1 h at room temperature, centrifuged, washed as described above, and suspended in goat anti-mouse IgG in CTCM (1:10) for 30 min. The pellet was washed and suspended as described above and examined with a fluorescence microscope.

Statistics. Data are presented as means of triplicate determinations ± the standard error of the mean in text and as bars in the figures. Comparisons between groups were analyzed by using the Student *t* test, with significance assumed to be a *P* value of <0.05.

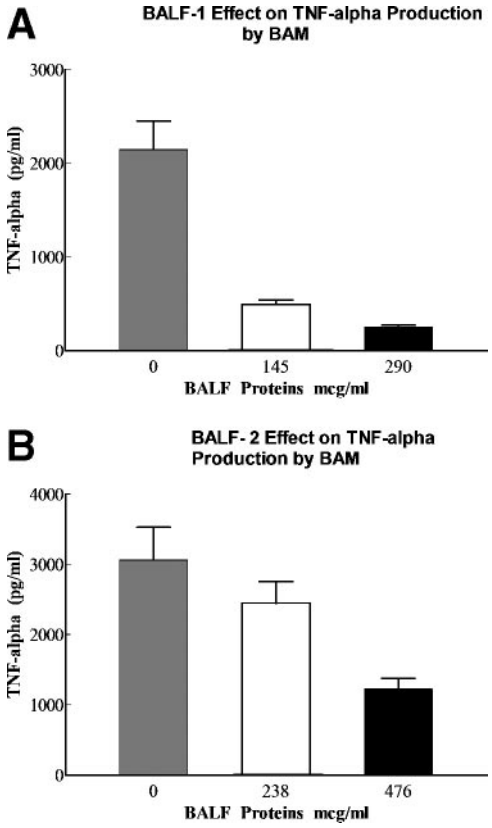

FIG. 1. Effect of BALF on BAM plus HK *B. dermatitidis* production of TNF-α. BAM-*B. dermatitidis* (2:1) production of TNF-α, as measured by ELISA (absorbance at 450 nm), was inhibited in a concentration-dependent manner. (A) Inhibition by BALF (BALF-1) at 145 and 290 μg/ml (both $P < 0.01$). (B) Inhibition by a second preparation of BALF (BALF-2) at 238 and 476 μg/ml ($P < 0.01$).

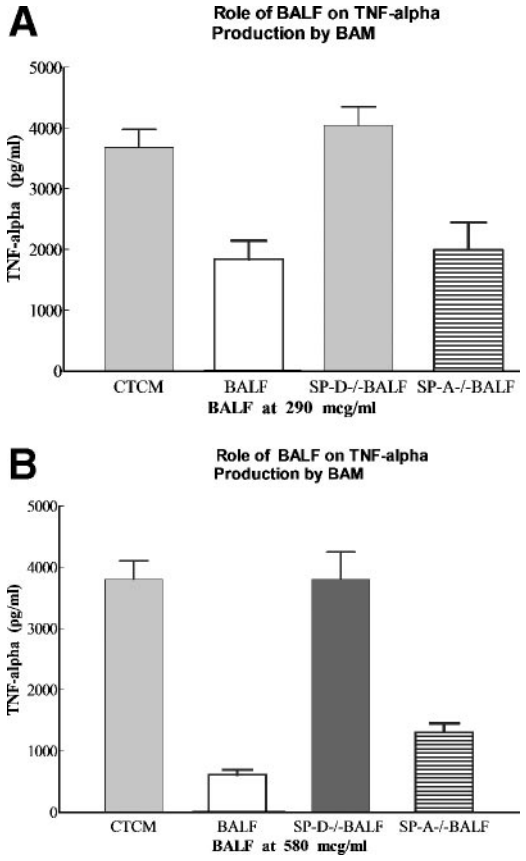

FIG. 2. Effect of normal, SP-$D^{-/-}$, and SP-$A^{-/-}$ BALF on production of TNF-α by BAM plus viable *B. dermatitidis*. BAM-*B. dermatitidis* (2:1) production of TNF-α measured by ELISA (absorbance at 450 nm) is shown. (A and B) Inhibition by different BALFs at 290 μg/ml (A) and inhibition by BALFs at 580 μg of protein/ml (B). BALF and SP-$A^{-/-}$ BALF inhibition (both $P < 0.05$ in panel A and $P < 0.01$ in panel B).

RESULTS

Inhibition of TNF-α production by BAM-*B. dermatitidis* by BALF. TNF-α production by BAM on exposure to HK *B. dermatitidis* was inhibited by 61% in the presence of normal (CD-1) BALF at 145 μg/ml or 78% by BALF at 290 μg/ml (both $P < 0.01$) (Fig. 1A). In a second experiment, with another BALF preparation, TNF-α production by BAM in response to HK *B. dermatitidis* was less but was inhibited by 21% in presence of BALF at 238 μg of protein/ml or 63% ($P < 0.01$) by BALF at 476 μg of protein/ml (Fig. 1B). These results suggest that BALF inhibits TNF-α production by BAM in a concentration-dependent manner.

In a separate experiment, heat-killed *B. dermatitidis* was coated with BALF as described in Materials and Methods. Whereas BALF at 290 μg/ml inhibited TNF-α production by 90%, the coated *B. dermatitidis* inhibited the stimulation of BAM for TNF-α production by 63% ($P < 0.05$) compared to stimulation of BAM with uncoated HK *B. dermatitidis* (data not shown). This result indicated that factor(s) in BALF bind to *B. dermatitidis* and interfere with the stimulation of BAM for TNF-α production. Studies of the nature of the possible coating material are described subsequently.

BALF twice absorbed with HK *B. dermatitidis*, prepared as described in Materials and Methods, was compared at 290 μg of protein/ml to unabsorbed BALF. The absorbed BALF had decreased ability to inhibit TNF-α production by BAM-HK *B. dermatitidis* compared to unabsorbed BALF: 23% versus 79% ($P < 0.05$). A similar result, 30% inhibition, was obtained in a separate experiment with a 156-μg/ml preparation of twice-absorbed BALF. This suggests that the material in BALF that inhibits the BAM production of TNF-α, in response to HK *B. dermatitidis*, binds to *B. dermatitidis* (during the absorption procedure) and is thus depleted from the BALF.

SP-D-deficient lavage fails to inhibit TNF-α from BAM after exposure to viable *B. dermatitidis*. TNF-α production by BAM to viable *B. dermatitidis* was inhibited by 51% in the presence of BALF (290 μg/ml) or 47% by BALF SP-$A^{-/-}$ (290 μg/ml) (both $P < 0.05$). Viable *B. dermatitidis* produced more TNF-α from the BAM controls than the experiments described above, thus the absolute amount with normal BALF is greater, and the percent inhibition is similar. There was no significant inhibition or enhancement by SP-$D^{-/-}$ BALF (290 μg/ml) compared to BAM-*B. dermatitidis* (Fig. 2A). A second experiment gave similar results with the three BALFs.

When the protein concentration of BALF was increased to 580 μg/ml, TNF-α production by BAM to *B. dermatitidis* was

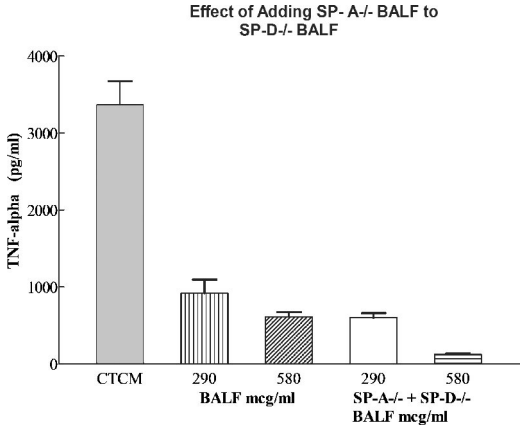

FIG. 3. Effect of normal BALF and SP-A$^{-/-}$ BALF added to SP-D$^{-/-}$ BALF on TNF-α production by BAM plus viable *B. dermatitidis*. Inhibition of BAM-*B. dermatitidis* (2:1) production of TNF-α by BALF at 290 and 580 μg of protein/ml was measured by ELISA (absorbance at 450 nm). Inhibition by SP-A$^{-/-}$ BALF added to SP-D$^{-/-}$ BALF in 1:1 at 290 and 580 μg/ml is shown. For normal BALF or a mixture of BALFs at 290 or 580 μg/ml, the *P* values for all four were <0.01 versus control. Comparison of BALF, or a mixture of BALFs, at 290 versus 580 μg/ml yielded *P* values of <0.05.

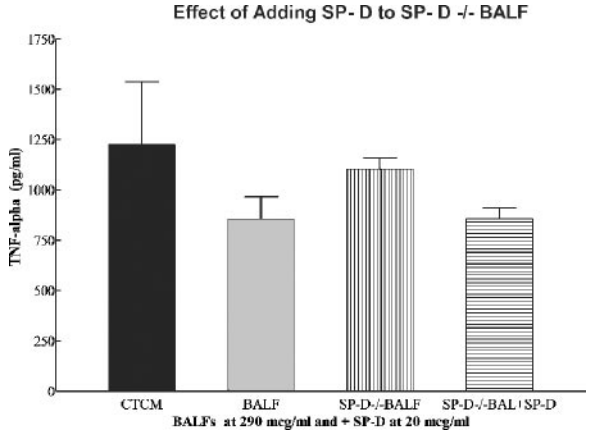

FIG. 4. Effect of adding SP-D to SP-D$^{-/-}$ BALF on TNF-α production by BAM-*B. dermatitidis*. BAM-*B. dermatitidis* (2:1) production of TNF-α, as measured by ELISA (absorbance at 450 nm), was inhibited by normal BALF (290 μg/ml) ($P < 0.05$), SP-D$^{-/-}$ BALF ($P > 0.05$), and SP-D added to SP-D$^{-/-}$ BALF at 20 μg/ml ($P < 0.01$).

inhibited 88% by BALF, 68% by SP-A$^{-/-}$ BALF (both $P < 0.01$), and 6% by SP-D$^{-/-}$ BALF ($P > 0.05$) compared to BAM-*B. dermatitidis* (Fig. 2B). A second experiment gave similar results with the three BALFs. These results suggest that SP-D, but not SP-A, in BALF inhibits TNF-α production by BAM-*B. dermatitidis*.

Inhibition of TNF-α production by BAM plus viable *B. dermatitidis* by normal BALF and SP-A$^{-/-}$ BALF added to SP-D$^{-/-}$ BALF. When SP-A$^{-/-}$ BALF was added to SP-D$^{-/-}$ BALF at 1:1 for a total protein concentration of 290 μg/ml, TNF-α production by BAM-*B. dermatitidis* was inhibited ($P < 0.01$) 79%, similar to 75% by normal BALF at 290 μg/ml ($P < 0.01$). A second experiment gave similar results. If the total BALF protein in 1:1 mixing experiments was 580 μg/ml, TNF-α production by the BAM-*B. dermatitidis*-BALF mixture was inhibited by 99% ($P < 0.01$) compared to BAM-*B. dermatitidis* without BALF (Fig. 3). A second experiment gave similar results. These findings indicate that SP-D$^{-/-}$ BALF lacks a factor(s) for inhibiting TNF-α production by BAM-*B. dermatitidis* that can be supplied by SP-A$^{-/-}$ BALF.

Inhibition of TNF-α production by BAM plus viable *B. dermatitidis* by normal BALF and SP-D added to SP-D$^{-/-}$ BALF. TNF-α production by BAM-*B. dermatitidis* was inhibited by 29% ($P < 0.05$) in presence of another batch of BALF (290 μg/ml), whereas in a concurrent study SP-D$^{-/-}$ BALF (290 μg/ml) failed to significantly inhibit TNF-α production (Fig. 4). TNF-α production was significantly (25%, $P < 0.01$) inhibited when SP-D$^{-/-}$ BALF (290 μg/ml) contained 20 μg of pure SP-D/ml (the maximum concentration available for study) versus BAM-*B. dermatitidis* plus SP-D$^{-/-}$ BALF (Fig. 4). A second experiment gave the same findings (30% inhibition). Lower SP-D concentrations did not inhibit TNF-α production. These findings indicate that SP-D$^{-/-}$ BALF lacks a factor for inhibiting TNF-α production by BAM-*B. dermatitidis* that can be supplied by pure SP-D.

Immunoblotting of BALF for SP-D. When normal BALF and SP-D$^{-/-}$ BALF were electrophoresed, blotted to polyvinylidene difluoride membrane, and probed for SP-D, only the former showed bands (Fig. 5A). In another experiment

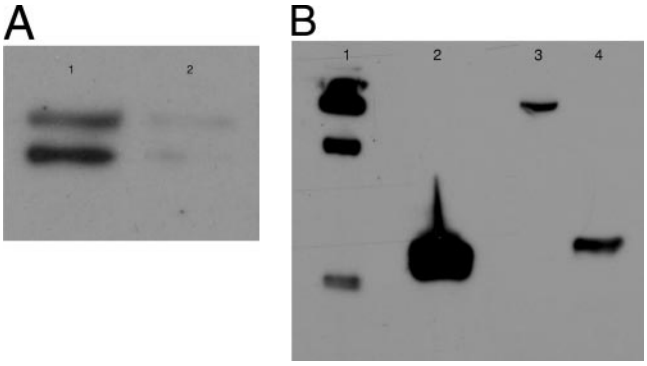

FIG. 5. Immunoblotting for SP-D. (A) Natural (nonreduced) BALF at 40 μg of protein/lane. Normal, lane 1; SP-D$^{-/-}$, lane 2. The dark band in lane 1 comigrates with purified nonreduced SP-D trimers. The lighter band is understood to be a small amount of trimer aggregates, stabilized by nondisulfide bonds. Minor species larger than trimers are frequently seen in protein preparations from natural sources. (B) SP-D at 2 μg of protein/lane: natural, lane 1; reduced lane 2. BALF at 20 μg of protein/lane: natural, lane 3; reduced, lane 4. In lane 1, the top band corresponds to the lighter band (aggregates) in lane 1 of Fig. 5A. SP-D is detected as single bands in BALF, the lesser amount of BALF studied in panel B apparently results in nondetection of the second band in natural BALF compared to panel A. This band in lane 3 and the corresponding band in lane 1 (SP-D trimers) comigrates with bovine serum albumin (about 98 kDa). The band in lane 4 and the corresponding band in lane 2 comigrate with a 36-kDa molecular mass marker and are considered to be reduced monomers. The intermediate band, between these two markers, in lane 1, comigrates with a 64-kDa molecular mass marker and is considered to represent dimers. The faintest band, at the bottom of lane 1, is understood to be unreduced monomers, which migrate more rapidly than reduced monomers because the lectin domain is folded in half by an intrachain disulfide band (with reduction, the protein unfolds and migrates as a larger protein in the presence of SDS). With the heavily loaded and exposed unreduced gels, delineation of the nature of the immunoreactive species is facilitated.

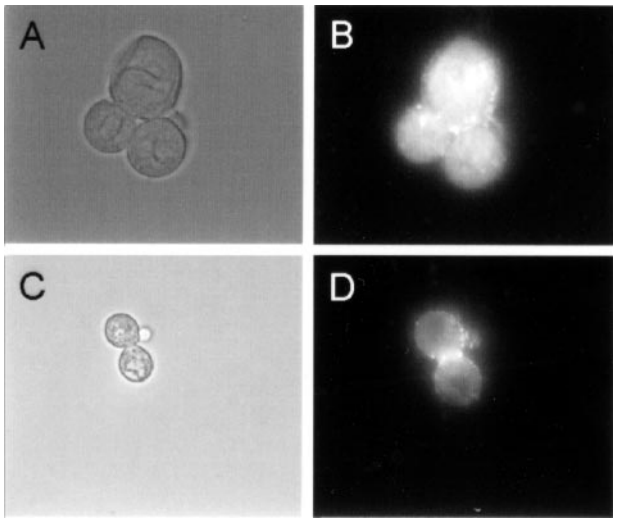

FIG. 6. Immunofluorescent staining of SP-D in BALF on *B. dermatitidis* using an indirect immunofluorescence technique with anti-SP-D antibody. Light microscopy in panels A and C shows *B. dermatitidis* not visible with fluorescence microscopy with control, SP-$D^{-/-}$ BALF. A diffuse cell surface staining pattern (B) and a pattern of staining focalized to budding region (D) with normal BALF are also shown.

where pure SP-D and BALF, each studied reduced and nonreduced, were immunoblotted for SP-D, reduced SP-D and reduced BALF gave single bands of similar molecular weights (Fig. 5B, lanes 2 and 4). In contrast, nonreduced SP-D showed four bands, one of which was seen in nonreduced BALF (Fig. 5B, lanes 1 and 3). Estimation of the area density of the band in reduced SP-D with that in reduced BALF at 10 times the concentration suggests that the SP-D in the BALF preparation approximated 0.1 μg.

Immunofluorescence staining of SP-D in BALF bound to *B. dermatitidis*. BALF, SP-$D^{-/-}$ BALF, and SP-$D^{-/-}$ BALF with pure SP-D added were incubated with viable *B. dermatitidis* and then incubated with rabbit anti-mouse SP-D, followed by fluoresceinated goat anti-rabbit antibody. In three experiments, bright green immunofluorescence was seen in the wall of *B. dermatitidis* with normal BALF. (In the absence of any antibodies under these conditions, *B. dermatitidis* appears red, a negative result, as was the case when the indirect antibody was applied without the monoclonal direct antibody). *B. dermatitidis* coated with SP-$D^{-/-}$ BALF was not stained; however, *B. dermatitidis* was stained when pure SP-D was added to SP-$D^{-/-}$ BALF, and the mixture was used for coating *B. dermatitidis* prior to application of the fluorescent indirect antibody system. These results show that SP-D in BALF bound to *B. dermatitidis*.

In the detailed photographic studies (Fig. 6) with HK *B. dermatitidis* and BALF, we noted two patterns of fluorescence (Fig. 6). One pattern was intense staining of *B. dermatitidis*, apparently SP-D covering the entire yeast surface (Fig. 6B). The second pattern (Fig. 6D) was fluorescence primarily at the budding regions of the cells.

β-Glucan and BAM TNF-α production and the effects of normal, SP-$A^{-/-}$, and SP-$D^{-/-}$ BALF. β-1,3-Glucan (250 μg/ml), a cell wall component of *B. dermatitidis*, stimulated TNF-α

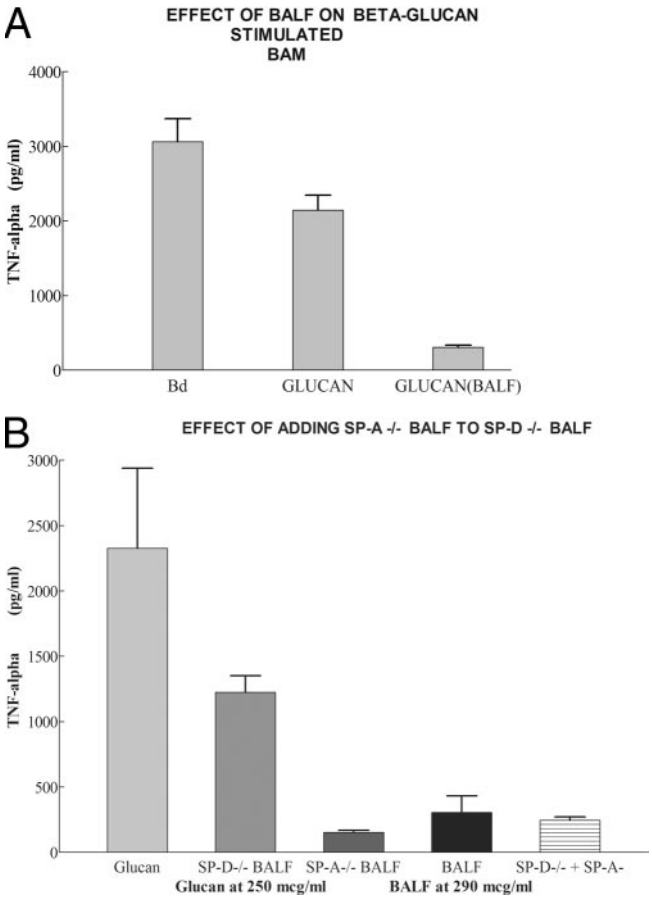

FIG. 7. Effect of BALF on β-glucan stimulated BAM on TNF-α production. TNF-α production by BAM plus β-glucan (250 μg/ml), measured by ELISA (absorbance at 450 μg/ml), is shown and compared to viable *B. dermatitidis*. (A) Inhibition ($P < 0.01$) by normal BALF (580 μg of protein ml). (B) Inhibition by 290 μg/ml of a second BALF preparation ($P < 0.01$) and BALF from SP-$D^{-/-}$ ($P < 0.05$), SP-$A^{-/-}$ ($P < 0.01$), and SP-$D^{-/-}$ added to SP-$A^{-/-}$ BALF in 1:1 mixture ($P < 0.01$).

production by BAM (Fig. 7A). Preliminary experiments showed this glucan concentration to be optimal. This concentration of glucan was less ($P < 0.01$) of a stimulus than the concentration of viable *B. dermatitidis* studied here. BALF at 580 μg/ml significantly inhibited ($P < 0.01$) TNF-α production (Fig. 7A) by glucan (250 μg/ml). With a second BALF preparation, 290 μg/ml could inhibit glucan-induced TNF-α production 97% (Fig. 7B) ($P < 0.01$). Production was inhibited 99% by BALF SP-$A^{-/-}$ ($P < 0.01$) and 49% by SP-$D^{-/-}$ BALF ($P < 0.05$), all at 290 μg of protein/ml (Fig. 7B). In a mixing experiment wherein SP-$A^{-/-}$ BALF was added 1:1 to SP-$D^{-/-}$ BALF for a total protein concentration of 290 μg/ml, TNF-α production by BAM with β-glucans was inhibited by 99% ($P < 0.01$) (Fig. 7B). The addition of polymyxin (5 μg/ml), to bind possible endotoxin contamination of the glucan preparation, did not affect the glucan results. These results suggest that factor(s) in BALF (more prominent in SP-$A^{-/-}$ BALF) could be binding to β-glucans in the cell wall and partly explain the inhibition of BAM TNF-α production by viable *B. dermatitidis*.

Immunofluorescent staining of β-glucan on *B. dermatitidis*. Viable *B. dermatitidis* was incubated with mouse monoclonal anti-β-1,3-glucan antibody and fluoresceinated indirect antibody as described in Materials and Methods. In three experiments, bright green immunofluorescence was seen in the wall of *B. dermatitidis*. Staining was more intense at the junction of the budding yeast with the parent yeast cell. These results demonstrate the β-glucan in *B. dermatitidis* cell wall and show glucan accessibility to a large protein molecule (antibody).

Binding of SP-D in BALF to β-glucan. BALF (290 μg/ml) was mixed with β-glucan (250 μg/ml) for 30 min before incubation with *B. dermatitidis* for 1 h. The treated *B. dermatitidis* was then incubated with rabbit anti-mouse SP-D and then goat anti-rabbit IgG conjugate. In the three experiments, *B. dermatitidis* was not stained with fluorescence when BALF mixed with β-glucan was used. However, *B. dermatitidis* was stained when BALF without β-glucan was used. This shows that β-glucan bound to SP-D in BALF and thus blocked SP-D from binding to *B. dermatitidis*.

DISCUSSION

BAM are highly phagocytic and express a broad range of receptors. The production of TNF-α is important in promoting host defense against *B. dermatitidis* (15), as well as other pathogenic fungi, such as *Cryptococcus neoformans* (11) and *A. fumigatus* (34), and intracellular bacterial pathogens such as *Mycobacterium tuberculosis* (21, 22). Levels of macrophage TNF-α production by strains of *B. dermatitidis* correlate with strain pathogenicity, in that virulent isolates stimulate less TNF-α than do nonvirulent strains (15). Although limiting inflammation and tissue destruction may be helpful to the host, it is also possible that *B. dermatitidis* has a novel offensive weapon in the pathogen-host interaction, namely, a means, in the presence of BALF, to blunt the defensive action engendered by host TNF-α production. Because TNF-α production is important in understanding the pathogenesis of *B. dermatitidis*, we explored the role of lung surfactant in enhancing or inhibiting TNF-α production by BAM in response to *B. dermatitidis*.

We found that BALF has a role in the interaction between viable *B. dermatitidis* and BAM, namely, inhibition of TNF-α production. The data presented indicate that if *B. dermatitidis* was heat killed and coated with BALF, BAM TNF-α production was similarly inhibited. We report that β-glucan stimulates TNF-α production by BAM, and TNF-α production is strongly inhibited in the presence of BALF. This suggests that glucan serves as a ligand for a fraction in BALF. There is increasing evidence that hydrophilic surfactant-associated proteins SP-A and SP-D play a first-line defensive role in the alveolar airspace and interact with alveolar macrophages during the recognition and clearance of pathogens responsible for pulmonary inflammation. Binding of SP-A and SP-D to the surfaces of microbes such as *Staphylococcus aureus* (16), influenza A virus (18), and *C. neoformans* (38) and the enhancement of phagocytosis via agglutination or receptor mediation has been well documented. These collectins may also have a direct cidal effect on fungi (33). SP-D has been shown to play a role in innate immunity through modulation of inflammation and clearance of organisms.

We affirmed the presence of SP-D in our BALF preparations and its absence from SP-$D^{-/-}$ BALF and found that SP-$D^{-/-}$ BALF did not significantly inhibit TNF-α production by BAM-*B. dermatitidis*. However, the mixing of SP-$D^{-/-}$ BALF and SP-$A^{-/-}$ BALF inhibited TNF-α production in this system. Pure SP-D added to SP-$D^{-/-}$ BALF had a similar effect. There are currently no commercial assays for measuring SP-D in murine BALF, but in comparing the area density of the immunoblots (Fig. 5B) of reduced pure SP-D and BALF, we estimate 290 μg of BALF protein/ml would contain approximately 1.5 μg of SP-D/ml. Differences between native and recombinant proteins or between species (14) could result in differences in SP-D potency in our assays.

β-Glucan appears to be responsible for at least part of the stimulus in *B. dermatitidis* to BAM for TNF-α production, and this stimulus could be inhibited by normal BALF or SP-$A^{-/-}$ BALF. We are not aware of prior studies showing the role of SP-D in the interaction between BAM and *B. dermatitidis*. However, studies have shown SP-D binds to *A. fumigatus* conidia, acting as an opsonin for macrophages (30). A significant increase in lung inflammation in *Pneumocystis carinii*-infected SP-$D^{-/-}$ mice and delayed early clearance of *P. carinii* has been reported (3). SP-D agglutinates *Haemophilus influenzae* and group B *Streptococcus* (GBS), and in SP-$D^{-/-}$ mice there was decreased association of GBS and *H. influenzae* with alveolar macrophages. Moreover, infection with GBS and *H. influenzae* significantly increased proinflammatory cytokines, TNF, interleukin-6, and MIP-2 in lung homogenates from SP-$A^{-/-}$ and SP-$D^{-/-}$ mice (28). SP-D is chemotactic to neutrophils and enhances the uptake of bacteria such as *Escherichia coli*, *Streptococcus pneumoniae*, and *S. aureus* (27).

It was noteworthy that when SP-$D^{-/-}$ BALF, lacking inhibitory influence on BAM TNF-α production, was added to SP-$A^{-/-}$ BALF, not only were the inhibitory factor(s) restored (as might be expected if the sole factor was SP-D supplied by the SP-$A^{-/-}$ BALF), but the activity of the mixture was greater than that of SP-$A^{-/-}$ BALF alone. This suggests some synergy between SP-$A^{-/-}$ and SP-$D^{-/-}$ BALF, as if SP-$A^{-/-}$ BALF also removed some blocker of activity in SP-$D^{-/-}$ BALF, or SP-$D^{-/-}$ BALF supplied a factor enhancing the activity (presumed to be SP-D) in SP-$A^{-/-}$ BALF, or possibly some other interaction occurred that is also presently unclear. Previous studies have suggested SP-$D^{-/-}$ BALF may contain increased lipids, as well as various cytokines, proteases, and oxidants, all of which may have immunomodulatory properties. Our finding provides an avenue for further research.

BALF-incubated *B. dermatitidis* showed SP-D on *B. dermatitidis*, whereas *B. dermatitidis* SP-D fluorescence, as expected, was absent after *B. dermatitidis* incubation with only SP-$D^{-/-}$ BALF and was restored when pure SP-D was added. These observations provide confirmation of an earlier report (13) that SP-D binds to *B. dermatitidis*. The staining patterns of SP-D on *B. dermatitidis* suggest the distribution of the ligand for SP-D on *B. dermatitidis* may be variable, possibly related to different phases of the cell cycle and focalization of β-glucan. The staining at the budding region warrants further study. Our immunofluorescence studies showed β-glucan in *B. dermatitidis* cell walls, confirming studies associating β-glucan with *B. dermatitidis* (12). β-Glucan could block the demonstration of SP-D on *B. dermatitidis* after BALF incubation, suggesting that SP-D is binding to β-glucan on *B. dermatitidis*. We propose that

SP-D blockade of glucan on *B. dermatitidis* is the mechanism for SP-D inhibition of TNF-α production by BAM-*B. dermatitidis*. We cannot exclude the possibility that other glycopolymers could bind SP-D at the fungal cell wall. As an extension of the present work, we have subsequently indicated, in a preliminary report using peritoneal macrophages [A. Koneti, E. Brummer, and D. A. Stevens, Evasion of innate immune response by *Blastomyces dermatitidis*: interaction with serum factor(s) inhibits TNF-α production by macrophages, Progr. 43rd Ann. Meet. Infect. Dis. Soc. Am., abstr. 198, San Francisco, 2005], that anti-glucan antibody treatment of *B. dermatitidis* abrogated *B. dermatitidis* stimulation of macrophage TNF-α production.

Another avenue for further research is our observation that SP-D$^{-/-}$ BALF has modest activity in inhibiting BAM TNF-α production when the stimulus is glucan but no activity when the stimulus is viable *B. dermatitidis*. This could mean that a factor, other than the more potent SP-D, in SP-D$^{-/-}$ BALF can be engaged when the stimulus is pure glucan, as opposed to when the glucan is complexed with other carbohydrate polymers or glycoproteins, as is the case in a fungal cell wall.

The receptor(s) on macrophages with which *B. dermatitidis* interacts for TNF-α production have not been defined. Recently, the macrophage receptor for 1,3-β-glucan, Dectin-1, has been identified and characterized (1, 6, 7). β-Glucan causes signaling through Dectin-1 on macrophages for TNF-α production (43). Although we have not investigated Dectin-1 on macrophages, we speculate that Dectin-1 interacts with β-glucan on *B. dermatitidis*; such interactions and signaling would account for TNF-α production.

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