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 β-glucans but not by mannans, implicating a lectin-like activity in recognition but distinct in specificity from that of $\alpha_M\beta_2$. Mice deficient in $\alpha_X\beta_2$ were more prone to systemic infection with the LD_{50} fungal inoculum decreasing 3-fold in $\alpha_X\beta_2$-deficient mice compared with wild-type mice. After challenging i.v. with 1.5×10^4 cell/g, 60% of control C57BL/6 mice died within 14 d compared with 100% mortality of $\alpha_X\beta_2$-deficient mice within 9 d. Organs taken from $\alpha_X\beta_2$-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 $\alpha_X\beta_2$ is important for protection against systemic *C. albicans* infections and macrophage subsets in the liver, Kupffer cells, and in the brain, microglial cells use $\alpha_X\beta_2$ 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 candidemia has also been suggested (13, 15, 16). The most prominent receptors on leukocytes used in fungal or microbial recognition are integrins of the β_2 subfamily (17, 18). This subfamily of leukocyte receptors is composed of four members that share a common β_2 subunit that associates noncovalently with one of four distinct but structurally homologous α subunits to form $\alpha_M\beta_2$ (Mac-1, CD11b/CD18, and CR3), $\alpha_L\beta_2$ (LFA-1 and CD11a/CD18), $\alpha_X\beta_2$ (p150,95 CD11c/CD18 and CR4), and $\alpha_D\beta_2$ (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 β_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) or complete absence (severe) of all four of the β_2 integrins because of mutations in the *ITGB2* (β_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 β-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 β_2 integrins (33, 34), they do not directly facilitate leukocyte migration, adhesion, or phagocytosis.

Of the β_2 integrins, $\alpha_M\beta_2$ has been specifically implicated in the recognition of *C. albicans*. Polymorphonuclear leukocytes and NK cells use $\alpha_M\beta_2$ 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 $\alpha_M\beta_2$ among *C. albicans* proteins (40). Pra1p is a mannoprotein (1, 41) and is expressed on the surface of the

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Abbreviations used in this article: D-PBS, Dulbecco's PBS; HEK293, human epithelial kidney 293; HEK293/$\alpha_X\beta_2$ cell, the HEK293 cell expressing $\alpha_X\beta_2$ on its surface; KO, knockout; LAD-1, leukocyte adhesion deficit, type 1; MHC-II, MHC class II; NIF, canine hookworm neutrophil inhibitory factor; OG, octyl-β-D-glucopyranoside; Pra1, pH-regulated Ag 1; SDA, Sabouraud Dextrose Agar; WT, wild-type; $\Delta\alpha_X$ mice, mice depleted in $\alpha_X\beta_2$.

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hyphae but not on the yeast form of *C. albicans* (3, 41). Expression of Pra1p 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 α_M subunits have been previously described (44), it appears that the clinical manifestations of selective loss of $\alpha_M\beta_2$ are less severe than when all four β_2-integrins are absent, which suggests that $\alpha_M\beta_2$ may share at least part of its surveillance functions with other β_2 integrins, most likely with the integrin $\alpha_X\beta_2$ (19, 45). Integrin $\alpha_X\beta_2$ is present on the surface of all leukocyte subsets that express $\alpha_M\beta_2$, with the exception of dendritic cells, which have CD11c (α_X) 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 $\alpha_X\beta_2$ are less well studied, and its functions in innate immunity are still unclear. It was shown that $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ in *C. albicans* pathogenicity and the effects of its elimination on host defense in vivo, using $\alpha_X\beta_2$-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 Pra1-depleted strain CAMB5-18 (*pra1::hisG/pra1::hisG iro1-ura3Δ/IRO1-URA3*) was also used. This strain was derived from the strain CAMB435 by reversion of the *iro1-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

α_X-Knockout (KO) mice ($\Delta\alpha_X\beta_2$) were provided by Dr. C. M. Ballantyne (Baylor College of Medicine, Houston, TX). This mouse line was generated in parallel with other β_2-KO lines (53). All these β_2-KO murine lines have been used in a number of studies in comparison with the $\Delta\alpha_M$ mice (54–56), and there are no separate publications only characterizing the $\Delta\alpha_X$ mice. In our laboratory, these mice were backcrossed for more than 12 generations into a C57BL/J6 background. Before experiments, the genotypes of all mice were confirmed by PCR of blood DNA samples. Age-matched C57BL/J6 mice, obtained from The Jackson Laboratory (Bar Harbor, ME), were used as controls (wild-type [WT] mice). All protocols involving mice were approved by the Institutional Animal Care and Use Committee in accordance with the Public Health Service policy, the Health Research Extension Act (PL99-158), and the Cleveland Clinic policy. All experiments on mice involving *C. albicans* infections were carried out in a BSL2 facility. The mice were maintained during experiments on a 12-h alternating light/dark cycle and supplied with food (diet number 2918; Harlan Teklad, Madison, WI) and sterilized water ad libitum.

Cells

Human epithelial kidney 293 (HEK293) cells expressing $\alpha_X\beta_2$ (HEK293/$\alpha_X\beta_2$ cells) were prepared as described previously (57–59). Briefly, human full-length α_X and β_2 cDNAs were blunt-end cloned into TOPO-pCDNA 3.1(+) expression vector (Life Technologies, Carlsbad, CA). Plasmids were sequenced to ensure appropriate insert orientation and cotransfected into HEK293 cells using the LipofectAMINE Plus reagent (Life Technologies). To prepare control mock-transfected cells, the vector alone was used. Transfected cells were selected using neomycin sulfate (Life Technologies), and cells expressing the integrin were detected and sorted by flow cytometry (FACS) using a FACStar cell sorter (BD Biosciences) and anti-human CD18 mAb (clone IB4). The sorted cells were subcloned, and $\alpha_X\beta_2$ expression was characterized by FACS. The cell lines obtained were routinely grown in a monolayer in DMEM/F12 medium, supplemented with 10% FBS (all from BioWhittaker). For experiments, the cells were harvested using enzyme-free Cell Dissociation buffer (Life Technologies), washed with HBSS, and resuspended in HBSS containing 100 mM HEPES (pH 7.4), 5 mM $CaCl_2$, and 5 mM $MgCl_2$ (HBSS/HEPES). Monocytes were isolated from human donor blood using the *Pan* Monocyte Isolation Kit (Miltenyi Biotec) following the manufacturer's instructions.

All studies 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 α_M I-domain, IgG1), OKM1 (anti-human α_M lectin domain, IgG2b), IB4 (anti-human β_2, 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 CELLine Bioreactor Flasks (Integra Biosciences, Hudson, NH) in the Cleveland Clinic Hybridoma Core. The mAbs were purified from conditioned media using recombinant protein G columns (Life Technologies).

The mAb clone 3.9 (anti-human α_X, IgG1) and clone N418 (anti-mouse α_X) were purchased from BioLegend (San Diego, 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 recombinant hookworm neutrophil 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 568. CD45 was stained 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 stain. The images were observed using a Leica DMR microscope equipped with ×10/0.4 and ×20/0.5 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 $\alpha_X\beta_2$*

Recombinant $\alpha_X\beta_2$ was isolated from HEK293/$\alpha_X\beta_2$ cells with the method previously used by us to purify $\alpha_M\beta_2$ (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 $CaCl_2$ and $MgCl_2$ and loaded onto a column of immobilized IB4 (anti-β_2). 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 $CaCl_2$/$MgCl_2$, bound protein was eluted with three column volumes of 20 mM sodium acetate buffer (pH 4.2), containing OG and Ca/Mg^{2+}. Immediately after elution, 100 μl 1 M HEPES-NaOH (pH 8) 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 Ca/Mg^{2+}. The proteins were biotinylated using Sulfo-NHS-LC-Biotin (Pierce), according to the manufacturer's protocol. To visualize $\alpha_X\beta_2$–*C. albicans* interaction, *C. albicans* strain SC5314 was allowed to germinate in RPMI 1640 medium for 2 h and then purified. Biotinylated $\alpha_X\beta_2$ 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 5×10^5 *C. albicans* yeast were added and incubated overnight 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/$\alpha_X\beta_2$ cells were added in HBSS/HEPES 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/$\alpha_X\beta_2$ 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. Data 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/$\alpha_X\beta_2$ cell migration assays were performed in serum-free RPMI 1640 (monocytes) or DMEM/F-12 (HEK293/$\alpha_X\beta_2$) medium (Life Technologies) using modified Boyden chambers (Costar Transwell inserts in a 24-well plate format; Corning) with tissue culture-treated polycarbonate filters of 5-μm (for monocytes) or 8-μm pores (for HEK293/$\alpha_X\beta_2$ cells) 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/$\alpha_X\beta_2$ cell suspensions. The assays were initiated by addition of 50 μl cell suspension (10^5 cells/well) to 150 μl media in the upper chambers, and the plates were placed in a humidified incubator at 37˚C and 5% CO_2 for 8 h. For inhibition experiments, selected Abs, NIF, and glycans 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 HEPES (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, suspended in 0.25 ml HBSS/HEPES (pH 7.4), and mixed with 3×10^5 (1:3 ratio), 7×10^5 (1:7 ratio), or 1.1×10^6 (1:11 ratio) HEK293/$\alpha_X\beta_2$ or mock-transfected HEK293 cells. In control experiments, the HEK293/$\alpha_X\beta_2$ cells were preincubated with 20 μg/ml anti-α_X mAbs 3.9 for 10 min at room temperature. The cell/fungal mixtures were incubated at 37˚C with slow shaking for 2 h. To determine the extent of killing/phagocytosis, aliquots of the cell/fungal suspension were taken every 20 min, diluted with HBSS/HEPES, and subsequently plated in serial dilutions on SDA plates. The CFU were counted manually on day 2 using a Bel-Art Products Colony Counter. Cells were not lysed before plating, and all fungal cells that remained ingested were recorded as "killed." Results were independently verified by a modification of the method of Lehrer et al. (40, 66). Briefly, at the experiment end point, an equal volume of 1% Tween 80 was added to the fungal/leukocyte mixture to lyse leukocytes, and *C. albicans* cell pellets obtained by centrifugation were resuspended in 0.25 ml 2.5 mM methylene blue (Sigma-Aldrich) in HBSS/HEPES. The number of viable (nonstained) cells was counted in a hemacytometer. Control samples contained *C. albican*s incubated without HEK293 cells. Results obtained by both methods of quantitation of fungal viability showed close correlation with variances in the 5–10% range.

Murine model of systemic disseminated candidiasis

The model of systematic candidiasis, described and applied to C57BL/6 by MacCallum and Odds (67), was used. Each experimental group contained 10 mice of 10–12 wk of age, and weights ranged from 19 to 21 g. C57BL/6 (control WT mice) and $\alpha_X\beta_2$-depleted mice ($\Delta\alpha_X$ mice) were injected with 10^5 or 3×10^5 *C. albicans* SC5314 strain in 0.1 ml sterile saline via the tail vein. Mice were returned to cages and monitored. To determine the degree of distress, we developed a scoring system, similar to one described for the determination of humane end points in a murine model of leukemia (68). The following symptoms were evaluated and scored as follows: coma, 15 points; weight loss of: 20%, 15 points, 15%, 11 points, and 10%, 8 points; abdominal swelling, 6 points; significant decrease in mobility, 4 points; hunched posture, 3 points; porphyrin "red tears," 3 points; head pressing, 2 points; and spiky coat, 2 points. Each group was monitored daily over the 14-d period at the same time each day. The score was noncumulative and was recalculated for each mouse every 24 ± 1.5 h. The primary end point was the number of mice surviving on day 14 within each experimental group. When mice scored 15 or more points before 14 d, the animals were euthanized. At day 14, all surviving mice were euthanized and subjected to pathological examination. To determine the extent of *C. albicans* invasion and fungal burden in individual organs, liver, spleen, heart, lungs, and kidney were harvested, weighed, and homogenized in 5 ml PBS, and serial dilutions of the homogenates were plated onto SDA plates for CFU quantitation. In separate experiments, mice from each group of five mice were euthanized after 16 or 40 h of infection, and their organs were removed and examined to determine the degree of the fungal invasion and organ fungal burdens (67).

Statistical analyses

Statistical significance was determined using paired log-rank and Cox regression for mouse survival data or Student *t* test in all other cases. For all statistical calculations the statistical package in SigmaPlot, version 12.0 (Jandel Scientific Software, Chicago, IL) was used. Differences between groups were considered significant with $p < 0.05$. Data are expressed as means ± SD unless otherwise noted.

Results

Integrin $\alpha_X\beta_2$ is required to control C. albicans *infection*

As a first step to assess the biological significance of $\alpha_X\beta_2$ in the context of the total host–pathogen relationship, transgenic mice that lack α_X ($\Delta\alpha_X$ mice) were used in a murine model of disseminated candidiasis. In this assay, mice of both WT (C57BL/6 mice) and $\Delta\alpha_X$ 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). $\alpha_X\beta_2$ elimination dramatically decreased mice survival; during these same 336 h, 50% of the $\Delta\alpha_X$-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 $\Delta\alpha_X$ 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 $\alpha_M\beta_2$ decreases resistance of brain and liver to C. albicans *invasion*

The substantial increase in susceptibility of the $\Delta\alpha_X$ mice to the *Candida* infection indicates that $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ deletion on the rate of fungal colonization, WT and the $\Delta\alpha_X$ 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

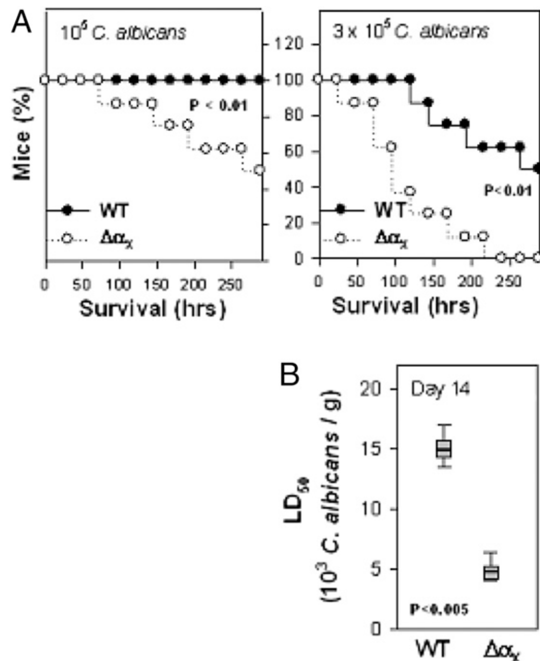

FIGURE 1. The effect of $\alpha_X\beta_2$ elimination on *C. albicans* virulence in a murine model of disseminated candidiasis. (**A**) The Kaplan–Meier (cumulative) graphic of murine survival. A total of 10^5 (*left panel*) or 3×10^5 (*right panel*) of strain SC5314 *C. albicans* were introduced in 100 μl D-PBS via tail vein injection into WT (●) or $\Delta\alpha_X$ (○) mice (n = 10 in each group). After administration, the mice were inspected on a 12 ± 2 h basis and euthanized when they became moribund (e.g., at 20% weight loss). (**B**) LD_{50} dose of *C. albicans*. The median survivors of both groups are calculated as medians (25th and 75th). The p values were calculated by log-rank test.

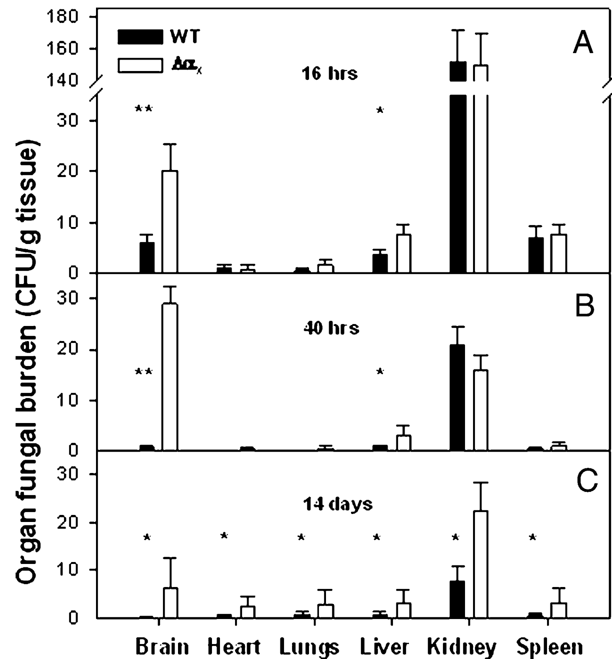

FIGURE 2. The effect of $\alpha_X\beta_2$ elimination on *C. albicans* dissemination. Challenged i.v. with SC5314 10^5 *C. albicans*, WT (■), or $\Delta\alpha_X$ (□) mice were euthanized after 16 h (**A**) or 40 h (**B**) of infection; their organs (brain, heart, lungs, liver, left kidney, and spleen) were removed, homogenized, and plated in serial dilution onto agar plates. (**C**) The organ fungal burdens of mice who survived to end point are presented. The results are presented as mean ± SD of two independent experiments (n = 3). $^*p < 0.05$, $^{**}p < 0.005$ (t test).

lungs at both early time points; but at day 14, differences in fungal burden in the surviving WT and $\Delta\alpha_X$ 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 $\Delta\alpha_X$ mice ($2 \times 10^4 \pm 3.2 \times 10^3$ CFU/g) compared with WT mice ($7.7 \times 10^3 \pm 1.2 \times 10^3$) (Fig. 2C). In contrast, fungal burdens in brains and livers recovered from $\Delta\alpha_X$ mice were substantially elevated compared with the corresponding WT organs: at 16 h, $\Delta\alpha_X$ brain had $2 \times 10^4 \pm 2.4 \times 10^3$ CFU/g tissue, whereas WT brains had $6.1 \times 10^3 \pm 7 \times 10^2$ CFU/g (10-fold raise; $p < 0.01$); $\Delta\alpha_X$ liver had $7.6 \times 10^3 \pm 1.3 \times 10^3$ CFU/g, 2-fold rise ($p < 0.05$), whereas WT liver had a fungal burden of $3.5 \times 10^3 \pm 360$ CFU/g (Fig. 2A). These differences increased over time: at 40 h, $2.9 \times 10^4 \pm 2.3 \times 10^3$ CFU/g $\Delta\alpha_X$ brain compared with 700 ± 120 CFU/g WT brain (40-fold rise; $p < 0.005$) and $3 \times 10^3 \pm 220$ CFU/g $\Delta\alpha_X$ 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 $\Delta\alpha_X$ and WT mice, respectively (Fig. 2C).

Residential macrophages require $\alpha_X\beta_2$ 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 $\Delta\alpha_X$ 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 $\Delta\alpha_X$ mice, the fungal colonies were numerous and showed evidence of extensive organ colonization (arrows in Fig. 3B, *right panel*).

To ensure that $\alpha_X\beta_2$ deletion affects only leukocytes, the sections of brains from WT (Fig. 3C) and $\Delta\alpha_X$ (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-α_X, 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 $\Delta\alpha_X$ mice do not organize but instead remained dispersed (Fig. 3C, 3D). These results indicate that subsets of brain residential leukocytes may use $\alpha_X\beta_2$ for localization to the fungus.

Activated monocytes use both $\alpha_M\beta_2$ and $\alpha_X\beta_2$ for fungal recognition

Previous studies demonstrated that NK lymphocytes (35, 61) and neutrophils (40) use integrin $\alpha_M\beta_2$ but not $\alpha_X\beta_2$ for *C. albicans* recognition. With our data on organ fungal burden suggesting that $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ involvement in leukocyte adhesion to *C. albicans*. Human peripheral blood monocytes were isolated, stimulated with PMA to activate their integ-

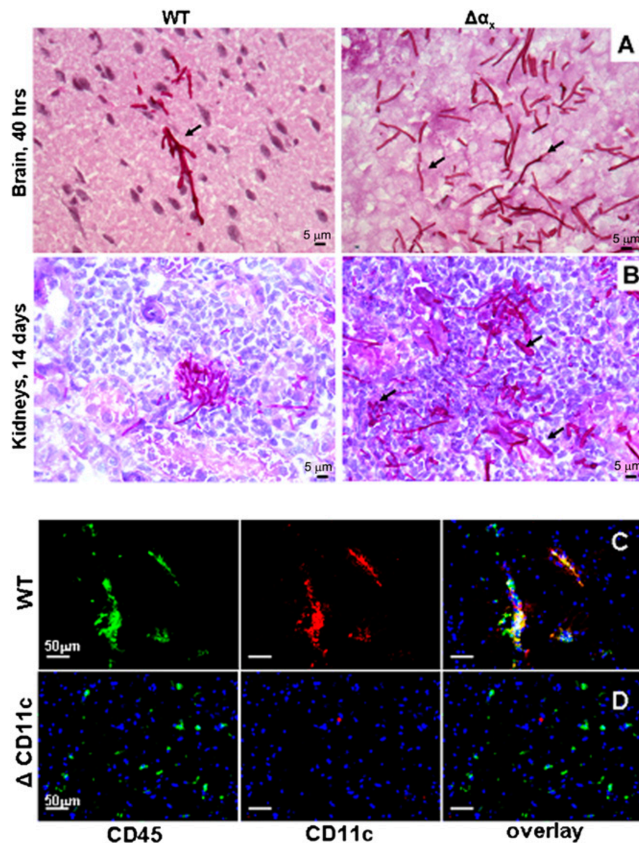

FIGURE 3. The effect of $\alpha_X\beta_2$ elimination on *C. albicans* invasion of the brain. Histological sections of murine brains taken after 16 h of infection (**A**) or of murine kidney obtained after 14 d of infection (**B**) from WT (*left two photos*) or from $\Delta\alpha_X$ mice (*right two photos*) were stained with hematoxylin. Arrows point on *C. albicans* hyphae (**A**) and on the signs of further organ colonization (B). Scale bars (A, B), 5 μm. Sections of brains taken from WT (**C**) or $\Delta\alpha_X$ (**D**) mice 40 h postinfection were stained with Alexa Fluor 488-labeled anti-CD45 mAbs (hematopoietic cells marker, *left panels*, green fluorescence) and Alexa Fluor 558-labeled anti-CD11c mAbs (anti-β_2, *middle panels*, red fluorescence). In the *right panels*, the overlay of the sample's green and red fluorescence is presented. The cellular nuclei are stained with DAPI (blue fluorescence). Scale bars (C, D), 50 μm.

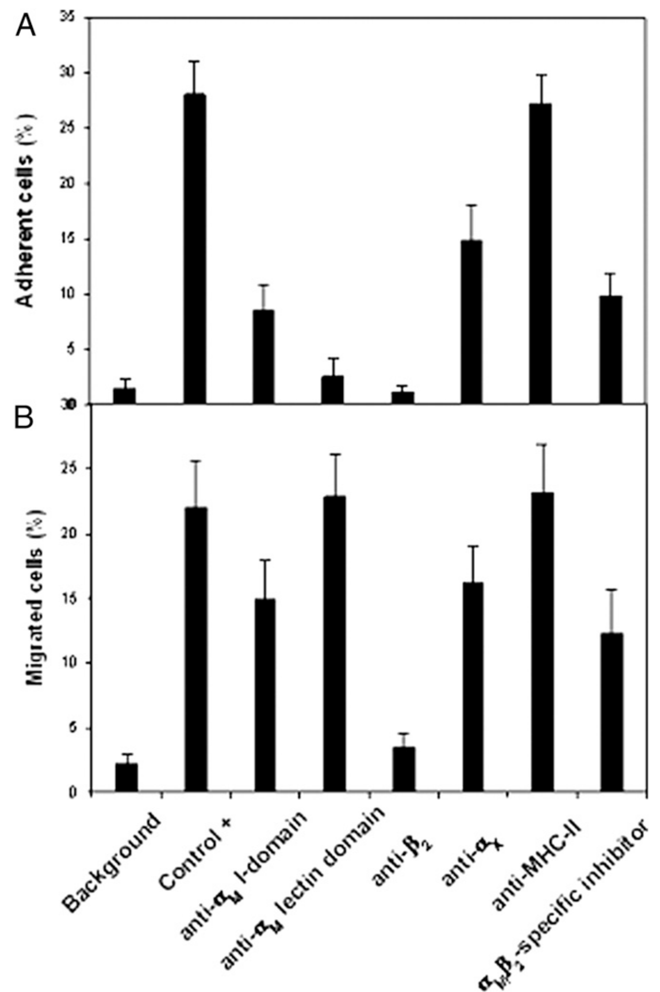

FIGURE 4. Effect of selected Abs and inhibitors on adhesive (**A**) and migratory (**B**) responses to *C. albicans* of PMA-activated human monocytes. Isolated human peripheral blood monocytes were preincubated for 10 min on ice in the presence of 5 mM PMA with or without inhibitors. (A) Adhesion to *C. albicans*. The plates were incubated at 37°C for 25 min, and then, nonadherent cells were removed by washing, and the adherent cells were quantified using the CyQUANT Cell Proliferation Assay kit. (B) Migration to *C. albicans*. A total of 5×10^5 monocytes/well were allowed to migrate overnight through polycarbonate membrane with 5-μm porosity to 10^6 pregerminated *C. albicans* of WT SC5314 strain. The number of migrated cells was quantified in the lower chamber and in the "fungal side" of the membrane using CyQUANT Cell Proliferation kits. As inhibitors, the following mAbs were used in 10 μg/ml concentration: anti-α_M I-domain (44a), anti-α_M lectin domain (OKM1), anti-β_2 (IB4), anti-α_X (3.9), irrelevant anti–MHC-II (W6/32), and 5 μM recombinant NIF. Migration or adhesion in the absence of *C. albicans* or in the absence of inhibitors was used as negative (background) and positive controls, respectively. Results 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, each in triplicate.

rins, and added to germinated SC5314 fungus. In the absence of inhibitors, 28 ± 6% of the activated monocytes adhered to the fungal hyphae as compared with only 2% monocyte adhesion to the fungal yeast form (defined as nonspecific adhesion). Both anti-α_M I-domain mAb 44a and the $\alpha_M\beta_2$-specific ligand NIF decreased monocyte adhesion to the fungal hyphae by ~3-fold, to 8 ± 4%. Anti-α_X mAb inhibited cell adhesion only ~50%, to 15 ± 6% of the total cells. The anti-α_M lectin domain mAb OKM1 and anti-β_2 mAb IB4 completely inhibited monocyte adhesion, whereas the irrelevant control, mAb W6/32, had no effect (Fig. 4A). A similar specificity was demonstrated in migration assays (Fig. 4B). When monocytes were allowed to migrate (through 5-μm pores) overnight to germinated *C. albicans*, anti-β_2 mAb completely inhibited the migration, anti-α_M I-domain mAb, and anti-α_X mAb inhibited 40–50%, and the control irrelevant anti-MHC class II (MHC-II) mAbs had no effect. However, a difference between migration and adhesion assays was noted. Anti-α_M lectin-domain mAb OKM1 completely inhibited adhesion of monocytes to the fungus, whereas this mAb had no effect on the monocyte migration to the fungus. This difference may reflect different roles of the fungal carbohydrates (e.g., β-glucans and mannans) recognized by the lectin domain of $\alpha_M\beta_2$ on the migratory and adhesive responses of monocytes. These results demonstrate that monocytes can use $\alpha_X\beta_2$ as well as $\alpha_M\beta_2$ to engage *C. albicans*, and taken together, the two integrins are the major mediators of monocyte adhesion and migration to the fungus.

Purified recombinant $\alpha_X\beta_2$ recognizes the hyphae of WT C. albicans *but not Pra1-deficient fungi*

To access the specificity of $\alpha_X\beta_2$–*C. albicans* interaction, a HEK293 cell line stably expressing the integrin was developed.

Full-length cDNAs of human α_X and β_2 were cotransfected into HEK293, and positive cells were sorted and subcloned. The established cell line (HEK293/$\alpha_X\beta_2$ cells) then was examined by FACS using a panel of Abs: anti-human α_X (mAb 3.9), anti-human β_2 (IB4), anti-human α_M I-domain (44a), anti-human α_M lectin domain (OKM1), and control irrelevant mAb W6/32 (anti-human MHC-II). As expected, α_X and β_2 were highly expressed on the HEK293/$\alpha_X\beta_2$ cell surface (Fig. 5A). Although α_X is highly homologous to α_M, the anti-α_M I-domain mAb 44a, which block binding of most ligands to $\alpha_M\beta_2$, did not recognize the HEK293/$\alpha_X\beta_2$ cells. Surprisingly, the anti-α_M lectin domain mAb OKM1, which inhibits the binding of polysaccharides such as β-glucans and mannans to $\alpha_M\beta_2$ (70), also showed weak reactivity with $\alpha_X\beta_2$ (Fig. 5A) but not with mock-transfected HEK293 cells (data not shown). Thus, the α_X subunit may contain structures similar to the α_M lectin domain and therefore may recognize fungal polysaccharides and serve as a pattern recognition receptor.

Both $\alpha_M\beta_2$ and $\alpha_X\beta_2$ integrins have many ligands in common. Because the *C. albicans* hyphae surface protein Pra1 was identified as a major *C. albicans* ligand for $\alpha_M\beta_2$ (40), we considered whether this protein might also serve as a ligand for $\alpha_X\beta_2$. We tested HEK293/$\alpha_X\beta_2$ cells as a source of $\alpha_X\beta_2$ protein and the ability of purified $\alpha_X\beta_2$ to recognize two *C. albicans* strains, SC5314 (WT) and Pra1-nul mutant (CAMB5-18). The yeast of both fungal strains were allowed to germinate overnight for maximal Pra1 expression in the WT strain and the fungal cell wall chitin and glucans were stained with blue fluorescent dye Blankophor (62). Labeled fungi then were incubated with FITC-labeled $\alpha_X\beta_2$ and examined by fluorescence microscopy. The microphotograph shown in Fig. 5B clearly demonstrates that soluble $\alpha_X\beta_2$ (green fluorescence) bound the hyphal form of WT fungus but not the rounded yeast form of *C. albicans* (Fig. 5B). No binding to the Pra1-deficient strain CAMB5-18 (Fig. 5C) was detected, indicating that $\alpha_X\beta_2$ binds to Pra1 or to a *C. albicans* hyphal constituent regulated by Pra1.

Upon expression of $\alpha_X\beta_2$ HEK293 cells acquire ability to recognize, bind, and phagocytose C. albicans

In the next set of experiments, the ability of $\alpha_X\beta_2$ to support HEK293 cell migration to *C. albicans* and adhesion to the fungi with subsequent phagocytosis was explored. HEK293 cells, expressing either $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ dependent. This conclusion was confirmed using a panel of $\alpha_X\beta_2$ and $\alpha_M\beta_2$ inhibitors. The adhesion of HEK293/$\alpha_X\beta_2$ was completely inhibited with anti-α_X (3.9) and anti-β_2 (IB4) mAbs, whereas the anti-α_M lectin domain mAb OKM1 inhibited 80 ± 10% of the cell adhesion. In contrast, the anti-α_M I-domain blocking mAbs 44a and NIF, a specific inhibitor of ligand binding to $\alpha_M\beta_2$ (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/$\alpha_X\beta_2$ cells by 50 ± 5%. Surprisingly, baker yeast mannans, which blocks HEK293/$\alpha_M\beta_2$ adhesion to *C. albicans* (36), did not block adhesion of HEK293/$\alpha_X\beta_2$ cells (Fig. 6A). In control experiments, neither β-glucan nor mannan was able to inhibit $\alpha_X\beta_2$-supported adhesion to another $\alpha_M\beta_2$ ligand—the fibrinogen peptide P2C, a nonglycosylated ligand that is also recognized by $\alpha_X\beta_2$ (59, 72) (results not shown). Both cell lines were not able to adhere to germinated Pra1-deficient *C. albicans* strain CAMB5-18 (40) (Fig. 6A), again indicating that *C. albicans* recognition by $\alpha_X\beta_2$ is Pra1 dependent.

Next, we tested migration of $\alpha_X\beta_2$ cells to *C. albicans* conditioned medium, a source of soluble Pra1 (40), in the presence or absence of integrin inhibitors. After 8 h, in the absence of inhibitors, 17 ± 4% $\alpha_X\beta_2$ cells migrated to fungal supernatant. The anti-β_2 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-α_X 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 α_V integrins on these cells. Anti-α_V mAb 272-17E6 inhibited migration of the cells to vitronectin to 4.4 ± 3.5% but had no affect on migration of the cells to the fungal supernatant. Echistatin, a snake venom disintegrin that inhibits ligand recognition by

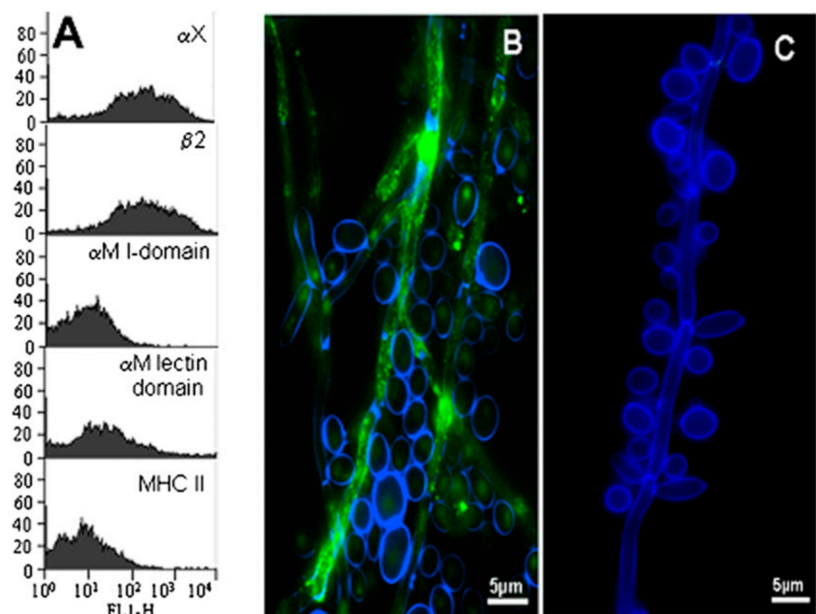

FIGURE 5. (**A**) Characterization of HEK293/$\alpha_X\beta_2$ cells by reactivity mAbs; (**B**, **C**) binding of purified $\alpha_X\beta_2$ to germinated *C. albicans*. (A) Characterization of HEK293/$\alpha_X\beta_2$ cells by FACS. A panel of Abs was used to examine the expression of $\alpha_X\beta_2$ Ags: anti-human α_X (mAb 3.9), anti-human β_2 (IB4), anti-human α_M I-domain (44a), anti-human α_M lectin domain (OKM1), and negative control irrelevant mAb W6/32 (anti-human MHC-II). Germinated *C. albicans* of WT strain SC5314 (B) or Pra1-depleted strain CAMB5-18 (C) labeled with Blankophore dye (blue fluorescence) was stained with and FITC-labeled purified $\alpha_X\beta_2$ (green fluorescence) and photographed using fluorescence microscopy. Scale bars (B, C), 5 μm.

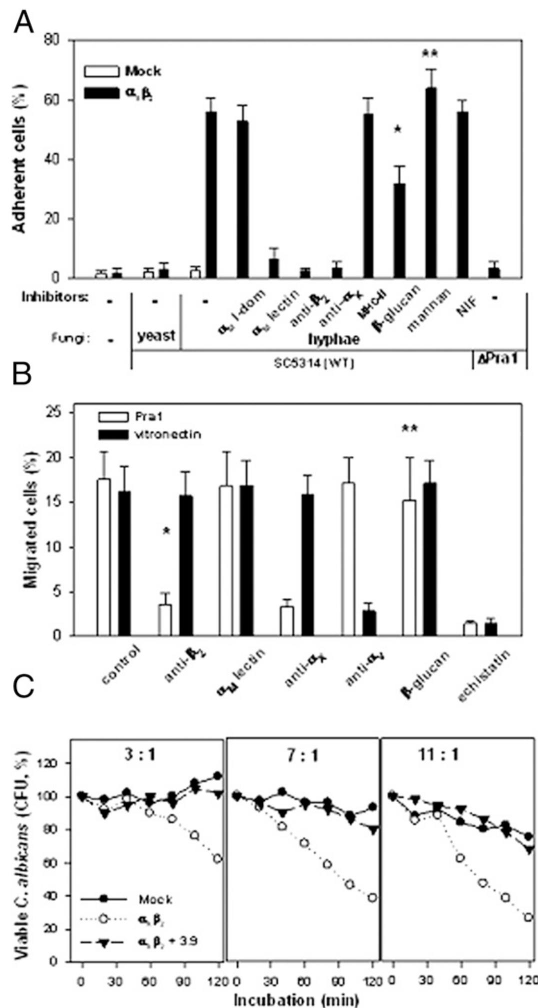

FIGURE 6. Upon expression on surface of HEK293 cells $\alpha_X\beta_2$ supports adhesive, migratory and phagocytic activities of the cells toward *C. albicans*. (**A**) HEK293/$\alpha_X\beta_2$ cell adhesion to *C. albicans*. A total of 5×10^5 HEK293/$\alpha_X\beta_2$ (■) or mock-transfected (□) cells were added to wells of tissue culture plates containing germinated (hyphae) or nongerminated (yeast) *C. albicans* of WT (SC5314) or ΔPra1 (CAMB5-18) strains. The plates were incubated at 37°C for 1 h, then nonadherent cells were removed by washing, and the adherent cells were quantified using the CyQUANT Cell Proliferation Assay kit. For inhibition assays, before addition to the plate wells, the cells were preincubated with 10 μg/ml of the Abs: anti-α_M I-domain (44a), anti-α_M lectin domain (OKM1), anti-β_2 (IB4), anti-α_X (3.9), and irrelevant anti–MHC-II (W6/32) or with 1 mM β-glucan, 1 mM mannan, or 5 μM recombinant NIF for 10 min at room temperature. (**B**) HEK293/$\alpha_X\beta_2$ cell migration to *C. albicans*. Cell migration was measured in Boyden chambers (Costar Transwell with 8-μm porosity in a 24-well format). A total of 10^5 HEK293/$\alpha_X\beta_2$ cells were added to the upper chamber, whereas lower chambers contained 10^6 germinated *C. albicans* cells of WT SC5314 strain (□) or 10 mM vitronectin (■) in serum-free DMEM/F-12 medium. The inhibitors mAbs IB4, OKM1, 3.9 (see above), anti-α_V (27217E6), and 1 mM β-glucan or echistatin were added with the cells to the upper chamber. Plates were incubated for 8 h in a humidified incubator at 37°C and 5% CO_2. The migrated cells were counted using the CyQUANT Cell Proliferation kit. (**C**) Phagocytosis of *C. albicans* by HEK293/$\alpha_X\beta_2$ cells. A total of 10^5 germinated *C. albicans* SC5314 strain cells were incubated with 3×10^5 (1:3 ratio), 7×10^5 (1:7 ratio), or 1.1×10^6 (1:11 ratio) mock-transfected HEK293 cells (●) or HEK293/$\alpha_X\beta_2$ cells in the presence (▲) or absence (○) of anti-α_X mAbs 3.9. Fungal survival was determined every 20 min by plating the sample aliquots onto SDA plates in serial dilutions. Results 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, each in triplicate. *$p < 0.05$ (*t* test), **$p > 0.05$.

most integrins, including the β_2 and α_V integrins (61), completely reduced cell migration to both *Candida* supernatant and vitronectin (Fig. 6B). Taken together, these data indicate that migration of $\alpha_X\beta_2$ cells to *C. albicans* proteins is integrin dependent, and $\alpha_X\beta_2$ is implicated in this response of the HEK293/$\alpha_X\beta_2$ cells. Notably, β-glucan and anti-α_M lectin domain mAb OKM1, which inhibited adhesion of $\alpha_X\beta_2$ cells to *C. albicans*, did not affect migration of these cells to the fungal extracellular proteins (Fig. 6B), suggesting that the lectin specificity of $\alpha_X\beta_2$ is involved in adhesion but not migration to *C. albicans*.

The ability of $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ cells with anti-α_X blocking mAb 3.9 completely inhibited the antifungal activity of the $\alpha_X\beta_2$ cells to levels observed with mock-transfected HEK293 cells ($p > 0.5$). These results indicate that $\alpha_X\beta_2$ is able to promote phagocytosis of *C. albicans*.

Discussion

In the present work, we demonstrate the importance of integrin $\alpha_X\beta_2$ for protection against *C. albicans* systemic infection by the innate immune system. Although the ability of $\alpha_X\beta_2$ 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 residential macrophages (see below).

Our data on the murine organ fungal burdens indicate that the $\alpha_X\beta_2$ elimination affects mainly the liver and brain, dramatically increasing invasion and propagation of the fungus in these organs. This effect of $\alpha_X\beta_2$ became evident at the earliest stages of infection: as early as16 h after the challenge, a 2-fold difference ($p < 0.05$) emerged in fungal burdens in the livers of $\Delta\alpha_X$ 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 $\alpha_X\beta_2$-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 β_2 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 α_X [also see (76)], and thus, $\alpha_X\beta_2$ 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 $\Delta\alpha_X$ 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 $\alpha_X\beta_2$-deficient mice, the $CD45^+CD11c^-$ cells in the brain remained diffusely distributed, suggesting that $\alpha_X\beta_2$ 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 monocytoid lineage (79, 80) and express both $\alpha_X\beta_2$ and $\alpha_M\beta_2$ integrins (81–83), and their importance for protection against *C. albicans* invasion has been demonstrated previously (84–87). The possible involvement of integrin $\alpha_X\beta_2$ in phagocytosis of *C. albicans* by microglial and Kupffer cells has been proposed (84, 88, 89). Taken together, these data suggest that $\alpha_X\beta_2$ but not $\alpha_M\beta_2$ is critical in antifungal activity of tissue-resident macrophage subsets. This conclusion is consistent with the previous report demonstrating that increased expression of $\alpha_X\beta_2$ 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, $\alpha_X\beta_2$ may become activated, whereas $\alpha_M\beta_2$ remains in an inactive conformation or vice versa, and therefore, these two β_2 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-$\alpha_X\beta_2$ mAbs block adhesion of PMA-activated monocytes only partially. The only anti-α_M mAb that blocks adhesion to the fungus is directed to the α_M lectin domain, and as we have shown, this mAb also cross-reacts with a previously unrecognized lectin domain within the α_X subunit.

β-Glucans and mannans are important immunomodulators, and their binding by leukocytes is implemented by integrin $\alpha_M\beta_2$ (90, 91). Upon ligation with the integrin, β-glucans activate $\alpha_M\beta_2$ and stabilize it in an intermediate active conformation (92).Unlike $\alpha_M\beta_2$, where the carbohydrate binding and sugar selectivity of its α_M-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 $\alpha_X\beta_2$. Therefore, our observation that activity of $\alpha_X\beta_2$ is modulated by fungal β-glucans is a novel finding of our present work. On the basis of ~70% homology between α_M and α_X and that the OKM1 mAb, which blocks glycan binding to α_M (70), weakly cross-reacts with α_X, we anticipate certain similarities in the sugar specificity of these integrin subunits. However, $\alpha_M\beta_2$ and $\alpha_X\beta_2$ demonstrated clear distinction in carbohydrate selectivity: although $\alpha_M\beta_2$ recognizes both β-glucans and mannans, the activity of $\alpha_X\beta_2$ 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/$\alpha_X\beta_2$ 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 $\alpha_X\beta_2$ and Pra1 was not tested directly. Therefore, we cannot exclude the possibility that another *C. albicans* hyphal protein that is regulated by Pra1 may serve as a ligand for $\alpha_X\beta_2$. However, the existing literature provides no evidence for such a molecule. Thus, our findings that purified $\alpha_X\beta_2$ interacts with *C. albicans* hyphae but not with the yeast form and that the HEK293/$\alpha_X\beta_2$ cells recognize and adhere to hyphae of WT *C. albicans* strain SC5314, but not of Pra1-deficient strain CAMB5-18, provide strong evidence that Pra1 serves as *C. albicans* ligand for $\alpha_X\beta_2$.

The integrin $\alpha_X\beta_2$ is usually present on the surface of leukocyte subsets together with another member of the β_2 integrin family, $\alpha_M\beta_2$, to which such primary antipathogen leukocyte activities, such as recognition of bacterial LPS and fungal mannoproteins, are traditionally ascribed (12, 17, 24). We speculate that $\alpha_M\beta_2$ and $\alpha_X\beta_2$ 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 $\alpha_X\beta_2$-deficient mice to *Candida* invasion and the $\alpha_X\beta_2$ requirement for fungal recognition and killing by macrophages clearly demonstrate that $\alpha_X\beta_2$ plays an independent role in the defense against fungal infections and does not simply serve as an auxiliary receptor for pathogens, secondary to $\alpha_M\beta_2$.

Taken together, these data clearly demonstrate the importance of $\alpha_X\beta_2$ in protection against *C. albicans* systemic infection, and this protective effect is mediated by subsets of tissue residential macrophages.

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Disclosures

The authors have no financial conflicts of interest.

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