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Histoplasma capsulatum Cell Wall β-Glucan Induces Lipid Body Formation through CD18, TLR2, and Dectin-1 Receptors: Correlation with Leukotriene B_4 Generation and Role in HIV-1 Infection[1]

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***Histoplasma capsulatum* (*Hc*) is a facultative, intracellular parasite of worldwide significance. Infection with *Hc* produces a broad spectrum of diseases and may progress to a life-threatening systemic disease, particularly in individuals with HIV infection. Resolution of histoplasmosis is associated with the activation of cell-mediated immunity, and leukotriene B_4 plays an important role in this event. Lipid bodies (LBs) are increasingly being recognized as multifunctional organelles with roles in inflammation and infection. In this study, we investigated LB formation in histoplasmosis and its putative function in innate immunity. LB formation in leukocytes harvested from *Hc*-infected C57BL/6 mice peaks on day 2 postinfection and correlates with enhanced generation of lipid mediators, including leukotriene B_4 and PGE_2. Pretreatment of leukocytes with platelet-activating factor and BLT1 receptor antagonists showed that both lipid mediators are involved in cell signaling for LB formation. Alveolar leukocytes cultured with live or dead *Hc* also presented an increase in LB numbers. The yeast alkali-insoluble fraction 1, which contains mainly β-glucan isolated from the *Hc* cell wall, induced a dose- and time-dependent increase in LB numbers, indicating that β-glucan plays a signaling role in LB formation. In agreement with this hypothesis, β-glucan-elicited LB formation was inhibited in leukocytes from 5-$LO^{-/-}$, $CD18^{low}$ and $TLR2^{-/-}$ mice, as well as in leukocytes pretreated with anti-Dectin-1 Ab. Interestingly, human monocytes from HIV-1-infected patients failed to produce LBs after β-glucan stimulation. These results demonstrate that *Hc* induces LB formation, an event correlated with eicosanoid production, and suggest a role for these lipid-enriched organelles in host defense during fungal infection.* *The Journal of Immunology,* **2009, 182: 4025–4035.**

Histoplasmosis is a pulmonary disease characterized by chronic granulomatosa and suppurative inflammatory reactions. The causative organism, *Histoplasma capsulatum* (*Hc*),[3] is a dimorphic fungus with a yeast-like morphology in tissue (1). Infection with *Hc* produces a broad spectrum of diseases, and although the vast majority of exposed individuals recover without relapse, it may progress to life-threatening systemic disease, particularly in individuals with AIDS (2). Indeed, in contrast to immunocompetent individuals in whom dissemination is uncommon, 95% of individuals with AIDS present disseminated disease even with highly active antiretroviral therapy (2, 3). Data from the literature have suggested that the increased susceptibility of HIV-infected subjects to opportunistic bacterial and fungal infections is related to defects in the 5-lipoxygenase (5-LO) metabolic capacity of alveolar macrophages (AM), mononuclear cells, and neutrophils (4–6).

Macrophages are the first cells to encounter *Hc* in the host (7), but the efficacy of the protective immune response requires numerous cellular and soluble effector mechanisms. In this context, macrophages and $CD4^+$ and $CD8^+$ T cells contribute to the clearance of *Hc* by releasing IL-12, IFN-γ, GM-CSF, and TNF-α, which inhibit yeast cell proliferation and promote the protective immune response (8–11). *Hc* infection and yeast cell wall fractions also induce the production of leukotrienes (LTs) in mice (12, 13), and we have demonstrated that these lipid mediators are essential for efficient pulmonary antifungal host defense and immunoregulation (13).

LTs are derived via the 5-LO pathway in the arachidonic acid metabolism (14). 5-LO localization is cell type-specific and also varies according to the activation state of the cell. 5-LO was shown to localize within the nuclear environment of AM, but was predominantly cytosolic in human neutrophils and resting peritoneal

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[3] Abbreviations used in this paper: *Hc*, *Histoplasma capsulatum*; ADRP, adipose differentiation-related protein; AM, alveolar macrophage; BALF, bronchoalveolar lavage fluid; DAPI, 4′,6′-diamidino-2-phenylindole; F1, yeast alkali-insoluble fraction; F2, yeast alkali-soluble fraction; F3, yeast galactomannan fraction; F1β-gluc, β-glucan from chitinase-treated F1; i.t., intratracheal(ly); LB, lipid body; 5-LO, 5-lipoxigenase; LT, leukotriene; PAF, platelet-activating factor; PC, peritoneal cavity; PM, peritoneal macrophage; PRR, pattern-recognizing receptor; WT, wild type.

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Table I. *Clinical characteristics of HIV patients*

Patient No.	Age (years)	Sex	$CD4^+$ T Cell Count (cells/μl)[a]	Ratio ($CD4^+/CD8^+$)[a]	Plasma HIV RNA Copy (eq/ml)[a,b]	On Therapy (HAART)[c]
1	28	Male	398	0.54	84	Yes
2	26	Female	356	0.49	10,994	No
3	25	Female	311	0.72	≤Lim. min.	No
4	38	Female	180	0.80	≤Lim. min.	Yes
5	36	Female	243	0.24	41,574	Yes
6	53	Female	133	0.17	1,255	Yes
7	31	Female	118	0.17	≤Lim. min.	No
8	26	Male	965	0.59	≤Lim. min.	No
9	53	Female	812	1.16	1,161	Yes
10	40	Male	603	1.07	≤Lim. min.	No
11	46	Male	981	0.86	≤Lim. min.	Yes
12	28	Male	978	0.77	1,342	No
13	21	Female	882	0.71	9,551	No
14	28	Female	541	1.76	≤Lim. min.	No

[a] Listed values were obtained at the time of apheresis.
[b] Lim. min., Minimal limit; eq/ml, viral RNA equivalent per milliliter.
[c] HAART, Highly active antiretroviral therapy.

macrophages (PM) (15). In addition to membranes, another lipid-bearing domain in cells consists of lipid bodies (LBs), which are nonmembrane-bound organelles abundant in inflammatory cells (16–20). LBs compartmentalize a diverse group of proteins involved in lipid metabolism, membrane trafficking and signaling (21), 5-LO and cyclooxygenase-2 pathways, and eicosanoid formation. An increase in LB numbers correlates with the concentrations of LTB_4, LTC_4, and PGE_2 released by leukocytes activated with the calcium ionophore A23187 (20, 22–24). Moreover, direct assessment of newly formed eicosanoids has characterized LBs as major sites of eicosanoid synthesis in inflammatory cells involved in allergic and infectious process (18, 25, 26).

In view of the importance of LTs in host defense mechanisms, the present study was undertaken to test the hypothesis that *Hc* infection induces LB formation as a complementary site for lipid mediator synthesis. We examined the LB formation induced in vivo and ex vivo by live and dead *Hc* and investigated the receptors and molecular mechanisms involved. Due to the clinical importance of our study for understanding the mechanisms of opportunistic fungal infection in immunodeficient patients, we also compared LB formation and the LTB_4 and PGE_2 synthesis capacities of cells from HIV-infected subjects with those from normal uninfected subjects. First, our study demonstrated that LB formation during murine *Hc* infection correlates with eicosanoid generation. Second, β-glucan (*Hc* cell wall fraction)-elicited LB formation in leukocytes is CD18, Dectin-1, and TLR2 dependent. Third, we revealed a role for endogenous platelet-activating factor (PAF) and LTB_4 in β-glucan-induced LB formation. Finally, our results suggest that LB formation is also required for LTB_4 release by PBMC from HIV-1-infected subjects.

Materials and Methods

Animals

Six- to 8-week old 5-$LO^{-/-}$ (129-$Alox5^{tm1Fun}$), $CD18^{low}$ (B6.129S-$Itgb2^{tm1bay}$), C3H/HePas ($TLR4^{lps\text{-}n}$), and C3H/HeJ ($TLR4^{lps\text{-}d}$) mice were obtained from The Jackson Laboratory. TLR2 knockout mice ($TLR2^{-/-}$) were donated by S. Akira (Osaka University, Osaka, Japan). All of the above strains and strain-matched wild-type (WT) C57BL/6 (WT to $CD18^{low}$) or sv129 (WT to 5-$LO^{-/-}$) mice of both sexes were bred in the Faculdade de Ciências Farmacêuticas de Ribeirão Preto (Universidade de São Paulo, Ribeirão Preto, Brazil). They were maintained in a room at 25°C with a light/dark cycle of 12 h and free access to food and water. Infected mice were kept in biohazard facilities in cages with a laminar flow. Experiments were approved by and conducted in accordance with guidelines of the Universidade de São Paulo Animal Care Committee (process no. 255/2005).

Parameters of human donors

Patients participating in this study were enrolled at the Hospital das Clínicas of Faculdade de Medicina, Ribeirão Preto, Brazil. The inclusion criteria in this study were patients infected with HIV-1 and not infected with *H. capsulatum*. Patients with previous antiretroviral therapy were divided into $CD4^+$ T cell counts of less than or greater than 500 cells/mm^3. Virological parameters were measured (HIV RNA copies/ml). The baseline characteristics of 14 (patient nos. 1–14) patients used in this study are shown in Table I. The control group was composed of healthy blood donors case matched to the HIV-1 positive cases following the criteria of age and sex. The inclusion criteria also considered samples that were negative according to serological tests used for blood donation screening: anti-HIV-1/2, anti-human T cell leukemia virus-1/2, anti-hepatitis C virus, hepatitis B surface Ag, anti-HB core, anti-syphilis, and Chagas diseases. This study was approved by and conducted in accordance with guidelines of the Universidade de São Paulo Human Care Committee (process no. 12837/2006). All patients and controls gave informed consent.

Fungal strain and culture conditions

The cultures of *Hc* and yeast cell preparations were performed as previously described (12). Live yeast cells were used when their viability was ≥90% as revealed by fluorescein diacetate (Sigma-Aldrich) and ethidium bromide (Sigma-Aldrich) staining.

Fungus and fractions of cell wall

Live and dead yeast and cell wall fractions of *Hc* were prepared as described (12, 13). At the end of purification, the alkali-insoluble sediment was named fraction 1 (F1, containing β-glucan and chitin). The alkali-soluble and acetic acid precipitated fraction was named fraction 2 (F2, containing α-glucan). The alkali-soluble and non-acetic acid-precipitated fraction was named fraction 3 (F3, containing galactomannan). A portion of F1 was enzymatically treated with chitinase (Sigma-Aldrich) (12) and after several washes the resulting precipitate was called β-glucan from F1 (F1β-gluc).

In vivo LB induction by Hc, F1, F2, F3 β-glucan, or F1β-gluc and the effects of PAF and LTB_4 antagonists

Mice were anesthetized i.p. with 2.5% 2,2,2-tribromoethanol and intratracheally (i.t.) infected with live yeast cells as described (13). Sublethal inocula (5×10^5 yeast cells/100 μl for C57BL/6 and $CD18^{low}$ mice and 3×10^6 yeast cells/100 μl for sv129 and 5-$LO^{-/-}$ mice) were used. Control mice received only 100 μl of PBS. On days 2, 7, and 14 following infection or PBS injections mice were killed in a CO_2 chamber. Cells from the bronchoalveolar space were collected and named bronchoalveolar lavage fluid (BALF) cells (13). During inhibitory studies, i.t. *Hc*-infected mice were orally treated twice with a BLT1 antagonist (CP-105,696) (Pfizer) (1 mg/kg 1 h before infection and 4 h before sacrifice), a PAF

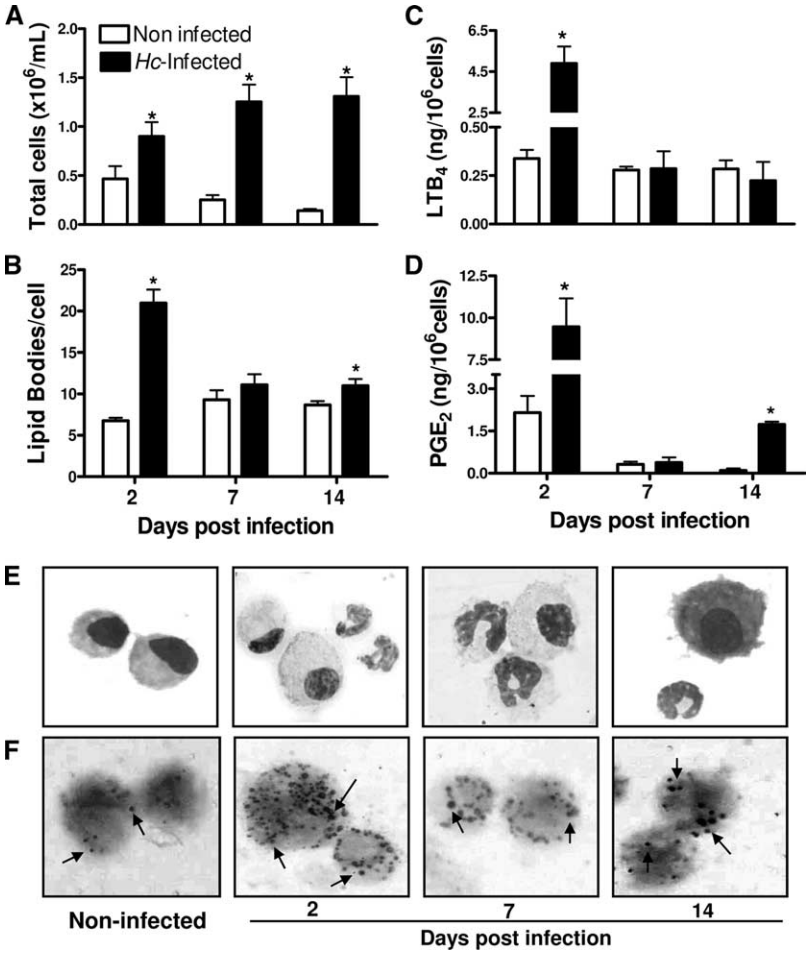

FIGURE 1. *Hc* infection induces LB formation in total leukocytes recruited to the bronchoalveolar space and primes the cells for eicosanoid generation. The number of total alveolar leukocytes was counted (*A*) and the cells were used for LB enumeration (*B*). BALF cells recovered from C57BL/6 animals at 2, 7, and 14 days post i.t. infection (5×10^5 *Hc* yeast/lung) were neutrophils and mononuclear cells. Controls were inoculated with 100 μl of PBS, and 97–100% of cells recovered were mononuclear cells. Immediately after recovery, the cells were stained with osmium tetroxide for LB records (*F*) or stimulated for 15 min with A23187 (0.5 μM) for LTB_4 (*C*) and PGE_2 (*D*) release. LB numbers were very low in noninfected cells but very prominent in leukocytes after 2 days postinfection, and decreased 7 and 14 days post infection. Light micrographic analyses after Panótico staining demonstrated the presence of neutrophils and mononuclear cells (*E*) in BALF of infected mice. Data represent the mean ± SEM from two separate experiments with six animals/group ($n = 12$). LB per cell were enumerated by light microscopy with a ×100 objective lens in 50 consecutively counted cells per animal, and totaled 600 cells per stimulus. The asterisk (∗) indicates statistically significant differences when cells from *Hc*-infected mice were compared with cells from noninfected mice.

antagonist (SR-27-417) (0.1 mg/kg 1 h before infection and 24 h before sacrifice), or both drugs at the same time. Animals were sacrificed 2 days postinfection and posttreatment. In another set of in vivo experiments, mice were injected i.p. with 100 μg of F1, F2, F3, or 10 μg of commercial β-glucan (Calbiochem) in 0.5 ml of PBS per cavity. Control groups received only PBS. At 4 h after the injection, animals were killed in a CO_2 chamber and cells from their peritoneal cavities (PC) were harvested by the injection of 3 ml of PBS. Cells were centrifuged for 10 min at $300 \times g$ and pellets were resuspended in 1 ml of Ca^{2+}/Mg^{2+}-free HBSS (Invitrogen). Total and differential cell counts from BALF and PC were performed as described previously (13). Cell viability was always >90%, as determined by trypan blue dye exclusion. Total leukocytes harvested from BALF and PC were prepared for LB enumeration or eicosanoid release as described below. As previously described, leukocytes from BALF of *Hc*-infected mice or PC inoculated with F1, F2, and F3 present neutrophils and mononuclear cells (12, 13).

In vitro lipid body induction and treatments

For the in vitro experiments, resident AM from BALF from naive mice (1×10^5 cells/well) or from human PBMC (5×10^5 cells/well) isolated over gradients of Ficoll-Paque PLUS (GE Healthcare) were resuspended in RPMI 1640 supplemented with 100 mg/ml gentamicin. Leukocytes were adhered to 8-well culture slides (BD Bioscience) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Nonadherent cells were removed after 2 h and adherent cells were incubated in RPMI 1640 supplemented with 10% FBS and 100 mg/ml gentamicin for 18 h. Adherent cells were mainly macrophages, as determined by cytocentrifuged smears stained by the Panótico method. Using C57BL/6 cells, we first determined the F1 dose response (0.1–1000 μg/ml) and time response (1, 4, 8, 16, and 24 h). Based on these results, we chose 100 μg/ml F1 and 8 h of stimulation as the optimal conditions for subsequent experiments. Therefore, macrophages were incubated with live or dead *Hc* (3:1; yeast:cell), F1, F2, F3 (100 μg/ml), β-glucan (0.1–1000 μg/ml), F1β-gluc (10 μg/ml), or PAF (10^{-6} M; Sigma-Aldrich) diluted in RPMI 1640 medium, and 0.5 ml was added to the wells. After 8 h (or 1 h with PAF) of incubation at 37°C, monolayers were recovered for LB enumeration. During inhibitory studies, mouse cells were pretreated for 1 h with a PAF receptor antagonist (SR-27417) (10^{-6} M), vehicle, anti-Dectin-1 Abs (mAb 2A11 at 2, 4, or 10 μg/ml; a gift from Dr. G. Brown (Faculty of Health Sciences, Cape Town, South Africa)) (27), or an irrelevant rat IgG2b isotype control (10 μg/ml). Human PBMC were stimulated with β-glucan (10 μg/ml) or F1 (100 μg/ml), as described above. The cells were prepared for LB evaluation, as described below.

LB staining and enumeration

Leukocytes on slides were fixed in 3.7% formaldehyde in Ca^{2+}/Mg^{2+}-free HBSS at pH 7.4. After staining, the slides were dried and mounted as described (18). Briefly, immediately after being harvested, total leukocytes from BALF of *Hc*-infected mice or PC of F1-, F2-, F3-, or β-glucan-inoculated mice were used for LB enumeration and eicosanoid release. Cells from naive mice were also collected and cultured to obtain adherent cells (mainly macrophages) for in vitro LB induction. Cytocentrifuged smears were fixed in 3.7% formaldehyde in Ca^{2+}/Mg^{2+}-free HBSS (pH 7.4) and rinsed in 0.1 M cacodylate buffer, 1.5% osmium tetroxide (30 min), and dH_2O. After the slides were immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, and restained in 1.5% osmium tetroxide (3 min), they were rinsed in dH_2O and dried for further analysis. The morphology of fixed cells was observed, and lipid bodies were enumerated as round osmiophilic structures by light microscopy with a ×100 objective lens in 50 consecutively scanned leukocytes in each slide. The operator responsible for counting was blinded to the codes for each slide (18, 20). Each slide represents one animal or one pool of cells. For each human subject, the cells were stimulated in triplicate, and 50 cells were counted in each (150 cells counted/individual) in a double-blinded manner.

LTB_4 and PGE_2 measurements

Leukocytes from mouse BALF and PC or human PBMC (1×10^6 cells/ml after treatment or infection) were resuspended in $Ca^{2+}Mg^{2+}$ HBSS and

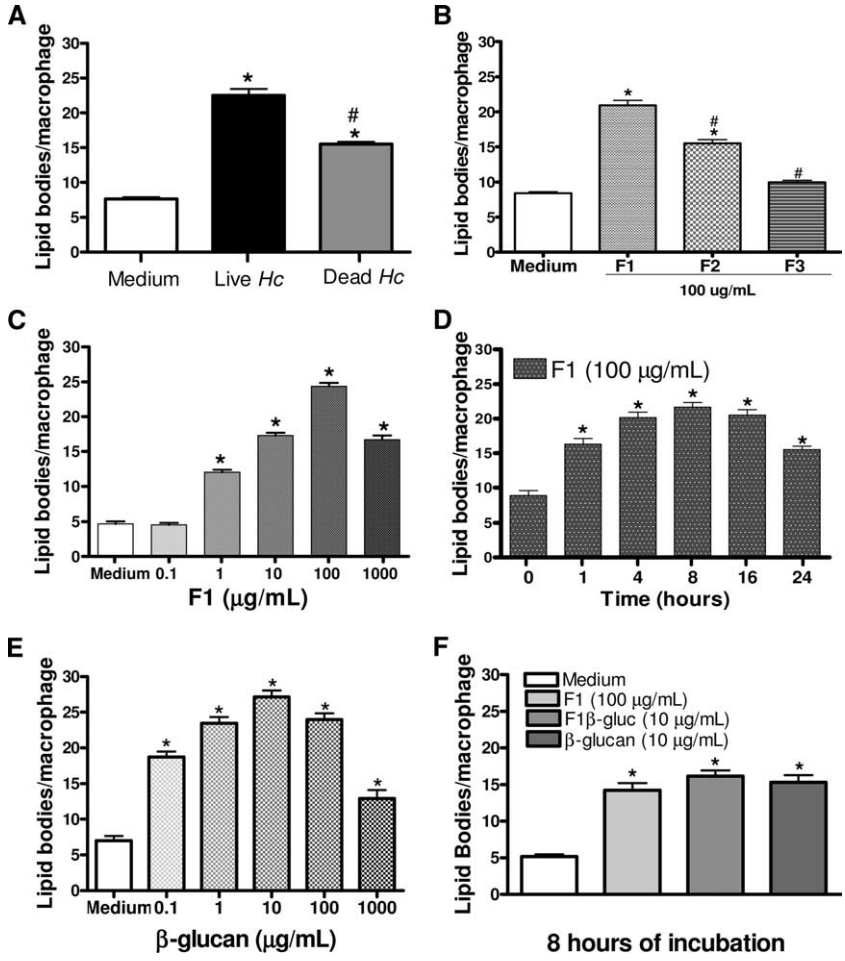

FIGURE 2. Live and dead *Hc*, F1, F2, F3, and β-glucan induced LB formation in alveolar macrophages in vitro. Adherent BALF macrophages recovered from naive C57BL/6 mice were incubated at 37°C for 8 h (except in the F1 time-response experiment) with live or dead *Hc* (*A*) or F1, F2, or F3 (*B*). In vitro F1 dose-response (0.1–1000 μg/ml) (*C*) and time-response (0–24 h of incubation with 100 μg/ml) (*D*) studies were performed. In addition, an 8-h dose-response analysis of LB formation induced by pure commercial β-glucan (0.1- 1000 μg/ml) was conducted (*E*). To demonstrate that LB formation was due to the presence of β-glucan, F1 treated with chitinase (F1β-gluc) was incubated for 8 h with cells (*F*). LBs were enumerated after osmium staining as described previously. The experiments were done twice, each with three separate pools of cells ($n = 6$/stimulus). Each bar represents the mean (±SEM) LBs per cell enumerated by light microscopy with a ×100 objective lens in 50 consecutively counted cells per well, totaling 300 cells per stimulus. Symbols indicate statistically significant differences when compared with RPMI 1640 medium (∗) or with live *Hc* or F1 (#).

stimulated for 15 min with 0.5 μM calcium ionophore A23187 (submaximal concentration) (Sigma-Aldrich). Reactions were stopped on ice and the samples were centrifuged for 10 min at 500 × *g* at 4°C. LTB_4 and PGE_2 in the supernatants were assayed by ELISA (GE Healthcare).

5-LO and adipose differentiation-related protein (ADRP) immunolocalization

BALF macrophages (5×10^5 cells/well) from naive mice were adhered to 8-well culture slides (BD Bioscience) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were incubated with *Hc* (3:1, yeast: cell), F1 (100 μg/ml), or RPMI 1640 medium. After 8 h of incubation at 37°C, monolayers were washed by gentle agitation with Ca^{2+} and Mg^{2+}-free HBSS and used for immunolocalization of 5-LO in LBs as described (28), with minor modifications. Cells were incubated for 1 h with 200 μl of anti-5-LO polyclonal antiserum (Cayman Chemical) (1/200 dilution in PBS) or with an isotype-matched rabbit IgG control (1/100 dilution in PBS), followed by incubation with an Alexa Fluor 488-labeled donkey anti-rabbit IgG Ab (Molecular Probes) (1/500 dilution) for 45 min and 4′,6′-diamidino-2-phenylindole (DAPI; Molecular Probes) (1/150 dilution) for nuclear (DNA) staining. To specifically localize LBs, a guinea pig anti-ADRP Ab (Research Diagnostics) (1/250 dilution) plus Cy3-labeled anti-guinea pig IgG Ab (The Jackson Laboratory) (1/500 dilution) were added. The images were obtained using a Leica DM5000B fluorescence microscope equipped with a DFC500 digital camera (Leica Microsystems). Images were edited using Adobe Photoshop CS 8.0 software (Adobe Systems).

Statistical analysis

Results were expressed as mean ± SEM. One-way ANOVA with Bonferroni posttests was used for multiple group analysis. Results were considered statistically significant with values of $p < 0.05$.

Results

Hc infection induces LB formation in leukocytes recruited to the bronchoalveolar space

The i.t. infection of C57BL/6 mice with live *Hc* induced leukocyte accumulation between 2 and 14 days postinfection in BALF mice (Fig. 1*A*). At 2 days postinfection, neutrophils were the predominant cells recovered, but mononuclear cell numbers increased and were in their greatest numbers at 14 days postinfection (Fig. *E*). When BALF cells were appropriately treated for lipid fixation and staining, an increase in LB formation was observed during all periods after infection, with the peak on day 2, when compared with cells from animals injected with only PBS (Fig. 1*B*). Because LBs are stores of arachidonic acid and contain eicosanoid-forming enzymes, we investigated whether increased numbers of leukocyte LBs from BALF of *Hc*-infected mice would lead to enhanced LTB_4 and PGE_2 production. The increase in LB numbers in BALF inflammatory cells collected 2 days postinfection with *Hc* correlated with the increased capacity of these cells to release LTB_4 (10-fold increase) and PGE_2 (5-fold increase) upon stimulation with the calcium ionophore A23187 (Fig. 1, *C* and *D*). These results suggest that during fungal infection, LBs are the sites of eicosanoid generation.

LB formation in resident BALF cells in vitro is mainly induced by β-glucan present in F1 from Hc cell wall

Previously published studies have demonstrated that resident AM have a higher capacity for producing LTs and to form LBs. We

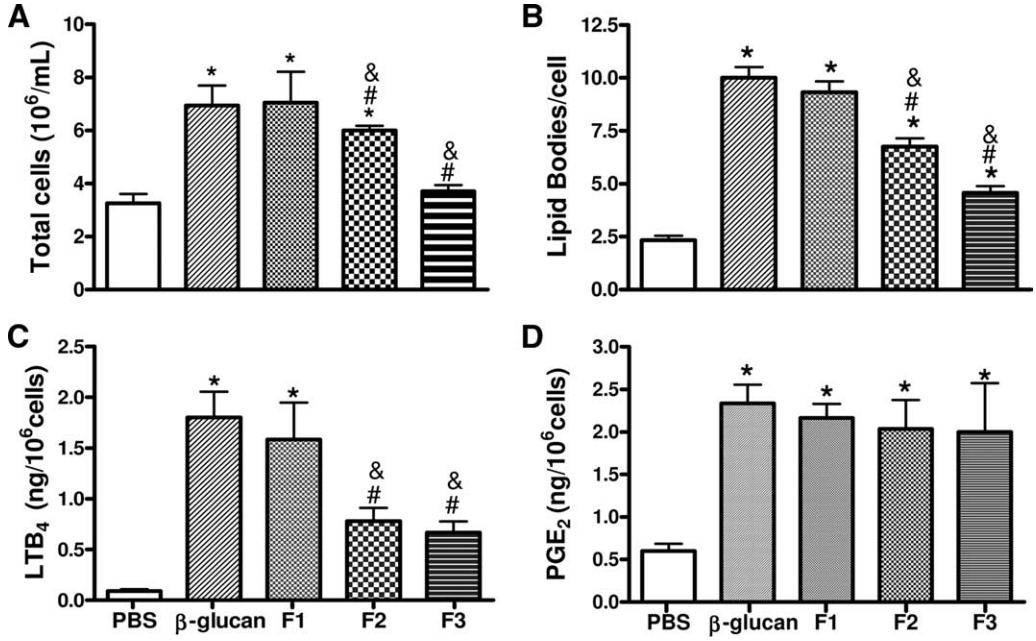

FIGURE 3. β-Glucan, F1, F2, and F3 induce LB formation in total leukocytes recruited to the PC and prime the cells for eicosanoid generation. The number of total peritoneal leukocytes (*A*) was counted and the cells were used for LB enumeration (*B*) or LTB_4 (*C*) and PGE_2 (*D*) release. C57BL/6 mice were inoculated i.p. with pure commercial β-glucan (10 μg/cavity), F1, F2, or F3 (100 μg/cavity), and the peritoneal cells recovered 4 h later were neutrophils and mononuclear cells. The control was inoculated with 100 μl of PBS and 96- 98% of the cells recovered were mononuclear cells. Total leukocytes were immediately stained with osmium tetroxide for LB enumeration or used for stimulation for 15 min with A23187 (0.5 μM) for LTB_4 and PGE_2 release. Eicosanoids were determined by ELISA. Data represent the mean (±SEM) from two separate experiments with six animals/group ($n = 12$). LBs per cell were enumerated by light microscopy with a ×100 objective lens in 50 consecutively counted cells per animal totaling 600 cells per stimulus. Symbols indicate statistically significant differences when compared with medium (*), F1 (#), and β-glucan (&).

used naive BALF adherent cells that present 95–98% of resident AM (29) to investigate the mechanisms of *Hc*-induced LB formation. As Fig. 2 shows, live or dead *Hc* yeast induced a 180 or 115% increase, respectively, in LB formation in BALF adherent cells in vitro after 8 h of incubation. In fact, dead *Hc*, which contains heat-denatured membrane proteins, induced significantly less LB formation when compared with live *Hc* yeast (Fig. 2*A*). The role of the different fractions of the *Hc* cell wall, F1 (β-glucan and chitin), F2 (α-glucan), and F3 (galactomannan) on LB formation was also investigated. As demonstrated in Fig. 2*B*, F1 induced LBs in higher numbers than F2 or F3. We observed that F1 induced a dose-dependent increase of LBs, with a maximum LB induction seen at an F1 concentration of 100 μg/ml (Fig. 2*C*). LB formation initiated within 1 h reached maximum levels 8 h after stimulation (Fig. 2*D*). In a similar manner, LB formation induced by commercial β-glucan was dose dependent but peaked after stimulation with 10 μg/ml commercial β-glucan (Fig. 2*E*). To demonstrate that the difference between the F1 and β-glucan potencies in inducing LB formation was a consequence of chitin contamination, F1 was treated with chitinase (F1β-gluc). As shown in Fig. 2*F*, chitinase-treated F1 elicited maximal LB formation at a concentration of 10 μg/ml, similar to what was observed with β-glucan.

F1 and β-glucan also induce LB formation in vivo

To study LB formation in vivo, F1, F2, F3 (100 μg/cavity), or pure commercial β-glucan (10 μg/cavity) was inoculated i.p., and PC leukocytes were harvested after 4 h. As shown in Fig. 3*A*, F1, F2, and F3 induced cell recruitment, with the highest cell numbers seen after F1 inoculation. However, when compared with pure commercial β-glucan, a 10-fold higher amount of F1 was required to induce a similar degree of cell recruitment

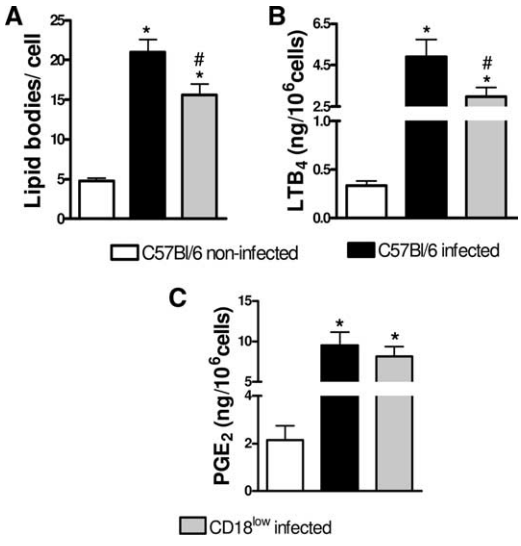

FIGURE 4. *Hc* infection induced both LB formation and priming for eicosanoid generation by a process dependent on β_2-integrin. Alveolar leukocytes from C57BL/6 or $CD18^{low}$ animals were obtained 2 days post i.t. infection with live *Hc* (5×10^5 yeast cells/lung). Cell were recovered and immediately stained with osmium for LB enumeration (*A*) and used for eicosanoid production after 15 min of stimulation with A23187 (0.5 μM). LTB_4 (*B*) and PGE_2 (*C*) in supernatants were measured by enzyme immunoassay. Data are expressed as means (±SEM) from two experiments with six animals per group ($n = 12$). LBs per cell were enumerated by light microscopy with a ×100 objective lens in 50 consecutively counted cells per animal, totaling 600 cells per stimulus. Symbols indicate statistically significant differences when compared with noninfected (*) or C57BL/6 mice (#).

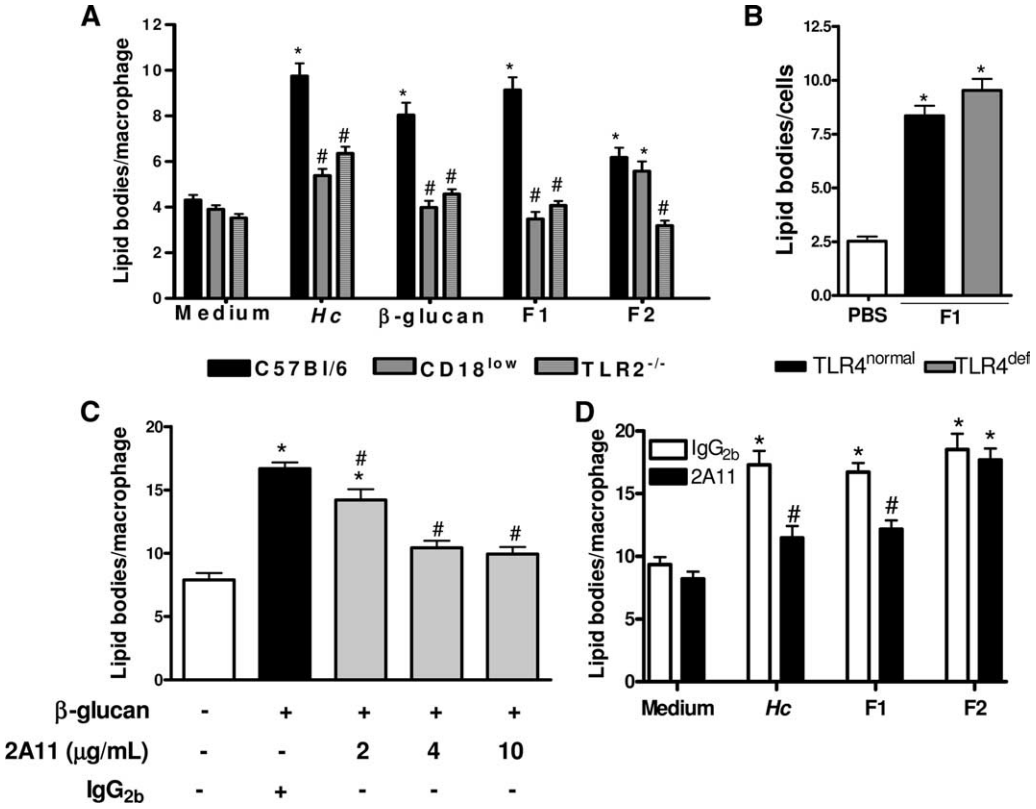

FIGURE 5. *Hc* infection and F1 and β-glucan stimulation induced LB formation by a process dependent on β_2-integrin, TLR2, and Dectin-1. Adherent BALF macrophages from naive C57BL/6, CD18^{low}, or TLR2$^{-/-}$ mice were collected for in vitro assays and then incubated with live *Hc* (3:1; yeast:cell), β-glucan (10 μg/ml), F1 (100 μg/ml), F2 (100 μg/ml), or medium for 8 h for LB induction (*A*). C3HeN (WT) and C3HeJ (TLR4^{deficient}) mice were inoculated with F1 (100 μg/cavity) or 100 μl of PBS, and the neutrophils and mononuclear cells recruited were recovered 4 h later for LB enumeration (*B*). BALF adherent macrophages from C57BL/6 naive mice were preincubated with different concentrations of anti-Dectin-1 (2A11) Ab or 10 μg/ml irrelevant IgG2b for 60 min and then stimulated with pure commercial β-glucan (10 μg/ml) for 8 h (*C*) for LB induction. In another set of experiments, macrophages were treated with 10 μg/ml anti-Dectin-1 (2A11) or 10 μg/ml IgG2b for 60 min and then incubated with live *Hc* (3:1; yeast:cell), F1 (100 μg/ml), F2 (100 μg/ml) or medium for 8 h for LB induction (*D*). In vivo experiments were performed twice with six animals each time. Data are expressed as means (±SEM) from twelve animals per stimulus. In vitro experiments were done twice with three separate pools of cells ($n = 6$ per stimuli), and LBs per cell were enumerated by light microscopy with a ×100 objective lens in 50 consecutively counted cells per slide or well, totaling 600 or 300 cells for the in vivo or in vitro assay, respectively. Symbols indicate statistically significant differences when compared: in *A*, with cells incubated only with medium (∗) and with cells from C57BL/6 mice (#); in *B*, with nonstimulated mice (∗); and in *C* and *D*, with cells without stimuli (∗) and with cells preincubated with IgG2b (#).

(Fig. 3*A*). Additionally, inflammatory cells recovered from PC after all stimuli presented an increase in LB formation. However, more pronounced increases in numbers of LBs were noted in cells recovered after β-glucan and F1 inoculation (Fig. 3*B*). As described above, an increased level of LTB_4 was seen in the supernatant of cells recovered from F1- or β-glucan-inoculated PC upon stimulation with A23187 (Fig. 3*C*), albeit less than that from BALF cells. In addition, PGE_2 production by cells from PC inoculated with F1, F2, F3, or β-glucan was at the same level, but higher than that observed in the supernatant of cells from PBS-inoculated PC (Fig. 3*D*).

β_2-Integrin, Dectin-1, and TLR2 but not TLR4 are key receptors for Hc-, F1-, and β-glucan-induced LB formation

The role of CD18, Dectin-1, and TLR in mediating pathogen recognition in the mechanism of LB formation was investigated. We observed that 2 days after in vivo *Hc* infection, the number of LBs in CD18^{low} (β_2-integrin-defective) cells was 30% lower than that in WT C57BL/6 cells (Fig. 4*A*). The decreased numbers of LBs in CD18^{low} cells correlated with a reduction in LTB_4 release (Fig. 4*B*) after A2318 priming, but not with PGE_2 production (Fig. 4*C*). Moreover, CD18^{low} AM incubated in vitro with live *Hc*, F1, and β-glucan presented fewer LBs compared with C57BL/6 cells (Fig. 5*A*). However, we did not observe any differences in LB formation between CD18^{low} and C57BL/6 cells stimulated with F2. AM from TLR2$^{-/-}$ mice presented fewer LBs when compared with WT (C57BL/6) cells regardless of stimuli (Fig. 5*A*). After F1 stimulation, no differences were observed in the number of LBs in TLR4^{deficient} cells when compared with WT C3H/HeN (TLR4^{normal}) cells (Fig. 5*B*). As expected, anti-Dectin-1 mAb inhibited β-glucan-, *Hc*,- and F1-induced LB formation (Fig. 5*C*), but it did not inhibit LB formation induced by F2 containing α-glucan (Fig. 5*D*).

LB formation induced by Hc, F1, or β-glucan depends on endogenous LTB_4 and PAF

To investigate the mediators involved in *Hc*-induced LB formation, BALF leukocytes from *Hc*-infected WT sv129 and 5-LO$^{-/-}$ mice were obtained. Cells collected from 5-LO$^{-/-}$ mice 2, 7, and 14 days postinfection presented a drastic decrease in the number of LBs in comparison with cells from sv129 *Hc*-infected mice (Fig. 6*A*). In addition, 5-LO$^{-/-}$ AM stimulated in vitro (8 h) with F1, F1β-gluc, or β-glucan presented fewer numbers of LB than cells from sv129 mice submitted to the same treatments (Fig. 6*B*). To

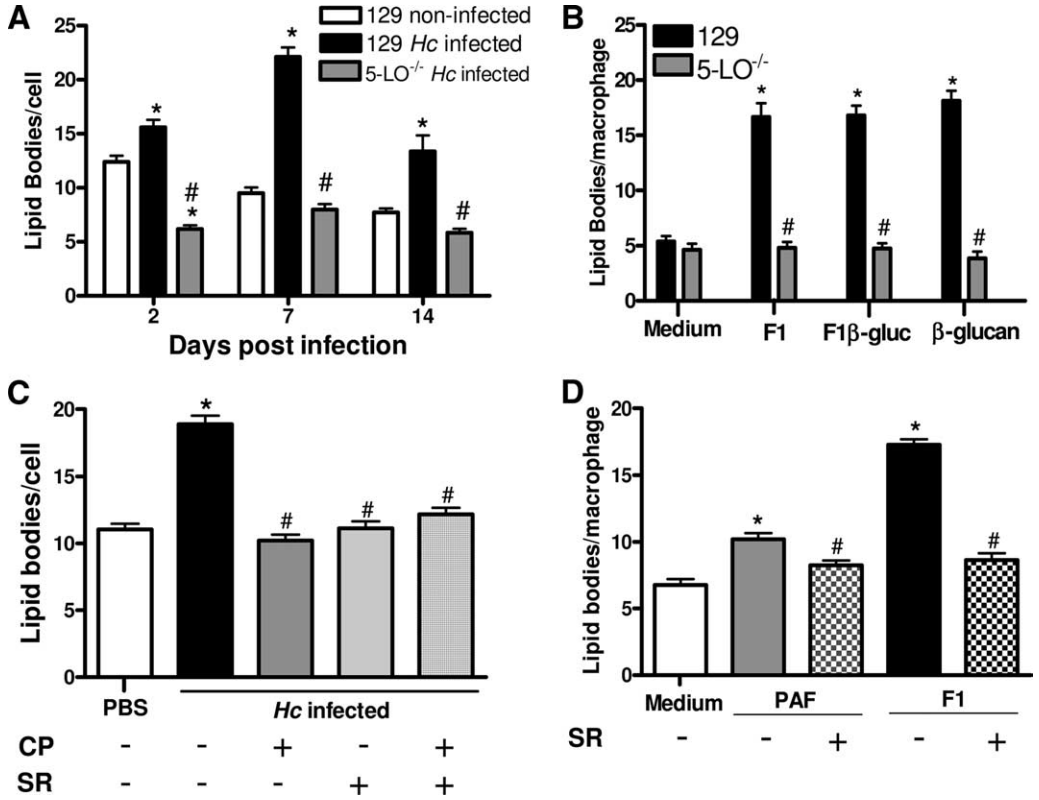

FIGURE 6. Live *Hc* or F1 induces LB formation by mechanisms dependent on PAF and 5-LO products. WT (sv129 strain) and 5-L0$^{-/-}$ mice were inoculated i.t. with *Hc* (3×10^6 cells/lung) or vehicle (100 μl) and LB formation was counted 2, 7, and 14 days postinfection in total BALF leukocytes (*A*). BALF adherent macrophages recovered from naive WT and 5-L0$^{-/-}$ mice were stimulated in vitro with F1 (100 μg/ml), F1-β-gluc (10 μg/ml), or pure commercial β-glucan (10 μg/ml) for 8 h to induce LB formation (*B*). C57BL/6 animals were i.t. inoculated with *Hc* (5×10^5 cells/lung) or PBS and treated as described in *Materials and Methods* with the PAF receptor antagonist SR-27-417 (SR; 0.1 mg/kg) or the BLT1 receptor antagonist CP-105,696 (CP; 1 mg/kg), or both. Cells recovered from BALF 2 days postinfection were neutrophils and mononuclear cells and were used for LB quantification (*C*). BALF adherent macrophages were pretreated in vitro with the PAF receptor antagonist SR-27-417 (SR; 10^{-6} M) or vehicle for 1 h at 37°C and then stimulated for 8 h with PAF (10^{-6}M) or F1 (100 μg/ml) to induce LB formation (*D*). The data are expressed as means (±SEM) from two experiments, each with six mice ($n = 12$) or three separate pools of cells ($n = 6$). LBs per cell were enumerated by light microscopy with a ×100 objective lens in 50 consecutively counted cells per slide or well, totaling 600 and 300 cells, respectively, for the in vivo or in vitro assays. Symbols indicate statistically significant differences: in *A*, noninfected mice compared with infected mice (*) and 5-LO mice compared with 129 mice (#); in *B*, cells in medium (*) and 5-LO (#) compared with stimulated sv129 cells; in *C*, infected mice compared with noninfected mice (*) and infected nontreated mice compared with infected mice treated with SR-27-417 (SR) and CP-105,696 (CP) (#); in *D*, with cells in medium (*) and PAF- or F1-stimulated cells with and without SR-27-417 (SR) (#).

evaluate LTB_4 and PAF involvement in LB formation in vivo, infected C57BL/6 mice were treated orally with the LTB_4 receptor antagonist CP-105,696 and/or the PAF receptor antagonist, SR-27417. Treatments with CP-105,696 and/or SR-27417 inhibited 40 and 38%, respectively, of LB formation 2 days postinfection (Fig. 6*C*). Subsequently, we investigated the PAF contribution on F1-induced LB formation in vitro. For this, C57BL/6 BALF cells were pretreated with SR-27417 before F1 stimulation. SR-27417 (10^{-6} M) inhibited LB formation induced by F1 and PAF (positive control) by 50 and 15%, respectively (Fig. 6*D*).

5-LO and ADRP colocalizes at LBs

We evaluated whether 5-LO, a key leukotriene-forming enzyme, was localized at LBs formed within 8 h of *Hc* infection or F1 stimulation. Compartmentalization of 5-LO and ADRP at LBs was analyzed by immunofluorescence. BALF AM of *Hc*-infected mice were stained with anti-5-LO Abs (Fig. 7*I*) and showed focal cytoplasmic staining in green. However, in F1-stimulated cells, 5-LO perinuclear membrane staining was observed in addition to the cytoplasmic labeling (Fig. 7*E*). *Hc*-infected or F1-stimulated cells exhibited strong ADRP-labeled LBs as red punctate intracytoplasmic inclusions (Fig. 7, *F* and *J*). As shown in Fig. 7*G*, after F1

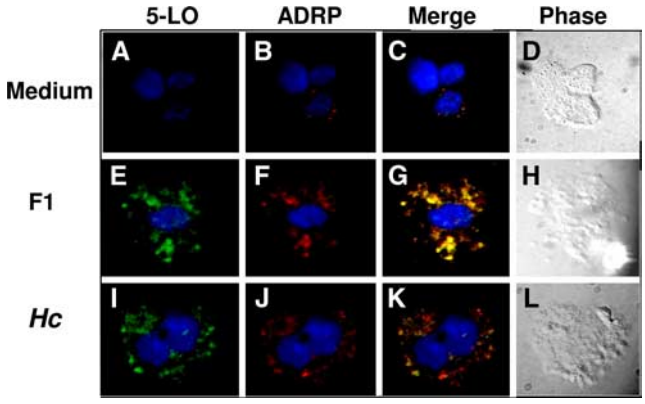

FIGURE 7. Intracellular localization of 5-LO and ADRP in alveolar adherent cells after in vitro *Hc* infection or F1 stimulation. BALF adherent macrophages were recovered from naive C57BL/6 mice and either infected with live *Hc* (3:1; yeast:cell), stimulated with F1 (100 μg/ml), or neither infected nor stimulated. After 8 h at 37°C, macrophages were labeled for ADRP-associated LBs (*B*, *F*, *J*) and 5-LO (*A*, *E*, *I*). Merged images (*C*, *G*, *K*) show colocalization of 5-LO in ADRP-associated LBs. The nuclei of the cells were staining with DAPI. The data are representative results from three separate experiments. The cells were observed using differential interference contrast-phase photomicrographs (*D*, *H*, and *L*). Original magnification: ×1000.

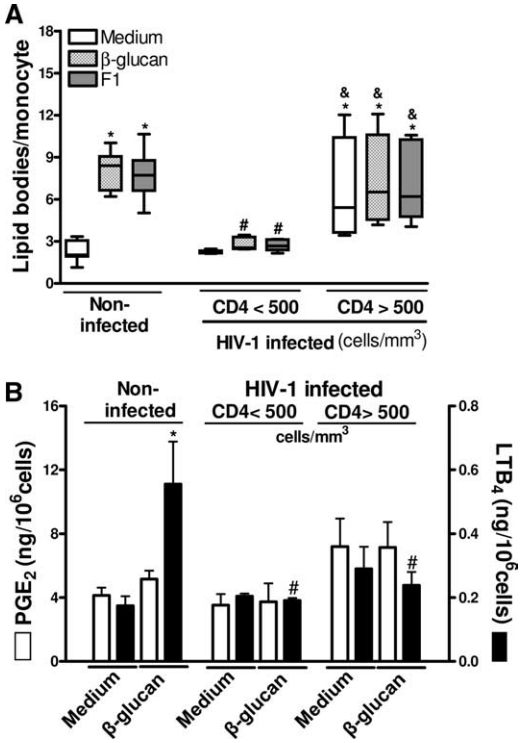

FIGURE 8. HIV-1-infected patient with a low number of $CD4^+$ T lymphocytes present an impaired capacity to produce LBs and to generate eicosanoids in response to F1 and β-glucan. Adherent mononuclear cells were stimulated in vitro with β-glucan (10 μg/ml), F1 (100 μg/ml), or medium for 8 h for LB enumeration (*A*) or stimulated for 15 min with A23187 (0.5 μM) for LTB_4 and PGE_2 release (*B*). The data are expressed as means (±SEM) from 14 noninfected subjects and 14 HIV-infected subjects (seven with <500/mm^3 $CD4^+$ T lymphocytes and seven with >500/mm^3 $CD4^+$ T lymphocytes). LBs per cell were enumerated by light microscopy with a ×100 objective lens in 50 consecutively counted cells per slide, totaling 150 cells per patient. LTB_4 and PGE_2 were measured by ELISA. Symbols indicate statistically significant differences: cells from noninfected subjects incubated with medium vs cells with ß-glucan or F1 (∗); β-glucan- or F1-stimulated cells from HIV-infected subjects compared with cells from noninfected subjects (#); and HIV-infected with >500/mm^3 CD4 compared with <500/mm^3 CD4 (&).

stimulation, cytoplasmic 5-LO matched perfectly with ADRP-stained LBs. However, after *Hc* infection, colocalization was less evident, perhaps due to different times in the biogenesis of LB formation and 5-LO expression (Fig. 7*K*). As expected, leukocytes from vehicle-inoculated animals exhibited discrete staining (Fig. 7, *A–C*). Nuclei of cells were observed after DAPI staining.

LB formation and eicosanoid generation by PBMC from HIV-infected patients are deficient

We investigated the ability of PBMC from HIV-infected patients to form LBs after β-glucan or F1 stimulation in vitro. The hematology and viral characteristics of these HIV-1-infected subjects are shown in Table I and the results are shown in Fig. 8. Notably, PBMC from HIV-1-infected patients with <500 $CD4^+$ T cells/mm^3 were unable to elicit either LB formation induced by F1 or β-glucan or eicosanoid generation when compared with PBMC from healthy donors (Fig. 8, *A* and *B*). Surprisingly, PBMC from patients with elevated $CD4^+$ T cell counts showed higher numbers of LBs in comparison with PBMC from patients with low $CD4^+$ T cell counts or control subjects. In addition, neither F1 nor β-glucan increased LB formation in these cells (Fig. 8*A*). The inability to form LBs was correlated to low production of LTB_4 (Fig. 8*B*).

Discussion

We have previously demonstrated that high levels of LTB_4 and LTC_4 are produced in the lung during pulmonary histoplasmosis. The pharmacological inhibition of these mediators was associated with the down-regulation of Th1 and the up-regulation of Th2 cytokine production, which results in impairments of antimicrobial mechanisms allowing fungus persistency, proliferation, and dissemination (13).

It is accepted that lipid mediators such as LTs are membrane-derived, mainly from the nuclear envelope (15). However, a mechanism that might contribute to the enhanced eicosanoid production is the compartmentalization of an arachidonate substrate with cytosolic phospholipase A_2 and eicosanoid-forming enzymes in lipid-rich cytoplasmic inclusions named lipid bodies or LBs (21). These organelles, although few in number, are normal constituents of leukocytes and are increased in either size or number in inflammatory cells (21). In this study, we showed that *Hc* infection and stimulation of leukocytes with *Hc* cell wall fractions induce increased numbers of LBs, which correlated with the enhancement of eicosanoid generation. In addition, the mechanisms involved in *Hc*-induced LB formation were investigated. Finally, because patients with AIDS present reduced 5-LO metabolism (5), we hypothesize that HIV-1-infected cells may present an absence of LB formation in association with reduced LTs generation during *Hc* infection.

We established that *Hc* infection induces a dramatic increase in LB numbers in BALF cells recruited 2 days after infection. The increase in LB numbers in BALF cells correlated with the high capacity of these cells to produce LTB_4 and PGE_2, strongly suggesting that LBs are sites of eicosanoid formation in this infection. LB induction in BALF macrophages can also be elicited in vitro by live and dead fungus, suggesting that cell wall compounds participate in the induction of lipid mediator synthesis. Because F1 (high β-glucan levels and low chitin levels) is the main component of the *Hc* yeast cell wall involved in leukocyte recruitment (12), we investigated whether β-glucan-enriched F1 was involved in LB biogenesis. We observed that F1 was capable of inducing a dose- and time-dependent increase in LB formation in AM in vitro and triggered LB formation in peritoneal cells in vivo. In vitro, LB formation occurs very rapidly and with low concentrations of F1, peaking with 100 μg/ml F1. Our results also suggested that β-glucan is the main component involved in LB formation and eicosanoid production. Indeed, when F1 was treated with chitinase (F1β-gluc), resulting in β-glucan of a higher purity, its capacity to induce LBs was increased by 10-fold; chitinase-treated F1 induced the same number of LBs as 10 μg/ml pure commercial β-glucan. These results suggest a great specify of interaction between β-glucan and their receptors. However, the contribution of others fungal components, such as F2, could not be ruled out.

Mechanisms involved in *Hc*-, F1-, and β-glucan-induced LB formation were investigated. The polysaccharide-rich cell wall is a major source of pathogen-associated molecular patterns and comprises the initial structure sampled by cells of the immune system in fungal infection. Identified pattern-recognition receptors (PRRs) important for the detection of fungal surface components include TLR2 and TLR4, the β_2-integrin family members such as the MAC-1/CR3 integrin (CD11b/CD18), lactosylceramide, and C-type lectins such as Dectin-1 (30–34); all of these PRRs appear to detect fungal-associated carbohydrates. To clarify the role of PRRs in LB formation and LT production in *Hc* infection, we investigated the participation of β_2-integrin, Dectin-1, and TLRs. When compared with BALF cells recovered from *Hc*-infected WT

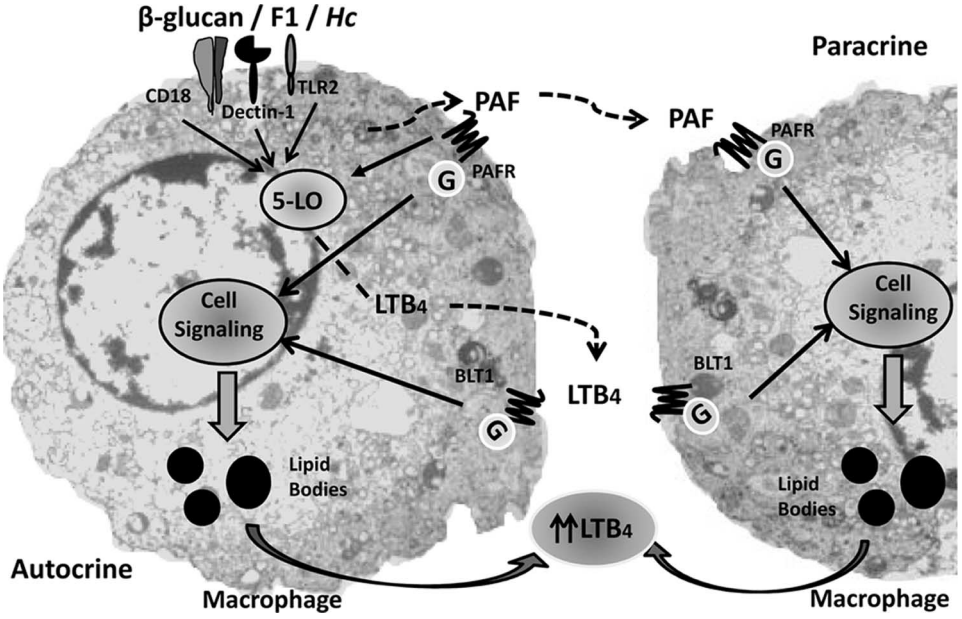

FIGURE 9. Schematic diagram showing LB formation induced by β-glucan, F1, and *Hc* in leukocytes. LB formation is dependent on PAF and 5-LO products released after β-glucan induction, present in F1 and *Hc*, which interact with CD18, TLR2, and Dectin-1 molecules resulting in PAF and LTB_4 release. PAF and LTB_4 interact with PAFR and BLT1 in an autocrine and paracrine manner, respectively, to amplify LBs formation.

C57BL/6 mice, BALF cells from $CD18^{low}$ mice produced significantly fewer numbers of LBs and LTB_4, with no alteration in PGE_2 production (Fig. 4). Moreover, $CD18^{low}$ macrophages stimulated in vitro with *Hc* or purified β-glucan or F1 had an impaired capacity to form LBs, suggesting a requisite role for β_2-integrin in these phenomena. Our results identifying β-glucan as the *Hc* cell wall compound that induces macrophage activation via β_2-integrin and lipid mediator production are relevant to understanding host defense in pulmonary mycosis. A role for β_2-integrin in eosinophil recognition of fungus and β-glucan was recently described (35), demonstrating that other cells may collaborate with macrophages in LT production and effective immune response against fungi, and perhaps in the induction of LB formation.

However, β_2-integrin does not appear to be the unique receptor involved in *Hc* and β-glucan cell interaction for the induction of LB formation, because cells treated with an anti-Dectin-1 mAb or cells obtained from $TLR2^{-/-}$ mice presented less LB formation after stimulation with *Hc* or commercial β-glucan or F1. Our data are in agreement with other studies demonstrating that Dectin-1 recognizes particulate and soluble β-glucan (27) for a number of fungal species (36). Our results demonstrating that β_2-integrin, TLR2, and Dectin-1 are required for *Hc*, F1,and β-glucan recognition and LB formation suggest that a complex interaction occurs between fungi and cells from the immune system and that this interaction appears to be necessary for signaling to take place. Others have demonstrated a similar cooperative phenomenon. Accordingly, CD11b/CD18-dependent mechanisms were shown to cooperate with TLR4 in LPS-induced LB formation (20). In contrast, F2 (α-glucan)-induced LB formation was independent of CD18 and Dectin-1 receptors, but dependent on TLR2. Additionally, phagocytosis of *Pseudallescheria boydii* is dependent on α-glucan and TLR2 (37), demonstrating a role for α-glucan in cell and pathogen interaction. Thus, we cannot rule out the participation of other cell wall fractions, such as F2 (α-glucan), in TLR2-dependent cell activation in histoplasmosis infection. The involvement of other glucan receptors in innate immunity also cannot be excluded and needs further investigation.

Similar to previous studies (18), we observed in this investigation that LB formation in response to *Hc* and F1 occurs independently of phagocytosis and is dependent on the activation of cellular receptors. Therefore, we investigated downstream mediators involved in LB biogenesis. LPS-induced LB biogenesis involves paracrine PAF production via innate immune receptor activation (TLR-4, CD14,and CD11b/CD18) (20), and PAF and PAF-like lipids are themselves able to induce LB formation (22, 23, 38, 39). Although there is no documentation regarding PAF release in histoplasmosis, we investigated the contribution of this mediator in LB formation in response to *Hc* and F1. Our data strongly supported PAF production during this mycosis and a role for the PAF receptor in *Hc*- and F1-induced LB formation in vivo and in vitro. Treatments with the compound SR-27417 (in vivo and in vitro) were able to inhibit *Hc*- and F1-mediated LB formation. Additionally, Bozza et al. (23) had pointed out the requisite role for 5-LO activity in the in vitro and in vivo induction of leukocyte LBs by PAF. In the same way, LB formation in histoplasmosis is also 5-LO dependent by a process related to LTB_4 production, suggesting an interaction between PAF and LTB_4 in this event. Furthermore, PAF and LTB_4 increase CD11b/CD18 expression in inflammatory cells (40, 41), suggesting a cooperative interaction in other events.

The functions of LBs in fungal infection were investigated. Evidence was provided that LBs are functionally active organelles, because the increased numbers of LBs in the BALF cells correlated with LTB_4 and PGE_2 production. Moreover, the decrease in LB formation in $CD18^{low}$ mice correlated with a decrease in LTB_4 generation. These results are very interesting, because we have previously demonstrated that LTs were the main mediators involved in leukocyte recruitment induced by *Hc* and F1 in vivo (12) and that LTs were essential for an effective immune response in *Hc* infection (13).

Leukotriene biosynthesis depends on a spatial/functional interaction between the 5-LO-activating protein FLAP and the terminal eicosanoid-forming enzymes (42), as well as the intracellular localization of these biosynthetic complexes (43, 44). In this context, we suggest that at least in the beginning of infection, LBs are an important source of LTs during histoplasmosis. Indeed, both *Hc* and F1 induce compartmentalization of 5-LO within newly formed LBs, as confirmed by the localization of 5-LO at the same site as that of ADRP, a protein characteristic of LBs. In F1-stimulated cells, 5-LO is localized either in LBs or in the nuclear membrane, not excluding the perinuclear pathway. The compartmentalization

of 5-LO within LBs was accompanied by priming for LTB_4 production by either *Hc*-infected or fungal cell wall fraction-stimulated cells. Those findings, taken together with recent observations that LBs are a major locale of LTB_4 synthesis during infection (26), points to a key role for LBs in LT production during histoplasmosis. Interestingly, 5-LO-derived mechanisms are involved in LBs biogenesis, and LBs are chiefly involved in the amplification of LTB_4 production during histoplasmosis, thus unveiling a positive feedback loop of leukotriene synthesis operating during histoplasmosis infection.

Infection with HIV-1 results in the development of AIDS by causing the depletion and dysfunction of $CD4^+$ T lymphocytes (45). One consequence of the depletion and dysfunction of $CD4^+$ T lymphocytes in AIDS is the decline of production of cytokines such as GM-CSF (46) and the inability of patients to elaborate 5-LO products, resulting in a marked impairment of LT synthesis (5). Moreover, chemotaxis, phagocytosis, and the killing of microorganisms by macrophages, monocytes, and neutrophils (47) are also reduced with the progression of AIDS, facilitating opportunistic infections such as that by *Hc* (48). Extending our mouse results and literature findings, we suggest that PBMC from $CD4^+$ T lymphocyte-depleted patients form only discrete amounts of LB, LTB_4, and PGE_2 following in vitro stimulation with F1 and β-glucan, due to an inability to express 5-LO and/or FLAP. As a consequence, these cells produce less LTB_4, and perhaps less PAF, for amplifying the signaling to LB formation and PAF and LTB_4 release by either an autocrine or paracrine mechanism. In contrast, in HIV patients with normal numbers of $CD4^+$ T cells, the number of LBs is larger and independent of the in vitro stimulation, and they also produce more PGE_2 (Fig. 8). We propose that in HIV-infected patients when the number of $CD4^+$ T cells is high, LBs contribute preferentially to PGE_2 instead of LTB_4 generation. This could have an adverse impact on host defense, because PGE_2 is an upmodulator of HIV growth (49) and also inhibits phagocytosis and microbial killing (50, 51). Consequently, LB organelles may have a harmful role in HIV infection; instead of being sources for LTB_4 that play an important role in host defense mechanisms against microorganisms (13, 52), they appear to be sites of PGE_2 production. The imbalance between LTB_4 and PGE_2 production could increase virus proliferation and occurrences of opportunistic infections, such as histoplasmosis. Under these circumstances, LB could be considered a target for cyclooxygenase-2 inhibition to decrease PGE_2 in HIV infection. However, more studies are needed to clarify if and how the HIV subverts eicosanoid synthesis pathways and directs them toward PGE_2 production.

In conclusion, *Hc*, through β-glucan present in the cell wall, induces rapid and persistent leukocyte LB formation by a CD18-, Dectin-1- and TLR2-dependent mechanism with the generation of downstream mediators such as LTB_4 and PAF (see schematic model in Fig. 9). Leukocyte LBs formed during *Hc* infection function as inducible intracellular domains involved in eicosanoid-forming enzyme compartmentalization with a direct impact on the capacity of leukocytes to generate increased amounts of eicosanoids. The absence of LBs in HIV-1 infected cells stimulated with β-glucan demonstrates the low level of LTB_4 generation. Taken together, our observations suggest that LBs carry out specific functions in eicosanoid generation in the course of fungal infection. These functions go beyond simply being lipid storage organelles and may have implications for the host immune defense during histoplasmosis.

Acknowledgments

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Disclosures

The authors have no financial conflict of interest.

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