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Integrin $\alpha_X\beta_2$ Is a Leukocyte Receptor for Candida albicans and Is Essential for Protection against Fungal Infections

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The opportunistic fungus Candida albicans is one of the leading causes of infections in immunocompromised patients, and innate immunity provides a principal mechanism for protection from the pathogen. In the present work, the role of integrin $\alpha_X\beta_2$ in the pathogenesis of fungal infection was assessed. Both purified $\alpha_X\beta_2$ and $\alpha_X\beta_2$-expressing human epithelial kidney 293 cells recognized and bound to the fungal hyphae of SC5314 strain of C. albicans but not to the yeast form or to hyphae of a strain deficient in the fungal mannoprotein, Pra1. The binding of the integrin to the fungus was inhibited by $\beta$-glucans but not by mannans, implicating a lectin-like activity in recognition but distinct in specificity from that of $\alpha_\text{M}\beta_2$. Mice deficient in $\alpha_\text{M}\beta_2$ were more prone to systemic infection with the LD$_{50}$ fungal inoculum decreasing 3-fold in $\alpha_\text{M}\beta_2$-deficient mice compared with wild-type mice. After challenging i.v. with $1.5 \times 10^4$ cells/g, 60% of control C57BL/6 mice died within 14 d compared with 100% mortality of $\alpha_\text{M}\beta_2$-deficient mice within 9 d. Organs taken from $\alpha_\text{M}\beta_2$-deficient mice 16 h postinfection revealed a 10-fold increase in fungal invasion into the brain and a 2-fold increase into the liver. These data indicate that $\alpha_\text{M}\beta_2$ is important for protection against systemic C. albicans infections and macrophage subsets in the liver, Kupffer cells, and in the brain, microglial cells use $\alpha_\text{M}\beta_2$ to control fungal invasion. The Journal of Immunology, 2012, 189: 2468–2477.

Candida albicans is a common opportunistic fungal pathogen. It is a dimorphic fungus existing as rounded yeast cells or as filamentous forms (1, 2). Although the yeast form can colonize mucosal membranes, it is thought that the filamentous form provides some protection to the microorganism against host defense systems, and the ability of C. albicans to rapidly and reversibly switch between yeast and filamentous morphologies is crucial to its pathogenicity (3–6). In recent years, Candida infections ranked as the fourth most common cause of nosocomial infections with immunocompromised patients being particularly susceptible (7, 8). Bloodstream fungal infections have an extremely high (30–70%, by different estimations) morbidity and mortality (8–11).

The innate immune system provides the principal protection against Candida infections. Polymorphonuclear leukocytes have been shown to be the primary components of the cellular immune defenses against Candida (12–14), and a protective role for macrophages in disseminated candidiasis has also been suggested (13, 15, 16). The most prominent receptors on leukocytes used in fungal or microbial recognition are integrins of the $\beta_2$ subfamily (17, 18). This subfamily of leukocyte receptors is composed of four members that share a common $\beta_2$ subunit that associates noncovalently with one of four distinct but structurally homologous $\alpha$ subunits to form $\alpha\beta_2$ (Mac-1, CD11b/CD18, and CR3), $\alpha_\text{M}\beta_2$ (LFA-1 and CD11a/CD18), $\alpha_\text{X}\beta_2$ (p150,95 CD11c/CD18 and CR4), and $\alpha_\text{E}\beta_2$ (CD11d/CD18) (19–23). These cell surface receptors are expressed on monocytes, granulocytes, macrophages, and NK cells and have been implicated in diverse protective responses mediated by these cells, including phagocytosis, cell-mediated killing, chemotaxis, and cellular activation. Specifically, the $\beta_2$ integrins mediate migration of leukocytes to sites of infection and adhesion to microorganisms with subsequent phagocytosis or killing of many pathogens (12, 17, 24). Patients with leukocyte adhesion deficiency-1 (LAD-1), a rare hereditary disease that is characterized by low expression (mild LAD-1) to complete absence (severe) of all four of the $\beta_2$ integrins because of mutations in the ITGB2 ($\beta_2$) gene (25, 26), are highly susceptible to a wide range of bacterial and fungal infections (27, 28) and the increased sensitivity of such patients to C. albicans infections has been discussed (29). Although other leukocyte pattern recognition receptors, which recognize fungal $\beta$-glucans (Dectin-1 and TLR2 (30, 31) and mannan-specific TLR4 (32)), also participate in fungal recognition and apparently are essential in leukocyte activation and notably in activation of $\beta_2$ integrins (33, 34), they do not directly facilitate leukocyte migration, adhesion, or phagocytosis.

Of the $\beta_2$ integrins, $\alpha_\text{M}\beta_2$ has been specifically implicated in the recognition of C. albicans. Polymorphonuclear leukocytes and NK cells use $\alpha_\text{M}\beta_2$ to adhere only to the filamentous form but not to the yeast form of C. albicans (35, 36). C. albicans pH-regulated Ag 1 (Pra1) (37), also known as fibrinogen binding protein 1 (38) or C. albicans 58-kDa mannoprotein (39), was identified as the major ligand of $\alpha_\text{M}\beta_2$ among C. albicans proteins (40). Pra1p is a mannoprotein (1, 41) and is expressed on the surface of the hyphae but not on the yeast form of C. albicans (3, 41). Express-
sion of Pralp is strongly pH dependent and is also regulated by nutrition and certain other fungal genes (37, 41, 42). Disruption of the PRA1 gene protects the fungus against leukocyte killing in vitro and in vivo, impedes the innate immune response to infection, and increases overall fungal virulence and organ invasion in vivo (29, 43).

Although mutations in αXβ2 subunits have been previously described (44), it appears that the clinical manifestations of selective loss of αXβ2 are less severe than when all four β2-integrins are absent, which suggests that αXβ2 may share part of its surveillance functions with other β2 integrins, most likely with the integrin αXβ2 (19, 45). Integrin αXβ2 is present on the surface of all leukocyte subsets that express αXβ2, with the exception of dendritic cells, which have CD11c (αX) as a major surface marker. These integrins are ~70% identical and also share a number of ligands, most notably fibrinogen (46), ICAM-1 (47), and iC3b, a component of the complement system (48). However, the functions of αXβ2 are less well studied, and its functions in innate immunity are still unclear. It was shown that αXβ2 is involved in macrophage-mediated phagocytosis of Mycobacterium tuberculosis (49) and Mycobacterium leprae (50) and may play a role in the development of gastric ulcers in chronic Helicobacter pylori infection (51).

The present study was undertaken to determine the role and significance of the αXβ2 in C. albicans pathogenicity and the effects of its elimination on host defense in vivo, using αXβ2-deficient mice in a model of systemic murine candidiasis.

Materials and Methods

C. albicans strains

C. albicans strain SC5314 was used in most in vitro and in vivo experiments. In some experiments, the Pral-depleted strain CAMB45-18 (pral1::hisGpral1::hisG iso1-ura3/ΔR01-URA3) was also used. This strain was derived from the strain CAMB45 by reversion of the iso1-ura3 deletion as previously described (52) and was characterized by us previously (29). All strains were routinely maintained on Difco Sabouraud Dextrose Agar (SDA) plates (BD Biosciences, Sparks, MD).

Animals

αX-Knockout (KO) mice (ΔαXβ2) were provided by Dr. C. M. Ballantyne (Baylor College of Medicine, Houston, TX). This mouse line was generated in parallel with other β2-KO lines (53). All these β2-KO murine lines have been used in a number of studies in comparison with the wild type (C57BL/6). They have been used in a number of studies in comparison with the wild type (C57BL/6). All experiments involving human blood cells were performed in accord with protocols and policies approved by the Institutional Review Board at Cleveland Clinic and with the Helsinki Declaration of 1975 as revised in 2000.

Abs and inhibitors

mAbs used in this study were as follows: 44a (anti-human αX-I-domain, IgG1), OKM1 (anti-human αX lectin domain, IgG2b), IB4 (anti-human β2, IgG2a). The hybridoma cell lines producing these mAbs were obtained from the American Type Culture Collection and adapted to growing in serum-free media in CELLLine Bioreactor Flasks (Integra Biosciences, Hudson, NH) in the Cleveland Clinic Hybridoma Core. The mAbs were purified from conditioned media using recombiant protein G columns (Life Technologies).

The mAb clone 3.9 (anti-human αX, IgG1) and clone N418 (anti-mouse αX) were purchased from San Diogo, CA, and mAb clone YW62.3 (anti-mouse CD45) was obtained from Serotec (Raleigh, NC).

Baker yeast β-glucan, mannan, and echistatin (60, 61) were purchased from Sigma-Aldrich (St. Louis, MO). The recombiant hoknwerk neu- troph inhibitory factor (NIF) was prepared as described previously (58).

Tissue section preparation and assaying

Brains and livers from experimental mice were snap-frozen in OCT. Brain sections (6 μm thick) were processed for immunohistochemical staining with the following Abs: hamster anti-mouse CD11c (BioLegend) followed by biotinylated anti-hamster Ab and streptavidin Alexa Fluor 488. CD45 staining was revealed using rat anti-mouse CD45 mAb (BD Biosciences) and rabbit–anti-rat Ab conjugated to Alexa Fluor 488. The slides were mounted using Vectashield mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). To visualize the extent of the fungal cell invasion and proliferation, the brain and liver sections were stained with periodic acid–Schiff (PAS) reagent. The images were captured using a Leica DMR microscope equipped with ×100/1.4 and ×200/0.5 NA objective lenses (Leica Microsystems, Wetzlar, Germany) and photographed with a Qimaging Retiga ExiFas camera (Qimaging, Burnaby, British Columbia, Canada) using ImagePro 5.1 software (Media Cybernetics, Silver Spring, MD). The images were processed with Adobe Photoshop 7.0 software (Adobe Systems, San Jose, CA).

C. albicans staining with soluble αXβ2

Recombinant αXβ2 was isolated from HEK293/αXβ2 cells with the method previously used by us to purify αXβ2 (40, 59). Briefly, 10 g cells was harvested, washed, and lysed with 1% Triton X-100 in TBS containing protease inhibitor mixture for mammalian cells (Sigma-Aldrich). The cell lysate was clarified by centrifugation, diluted with TBS containing CaCl2 and MgCl2, and loaded onto a column of immobilized IB4 (anti-β2). To prepare the immunoadsorbent, purified IB4 mAbs were coupled to cyanogen bromide-activated Sepharose 4B (GE Biosciences, Piscataway, NJ) following the manufacturer’s protocol to final concentrations of 2.2–2.8 mg immobilized proteins per 1 ml swollen gel. After washing with TBS containing 10 mM octyl-β-D-glucopyranoside (OG; Calbiochem, San Diego, CA) and CaCl2/MgCl2, bound protein was eluted with three column volumes of 20 mM sodium acetate buffer (pH 4.2), containing OG and Ca2+ and Mg2+. Immediately after elution, 100 μl 1 M HEPES-NaOH (pH 6) was added to each 1 ml of the column eluate to neutralize the acidic pH. Protein fractions were pooled and dialyzed against HEPES-NaCl, OG, and CaCl2. The proteins were biotinylated using Sulfo-NHS-LC-Biotin (Pierce), according to the manufacturer’s protocol. To visualize αXβ2–C. albicans interaction, C. albicans strain SC5314 was allowed to germinate in RPMI 1640 medium for 1 h and then purified. Biotinylated αXβ2 was added to obtain a 1 μg/ml concentration, and the samples were incubated for 1 h at 37˚C. After incubation, the fungi were washed with Dulbecco’s PBS (D-PBS) and incubated with FITC–streptavidin conjugate for 30 min at room temperature. Subsequently, the fungi were again washed with D-PBS. Blankophor, the β-glucan, and chitin–specific dye were added, and the mixture was incubated for an additional 30 min (62). Finally, the fungi were washed with D-PBS and analyzed by fluorescence microscopy (Leica Microsystems) at a magnification of ×800.
Cell adhesion assays

Cell adhesion assays were performed as described previously (40, 63). Briefly, to determine cell adhesion to fungal hyphae, 48-well Costar tissue culture plates (Corning, Corning, NY) were precoated with polyvinylpyrrolidone (PVP; Sigma-Aldrich) and washed with HBSS, and aliquots of 1×10^7 C. albicans yeast and resident peritoneal neutrophils at 37°C to germinate. For adhesion to C. albicans yeast, the fungi were incubated in YNB broth to prevent germination. After incubation, the supernatant was removed and adherent fungi were carefully washed with HBSS. A total of 10^5 PMA-activated peripheral blood human monocytes or HEK293/αXβ2 cells were added in HBSS/HPEPS and assay plates were incubated at 37°C for 1 h. Control wells were coated with PVP only. Each experimental point was in triplicate. Subsequently, plates were washed, and the number of adherent cells in each well was quantified using the CyQUANT Cell Proliferation Assay Kit (Life Technologies) as described previously (40, 63). For inhibition assays, before addition to the plate wells, the HEK293/αXβ2 cells or isolated monocytes were preincubated with 10–20 μg/ml selected Abs, 1 mM β-glucan, 1 mM mannan, or 5 μM echistatin for 10 min at room temperature. From cell adhesion and migration (see below) are presented as percentage (mean ± SE) of total cells (to which was assigned the value of 100%) and represent the results of three independent experiments.

Cell migration assays

Human peripheral blood monocytes and HEK293/αXβ2 cell migration assays were performed in serum-free RPMI 1640 (monocytes) or DMEM/F-12 (HEK293/αXβ2) medium (Life Technologies) using modified Boyden chambers (Costar Transwell inserts in a 24-well plate format; Corning) with 8-μm polycarbonate filters (Corning). The upper chamber, which contained final volumes of 200 μl cell suspension, with 10^6 cells, present on the undersurface of the membrane as well as in the lower chamber, were quantified using the CyQUANT Cell Proliferation Kit as described previously (40, 63–65). The lower chambers contained 600 μl media with 10^6 C. albicans yeast, which were germinated overnight prior to beginning the analyses. The upper chambers contained final volumes of 200 μl HEK293/αXβ2 cell suspensions. The assays were initiated by addition of 50 μl cell suspension (10^6 cells/ml) to 100 μl media in the upper chamber, and the plates were placed in a humidified incubator at 37°C and 5% CO2 for 8 h. For inhibition experiments, selected Abs, NIF, and glcyans were added simultaneously with the cells to the upper chamber. After migration, nonmigrated cells were removed from upper chamber using cotton swabs. The migrated cells, present on the undersurface of the membrane as well as in the lower chamber, were quantified using the CyQUANT Cell Proliferation Kit as described above and previously (63, 64).

Killing (phagocytosis) assay

A total of 10^5 C. albicans SC5314 strain cells in 0.25 ml high glucose RPMI 1640 medium containing 0.1 M HPEPS (pH 7.8) were allowed to germinate in plastic tubes at 37°C for 1 h with slow agitation. The fungal cells were collected by centrifugation, washed twice with D-PBS, and suspended in 0.25 ml HBSS/HPEPS (pH 7.4), and mixed with 3 × 10^3 (1.3 ratio), 3 × 10^4 (1:3 ratio), or 1 × 10^5 (1:11 ratio) HEK293/αXβ2 or monocytes in 100 μl media in the upper chambers, and the plates were placed in a humidified incubator at 37°C and 5% CO2 for 8 h. For inhibition experiments, selected Abs, NIF, and glcyans were added simultaneously with the cells to the upper chamber. After migration, nonmigrated cells were removed from upper chamber using cotton swabs. The migrated cells, present on the undersurface of the membrane as well as in the lower chamber, were quantified using the CyQUANT Cell Proliferation Kit as described above and previously (63, 64).

Results

Integrin αXβ2 is required to control C. albicans infection

As a first step to assess the biological significance of αXβ2 in the context of the total host–pathogen relationship, transgenic mice that lack αX (ΔαX mice) were used in a murine model of disseminated candidiasis. In this assay, mice of both WT (C57BL/6 mice) and ΔαX lines were challenged with 10^5 or 3 × 10^5 C. albicans inocula via tail vein injection. All WT mice inoculated with 10^5 (0.5 × 10^−4/g) C. albicans survived 14 d (336 h). αXβ2 elimination dramatically decreased mouse survival; during these same 336 h, 50% of the ΔαX-mice reached the predetermined end point (see Materials and Methods for a complete list of “mortality” criteria) with a median survival time 264 h (Fig. 1A, left panel). A similar level of mortality occurred in WT mice only after introduction of a 3-fold higher inoculum (Fig. 1B). After challenge with the 3 × 10^5 C. albicans cells (~1.5 × 10^−4/g) inoculum, ~50% of WT mice reached the end point within 12 d with a median survival time at 252 ± 12 h. In contrast, all ΔαX mice reached the end point with this inoculum within the first 9 d with a median survival time of 112 ± 8 h (p < 0.01; log-rank test) (Fig. 1A, right panel).

Elimination of αXβ2 decreases resistance of brain and liver to C. albicans invasion

The substantial increase in susceptibility of the ΔαX mice to the Candida infection indicates that αXβ2 plays a significant role in antifungal protection and innate immunity. This interpretation was further corroborated by pathological examination of infected mice. To determine the impact of αXβ2 deletion on the rate of fungal colonization, WT and the ΔαX mice were challenged i.v. with 10^5 C. albicans, and selected organs (brain, kidney, lung, heart, spleen, and liver) were recovered 16 and 40 h postinfection and at day 14 from mice that survived. Tissue targeting and invasion, fungal dissemination, and organ fungal burden were assessed in recovered organs. Consistent with previous studies (69), high fungal burden was present in the kidneys at all times postinfection and was similar at 16 and 40 h (p = 0.96; Student t test) in both mouse strains (Fig. 2A, 2B). There was no significant difference (p > 0.05) in fungal burdens in the spleen, heart, and
lungs at both early time points; but at day 14, differences in fungal burden in the surviving WT and ΔακX mice become evident (p < 0.05). At the day 14 survival point, fungal burden in the kidney was significantly elevated (2.8-fold difference; p < 0.01) in ΔακX mice (2 × 10^5 ± 3.2 × 10^4 CFU/g) compared with WT mice (7.7 × 10^3 ± 1.2 × 10^3) (Fig. 2C). In contrast, fungal burdens in brains and livers recovered from ΔακX mice were substantially elevated compared with the corresponding WT organs: at 16 h, ΔακX brain had 2 × 10^5 ± 2.4 × 10^3 CFU/g tissue, whereas WT brains had 6.1 × 10^4 ± 7.7 × 10^4 CFU/g (10-fold raise; p < 0.01); ΔακX liver had 7.6 × 10^3 ± 1.3 × 10^3 CFU/g, 2-fold rise (p < 0.05), whereas WT liver had a fungal burden of 3.5 × 10^3 ± 360 CFU/g (Fig. 2A). These differences increased over time: at 40 h, 2.9 × 10^5 ± 2.3 × 10^4 CFU/g ΔακX brain compared with 700 ± 120 CFU/g WT brain (40-fold rise; p < 0.005) and 3 × 10^5 ± 220 CFU/g ΔακX liver compared with 480 ± 110 CFU/g WT liver (6-fold rise; p < 0.01) (Fig. 2B). These differences were sustained at day 14 in all surviving mice: 6400 ± 1820 versus 200 ± 160 CFU/g (32-fold; p < 0.01) and 3100 ± 1200 versus 700 ± 400 CFU/g (4-fold; p < 0.01) for brain and liver of ΔακX and WT mice, respectively (Fig. 2C).

Residential macrophages require ακβ2 to control C. albicans invasion in vivo

The results of fungal burden studies were further confirmed in histological sections of tissues from the infected organs. In the sections of WT brains obtained at 40 h postinfection, staining with periodic acid–Schiff reagent revealed only several scattered fungal hyphae (Fig. 3A, left panel). In contrast, in the brains of ΔακX mice 40 h postinfection, C. albicans formed a visible network of numerous fungal hyphae (Fig. 3A, right panel). After 14 d of infection, in the kidney sections of WT mice, C. albicans formed single scattered colonies (Fig. 3B, left panel), whereas in kidney sections of ΔακX mice, the fungal colonies were numerous and showed evidence of extensive organ colonization (arrows in Fig. 3B, right panel).

To ensure that ακβ2 deletion affects only leukocytes, the sections of brains from WT (Fig. 3C) and ΔακX (Fig. 3D) mice at 40 h of infection were immunostained with Abs against the common hematopoietic cell surface marker CD45 (anti-Ly5, labeled with Alexa Fluor 488, green fluorescence), anti-CD11c (anti-ακ, labeled with Alexa Fluor 568, red fluorescence), and DAPI (to visualize nuclei, blue fluorescence). The fluorescence overlays demonstrate that only hematopoietic cells (leukocytes) are CD11c^+ within this organ. The images also show that CD45^+CD11c^+ cells in the brain of the WT mice group form filamentous structures most likely along fungal hyphae. In contrast, CD45^+CD11c^+ cells in the brains of the ΔακX mice do not organize but instead remained dispersed (Fig. 3C, 3D). These results indicate that subsets of brain residential leukocytes may use ακβ2 for localization to the fungus.

Activated monocytes use both ακβ2 and ακβ2 for fungal recognition

Previous studies demonstrated that NK lymphocytes (35, 61) and neutrophils (40) use integrin ακβ2 but not ακβ2 for C. albicans recognition. With our data on fungal burden suggesting that ακβ2 deficiency affected residential tissue macrophages, the microglia in a brain, and Kupffer cells a in liver, we chose monocytes as representative primary cell to assess ακβ2 involvement in leukocyte adhesion to C. albicans. Human peripheral blood monocytes were isolated, stimulated with PMA to activate their integ-
To access the specificity of αβ2 interaction, a HEK293 cell line stably expressing the integrin was developed. Purified recombinant αβ2 recognizes the hyphae of WT C. albicans but not Prαl-deficient fungi.

To access the specificity of αβ2–C. albicans interaction, a HEK293 cell line stably expressing the integrin was developed.
Full-length cDNAs of human αX and β2 were cotransfected into HEK293, and positive cells were sorted and subcloned. The established cell line (HEK293/αXβ2 cells) then was examined by FACS using a panel of Abs: anti-human αX (mAb 3.9), anti-human β2 (IB4), anti-human αX I-domain (44a), anti-human αX lectin domain (OKM1), and control irrelevant mAb W6/32 (anti-human MHC-II). As expected, αX and β2 were highly expressed on the HEK293/αXβ2 cell surface (Fig. 5A). Although αX is highly homologous to αM, the anti-αX I-domain mAb 44a, which block binding of most ligands to αXβ2, did not recognize the HEK293/αXβ2 cells. Surprisingly, the anti-αX lectin domain mAb OKM1, which inhibits the binding of polysaccharides such as β-glucans and mannans to αXβ2 (70), also showed weak reactivity with αXβ2 (Fig. 5A) but not with mock-transfected HEK293 cells (data not shown). Thus, the αX subunit may contain structures similar to the αX lectin domain and therefore may recognize fungal polysaccharides and serve as a pattern recognition receptor.

Both αXβ2 and αXβ2 integrins have many ligands in common. Because the C. albicans hyphae surface protein Pral was identified as a major C. albicans ligand for αXβ2 (40), we considered whether this protein might also serve as a ligand for αXβ2. We tested HEK293/αXβ2 cells as a source of αXβ2 protein and the ability of purified αXβ2 to recognize two C. albicans strains, SC5314 (WT) and Pral-1-nul mutant (CAMB5-18). The yeast of both fungal strains were allowed to germinate overnight for maximal Pral expression in the WT strain and the fungal cell wall chitin and glucans were stained with blue fluorescent dye Blanophore dye (blue fluorescence) and photographed using fluorescence microscopy. Scale bars (B, C), 5 μm.

Upon expression of αXβ2 HEK293 cells acquire ability to recognize, bind, and phagocytose C. albicans

In the next set of experiments, the ability of αXβ2 to support HEK293 cell migration to C. albicans and adhesion to the fungi with subsequent phagocytosis was explored. HEK293 cells, expressing either αXβ2 or mock transfected, were added to plates with germinated C. albicans of SC5314 or CAMB5-18 strains. In some cases, plates with nongerminated fungi were also tested. After 30 min, unbound cells were washed away, and adherent cells were quantified using the CyQUANT fluorescent dye. In the absence of inhibitors, 58 ± 5% of αXβ2 cells adhered to the hyphal form of SC5314 strain, whereas they failed to adhere to the yeast form of this fungal strain. The control mock-transfected HEK293 cells did not adhere to any form of C. albicans SC5314 strain (Fig. 6A), suggesting that the adhesion is αXβ2 dependent. This conclusion was confirmed using a panel of αXβ2 and αXβ2 inhibitors. The adhesion of HEK293/αXβ2 was completely inhibited with anti-αX (3.9) and anti-β2 (IB4) mAbs, whereas the anti-αX lectin domain mAb OKM1 inhibited 80 ± 10% of the cell adhesion. In contrast, the anti-αX I-domain blocking mAbs 44a and NIF, a specific inhibitor of ligand binding to αXβ2 (58, 71) (NIF) as well as control W6/32 anti–MHC-II mAb, were ineffective. Soluble β-glucans at a concentration of 1 μg/ml inhibited adhesion of the HEK293/αXβ2 cells by 50 ± 5%. Surprisingly, baker yeast mannans, which blocks HEK293/αXβ2 adhesion to C. albicans (36), did not block adhesion of HEK293/αXβ2 cells (Fig. 6A). In control experiments, neither β-glucan nor mannans was able to inhibit αXβ2-supported adhesion to another αXβ2 ligand—the fibrinogen peptide P2C, a nonglycosylated ligand that is also recognized by αXβ2 (59, 72) (results not shown). Both cell lines were not able to adhere to germinated Pral-1-deficient C. albicans strain CAMB5-18 (40) (Fig. 6A), again indicating that C. albicans recognition by αXβ2 is Pral dependent.

Next, we tested migration of αXβ2 cells to C. albicans conditioned medium, a source of soluble Pral (40), in the presence or absence of integrin inhibitors. After 8 h, in the absence of inhibitors, 17 ± 4% αXβ2 cells migrated to fungal supernatant. The anti-β2 mAb IB4, at 20 μg/ml, reduced cell migration to fungal supernatant to 4 ± 1%. The same effect was observed in the presence of anti-αX mAb 3.9, which also inhibited migration to C. albicans supernatant. As a control, we also measured migration of the cells to vitronectin, which is mediated primarily by endogenous αX integrins on these cells. Anti-αX mAb 272-176 inhibited migration of the cells to vitronectin to 4.4 ± 3.5% but had no effect on migration of the cells to the fungal supernatant. Echistatin, a snake venom disintegrin that inhibits ligand recognition by

FIGURE 5. (A) Characterization of HEK293/αXβ2 cells by reactivity mAbs; (B, C) binding of purified αXβ2 to germinated C. albicans. (A) Characterization of HEK293/αXβ2 cells by FACS. A panel of Abs was used to examine the expression of αXβ2 Ags: anti-human αX (mAb 3.9), anti-human β2 (IB4), anti-human αX I-domain (44a), anti-human αX lectin domain (OKM1), and negative control irrelevant mAb W6/32 (anti-human MHC-II). Germinated C. albicans of WT strain SC5314 (B) or Pral-1-depleted strain CAMB5-18 (C) labeled with Blanophore dye (blue fluorescence) was stained with FITC-labeled purified αXβ2 (green fluorescence) and photographed using fluorescence microscopy. Scale bars (B, C), 5 μm.
most integrins, including the β₂ and αₓ integrins (61), completely reduced cell migration to both Candida supernatant and vitronectin (Fig. 6B). Taken together, these data indicate that migration of αₓβ₂ cells to C. albicans proteins is integrin dependent, and αₓβ₂ is implicated in this response of the HEK293/αₓβ₂ cells. Notably, β-glucan and anti-α₂ lectin domain mAb OKM1, which inhibited adhesion of αₓβ₂ cells to C. albicans, did not affect migration of these cells to the fungal extracellular proteins (Fig. 6B), suggesting that the lectin specificity of αₓβ₂ is involved in adhesion but not migration to C. albicans.

The ability of αₓβ₂ to promote antifungal activity of HEK293 cells was also tested. Because fibroblasts such as HEK293 possess weak endogenous antifungal activity and are able to internalize pathogens by passive endocytosis (73, 74), we wished to distinguish passive nonspecific endocytosis from specific phagocytosis, and various ratios of fungi/cells were tested in killing assays. Germinated C. albicans cells of the SC5314 strain were coincubated with αₓβ₂ or mock-transfected HEK293 at fungi/HEK293 cell ratios of 1:3, 1:7, or 1:11 for 2 h. Aliquots were taken every 20 min, and the amounts of viable fungi remaining were quantified as CFU by plating aliquots in a series of dilutions onto agar plates. Only 58 ± 6% of the initial fungi remained viable after a 2-h incubation with the αₓβ₂ cells at the 1:3 ratio. At a 1:7 ratio, the amount of surviving fungi decreased to 42 ± 5% and fell to 28 ± 6% at a 1:11 ratio. In contrast, with mock-transfected HEK293 cells, fungal survival was 76 ± 6 and 82 ± 5% at the 1:11 and 1:7 ratios, respectively, and all fungi survived at the ratio 1:3 (Fig. 6C). Preincubation of αₓβ₂ cells with anti-α₂ blocking mAb 3.9 completely inhibited the antifungal activity of the αₓβ₂ cells to levels observed with mock-transfected HEK293 cells (p < 0.5). These results indicate that αₓβ₂ is able to promote phagocytosis of C. albicans.

Discussion
In the present work, we demonstrate the importance of integrin αₓβ₂ for protection against C. albicans systemic infection by the innate immune system. Although the ability of αₓβ₂ to recognize C3bi and thereby to assist in the elimination of opsonized particles has been described previously (48), to our knowledge, our study is the first demonstration of involvement of this integrin in direct pathogen recognition and elimination by leukocytes as well as its critical importance in the control of fungal invasion to brain and liver by certain subsets of tissue resident macrophages (see below).

Our data on the murine organ fungal burdens indicate that the αₓβ₂ elimination affects mainly the liver and brain, dramatically increasing invasion and propagation of the fungus in these organs. This effect of αₓβ₂ became evident at the earliest stages of infection; as early as 16 h after the challenge, a 2-fold difference (p < 0.05) emerged in fungal burdens in the livers of Δα₂ mice and WT mice and a 10-fold difference (p < 0.01) in the brains. At 40 h postinfection, this difference in the susceptibility of the αₓβ₂-deficient mice and WT animals reached 6-fold (p < 0.01) in the liver and >40-fold (p < 0.005) in the brain.

The integrins of the β₂ subfamily, known collectively as “leukocyte integrins,” are expressed predominantly on the surface of leukocytes (23, 75). In our experiments, immunostaining of infected brain and liver sections revealed that only the CD45+ hematopoietic cells in these tissues express αₓ [also see (76)], and thus, αₓβ₂ elimination is likely to affect leukocyte function only.

The kidney and the brain are the primary targets for C. albicans during systemic infection. The fungi invade these organs directly from the bloodstream, and invasion can start during the first minutes postinfection (67). The blood immune mechanisms (e.g., monocytes, neutrophils, NK lymphocytes, and cells of the blood-
brain barrier) provide little protection from neuroinvasion during the initial stages of systemic infection. Blood cells can clarify the bloodstream of sublethal doses of *C. albicans* only after 20 h of infection, and, in the case of near-lethal doses, fungal CFUs can be detected in the blood even after 24–30 h postinfection (67). The i.v. route for the fungal injection bypasses possible contact of the fungi with tissue macrophages. To circumvent the blood–brain barrier, *C. albicans* uses a unique mechanism of invasion: upon binding to gp96 heat shock protein and/or to N-cadherin on the surface of normally nonphagocytic brain microvascular epithelial cells, fungi stimulate their own uptake (77, 78). Therefore, in our model, the difference in organ fungal burdens of WT and ΔαX in mice appears to be due to differences in activity of the organ-resident macrophage subsets, microglial cells in brain, and Kupffer cells in liver.

Existing literature present extensive evidence that microglia play the principal role in the protection against *C. albicans* intracerebral infections. Direct proof of their crucial role was provided by the demonstration that intracerebral transfer of microglial cells provides complete protection (100% survival) against subsequent intracerebral challenge with a lethal inoculum of the fungus. After i.v. challenge with near-lethal *C. albicans* inoculums, the concentration of fungal CFUs in the brain rapidly increases and reaches maximal level at ~24 h infection. Then, the fungal burden in brain stabilizes and remains at this level until days 7–8 with a subsequent slow decline (67). This time course implies that 24 h is sufficient for microglial activation and conversion to “brain macrophages,” and the migration to the fungus to contain infection and corresponds well with our data, demonstrating that after 40 h most brain CD45−CD11c− cells in WT have migrated and assembled around the hyphal-like structures of *C. albicans*. In αXβ2-deficient mice, the CD45−CD11c− cells in the brain remained diffusely distributed, suggesting that αXβ2 is required for these cells to migrate to and recognize *C. albicans*.

Kupffer cells are the specialized phagocytic cells found on the luminal surface of hepatic sinusoids (79). These cells are of monocyteoid lineage (79, 80) and express both αXβ2 and αXβ1 integrins (81–83), and their importance for protection against *C. albicans* invasion has been demonstrated previously (84–87). The possible involvement of integrin αXβ2 in phagocytosis of *C. albicans* by microglial and Kupffer cells has been proposed (84, 88, 89). Taken together, these data suggest that αXβ2 but not αXβ1 is critical in antifungal activity of tissue-resident macrophage subsets. This conclusion is consistent with the previous report demonstrating that increased expression of αXβ2 results in enhanced phagocytosis of *M. tuberculosis* by human macrophages (49).

Integrins on the surface of nonstimulated leukocytes are expressed in inactive “closed” conformation and require activation to recognize their ligands with high affinity. During inflammation, various physiological agonists induce activation of specific integrins. Thus, αXβ2 may become activated, whereas αXβ2 remains in an inactive conformation or vice versa, and therefore, these two β2 integrins may differentially participate in leukocyte function despite both being expressed on the leukocyte surface (19). Activation of peripheral blood monocytes in vitro with PMA results in activation of all leukocyte integrins. For this reason, anti-αXβ2 mAbs block adhesion of PMA-activated monocytes only partially. The only anti-αX mAb that blocks adhesion to the fungus is directed to the αM lectin domain, and as we have shown, this mAb also cross-reacts with a previously unrecognized lectin domain within the αX subunit.

β-Glucans and mannans are important immunomodulators, and their binding by leukocytes is implemented by integrin αXβ2 (90, 91). Upon ligation with the integrin, β-glucans activate αXβ2 and stabilize it in an intermediate active conformation (92). Unlike αMβ2, where the carbohydrate binding and sugar selectivity of its αM-lectin domain are well characterized (e.g., (93–95)), there is no evidence in the literature for recognition of fungal glycans or bacterial LPS by αXβ2. Therefore, our observation that activity of αXβ2 is modulated by fungal β-glucans is a novel finding of our present work. On the basis of ~70% homology between αM and αX and that the OKM1 mAb, which blocks glycan binding to αM (70), weakly cross-reacts with αX, we anticipate certain similarities in the sugar specificity of these integrin subunits. However, αXβ2 and αMβ2 demonstrated clear distinction in carbohydrate selectivity: although αXβ2 recognizes both β-glucans and mannans, the activity of αXβ2 appears to be modulated by β-glucans but not by mannans. In our experiments, mannans, unlike β-glucans, were not able to inhibit adhesion of HEK293/αXβ2 to *C. albicans* hyphae. The observed differences in sugar selectivity of the integrins may play an important role in the regulation of leukocyte activation and differentiation (95, 96).

In the present work, direct interaction between purified αXβ2 and Pral was not tested directly. Therefore, we cannot exclude the possibility that another *C. albicans* hyphal protein that is regulated by Pral may serve as a ligand for αXβ2. However, the existing literature provides no evidence for such a molecule. Thus, our findings that purified αXβ2 interacts with *C. albicans* hyphae but not with the yeast form and that the HEK293/αXβ2 cells recognize and adhere to hyphae of WT *C. albicans* strain SCA314, but not of Pral-deficient strain CAMB-18, provide strong evidence that Pral serves as *C. albicans* ligand for αXβ2.

The integrin αXβ2 is usually present on the surface of leukocyte subsets together with another member of the β2 integrin family, αMβ2, to which such primary antipathogen leukocyte activities, such as recognition of bacterial LPS and fungal mannanproteins, are traditionally ascribed (12, 17, 24). We speculate that αMβ2 and αXβ2 integrins may play complementary roles in executing cellular immunity or that different cellular agonists may favor activation and utilization of one particular integrin. Our data showing significantly reduced resistance of αXβ2-deficient mice to *Candida* invasion and the αXβ2 requirement for fungal recognition and killing by macrophages clearly demonstrate that αXβ2 plays an independent role in the defense against fungal infections and does not simply serve as an auxiliary receptor for pathogens, secondary to αMβ2.

Taken together, these data clearly demonstrate the importance of αXβ2 in protection against *C. albicans* systemic infection, and this protective effect is mediated by subsets of tissue residential macrophages.

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Disclosures

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References