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Superantigen-Like Effects of a *Candida albicans* Polypeptide

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Abstract

The amino terminal sequence of the *Candida albicans* cell wall protein Int1 exhibited partial identity with the major histocompatibility complex (MHC) class II binding site of the *Mycoplasma arthritidis* superantigen MAM. Int1-positive *C. albicans* blastospores activated human T lymphocytes and expanded Vβ subsets 2, 3, and/or 14; Int1-negative strains were inactive. Release of interferon-γ (IFN-γ) but not of tumor necrosis factor–α or interleukin-6 was Int1 dependent; interleukin-4 and interleukin-10 were not detected. T lymphocyte activation, Vβ expansion, and IFN-γ release were associated with a soluble polypeptide that encompassed the first 263 amino acids of Int1 (Pep_{263}). Monoclonal antibody 163.5, which recognizes an Int1 epitope that overlaps the region of identity with MAM, significantly inhibited these activities when triggered by Int1-positive blastospores or Pep_{263} but not by staphylococcal enterotoxin B. $Histidine_{263}$ was required. Pep_{263} bound to T lymphocytes and MHC class II and was detected in the urine of a patient with *C. albicans* fungemia. These studies identify a candidal protein that displays superantigen-like activities.

Candida species have emerged as the fourth most common cause of nosocomial bloodstream infections, and *Candida albicans* typically predominates [1–4]. Although mucosal colonization is usually not a direct cause of mortality, entry of *C. albicans* into the bloodstream is associated with fatality rates of 10%−49% among patients with thermal burns, cardiopulmonary bypass or abdominal surgery, neutropenia or neutrophil dysfunction, or extreme prematurity (birth weight, <1000 g) [5]. Although most patients with candidemia have a prolonged course with an incremental cost of treatment ranging from $40,000 to $90,000 per patient [6,7], a few rapidly progress to hypotension and death accompanied by concentrations of proinflammatory cytokines (i.e., interferon-γ [IFN-γ], tumor necrosis factor–α [TNF-α], and interleukin-6 [IL-6]) that exceed those found in bacterial infections [8–10].

With other opportunistic pathogens, such as *Aspergillus fumigatus*, a fulminantly fatal course may be facilitated by toxins, including aflatoxin or gliotoxin. Neither toxin has been identified in *C. albicans* [11], although provocative evidence for a superantigen-like toxin derives from the observation that human peripheral blood mononuclear cells (PBMCs) incubated with *C. albicans* skin test antigen increased gene transcripts for Vβ subsets 5.1 and 5.2 [12]. The amino terminus of the *C. albicans* cell wall protein Int1 (relative molecular mass, 220 kDa), named for its integrin-like sequence and putative roles in adhesion, morphogenesis, and virulence [13,14], displays 56% identity with the major histocompatibility complex (MHC) class II binding site of the well-characterized superantigen MAM from *Mycoplasma arthritidis* [15]

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(figure 1). We therefore investigated the possibility of Int1-dependent superantigen activity in *C. albicans*.

MATERIALS AND METHODS

Strains

C. albicans strains included CAF2 and its isogenic Int1-negative mutant CAG3 [14], as well as a second Int1-negative mutant (VBIDM2) constructed with polymerase chain reaction (PCR)–mediated mutagenesis [16] and expressing only the first 21 amino acids of Int1 (table 1).

PCR-mediated disruption of INT1

Materials and methods are specified in the appendix, which is only available in the electronic edition of the journal.

Antibodies

The following antibodies were obtained: anti-human CD3, CD4, CD8, CD25, CD69, HLA-DR/IgG2a, and secondary conjugates (Becton Dickinson); anti-human TCR Vβ subsets (Beckman Coulter); and IgG1 isotype control for monoclonal antibody (mAb) 163.5 (Becton Dickinson). Typically, 10 μL were used to stain 10^6 cells. The immunizing peptide for mAb 163.5 (Inhibitex) was C-VNSEPEALTDMKLKRENFSNLSLDEKVNLY coupled to ovalbumin.

Activation of human PBMCs by C. albicans

Organisms were grown to saturation (optical density at 600 nm [OD_{600}] = 0.5) in yeast extract/peptone/dextrose (YPD) medium with shaking at 30°C overnight; subcultures grown to early exponential phase in fresh YPD at 30°C (OD_{600} = 0.25) were washed twice in phosphate buffered saline (PBS) containing 0.2 μg/mL of amphotericin B (Invitrogen) to eliminate filamentous growth of *C. albicans* [23,24]. Preliminary experiments showed that this concentration of amphotericin B did not inhibit the response of PBMCs to staphylococcal enterotoxin B (SEB) or Int1-positive *C. albicans*.

Whole blood was collected from adult donors by venipuncture, using heparinized vacutainer tubes (Becton Dickinson). PBMCs were isolated by standard methods, using Histopaque (Sigma). PBMCs washed with Hanks balanced salt solution were resuspended at 2.5×10^6 cells/mL in medium containing RPMI1640 supplemented with 2 mmol/L of L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 1 mmol/L of sodium pyruvate, 0.1 mmol/L of nonessential amino acids, 20 mmol/L of Hepes, 10% certified fetal bovine serum, and 0.2 μg/mL of amphotericin B. In experiments with anti–HLA-DR antibodies, 10 μg/mL of azide free anti-HLA-DR or mouse IgG2a isotype control was added to PBMCs prior to plating.

A total of 5×10^5 blastospores in PBS were inoculated into each well of a 96-well plate and incubated at 37°C for 30 min. PBS manually aspirated from the culture wells containing adherent blastospores was replaced with 200 μL of complete medium containing 5×10^5 PBMCs, after ascertaining microscopically that wells contained approximately equal numbers of Int1-positive or Int1-negative yeast cells. SEB (Sigma; concentration, 10−100 ng/mL) served as a control. Plates were incubated at 37°C in 5% CO_2.

Flow cytometry

For all experiments except those with data reported in figure 2*B*, PBMCs were harvested for antibody staining on the fourth day of coculture and were analyzed by flow cytometry for

expression of the specified marker. Fluorochrome-labeled antibodies and conjugates were used in 2-color, 3-color, or 4-color flow cytometry with a FACSCalibur (BD Biosciences). Data were analyzed with Win-MDI software, version 2.8 (available at: http://facs.scripps.edu/software.html). Each experiment was performed in duplicate or triplicate wells; 30,000 cells from each well were analyzed.

Paraformaldehyde fixation of antigen-presenting cells and purification of T cells

PBMCs were plated in petri dishes at 37°C for 1 h. Adherent antigen-presenting cells were harvested by incubating cells at 4°C for 1 h in Ca^{2+}-free and Mg^{2+}-free PBS that contained 1 mmol/L EDTA and 0.5% glucose. T cells were purified from the nonadherent population to ≥95% purity, using a human T cell enrichment column kit (R&D Systems). PBS-washed antigen-presenting cells were resuspended in either complete medium or 0.06% paraformaldehyde (Sigma). After 5 min in paraformaldehyde at 37°C, ice cold 0.06% glycyl glycine was added (Sigma) [25]. After 2 PBS washes, paraformaldehyde-treated antigen-presenting cells were incubated in complete medium (CM; 0.17% Difco yeast nitrogen base, 0.5% NH_4SO_4, 2% glucose, and 1.5% Bacto-agar) at 37°C for an additional 30 min to remove residual paraformaldehyde before resuspension in fresh medium. Purified T cells and antigen-presenting cells were combined at a ratio of 2.3:1.

Cytokine measurement

Supernatants from T cell activations were analyzed for TNF-α, IFN-γ, IL-6, IL-4, and IL-10 by use of OptEIA ELISA II kits (BD Biosciences), according to the manufacturer's instructions.

Intracellular staining for IFN-γ

PBMCs cocultured with Int1-positive *C. albicans*, Int1-negative *C. albicans*, SEB, or medium alone were treated with brefeldin A (Golgi-Plug [BD Biosciences]) for 5 h before staining. Washed cells were stained for CD25, followed by intracellular staining for IFN-γ, according to the manufacturer's protocol (Cytofix/Cytoperm kit [BD Biosciences]).

Blockade of function with mAb 163.5

mAb 163.5 or an irrelevant mouse IgG1 was added to tissue culture wells daily at concentrations of 25 or 50 μg/mL.

Construction of 6XHis-Pep_{263} expression vector, mutagenesis of Pep_{263} codon 251, expression and purification of Pep_{263} and truncation mutants, and biotinylation of Pep_{263}

Materials and methods are specified in the appendix.

Binding of Pep_{263} to T lymphocytes and MHC class II molecules

A total of 5×10^5 freshly isolated human PBMCs were incubated with 10 μg of biotinylated Pep_{263} and 5×10^5 Int1-negative blastospores (VBIDM2) for 4 days at 37°C. In control incubations, Pep_{263} or Int1-negative blastospores were omitted. All cells in each experimental mixture were washed with 1× PBS/3% BSA/0.02% sodium azide (FACS buffer), pelleted by centrifugation at 500 *g*, and stained for 25 min at room temperature with 10 μL of CD3-FITC (BD Pharmingen) and 10 μL of a 1:10 dilution of streptavidin–phyco-erythrin (PE) (BD Pharmingen). In the absence of biotinylated Pep_{263}, no binding of streptavidin-PE to T lymphocytes was observed.

Experiments to measure the binding of biotinylated Pep_{263} to antigen-presenting cells were performed in the conditions described above, save that adherent antigen-presenting cells were detached from the wells by use of Versene (0.2 g/L EDTA-4Na in PBS [Gibco BRL]). Staining reagents included FITC-conjugated HLA-DR (BD; 10 μL for 10^6 cells) for class II molecules;

mannosylated BSA-FITC (Sigma; 10 μL of 1 mg/mL) for mannose receptors on antigen-presenting cells, and streptavidin-PE for biotinylated Pep_{263} (10 μL of a 1:10 dilution). In the absence of biotinylated Pep_{263}, no binding of streptavidin-PE to antigen-presenting cells was observed.

Western immunoblotting

Five milliliters of urine from a 43-day old premature male of 24 weeks' gestation with *C. albicans* fungemia was centrifuged at 2500 *g* at 4°C for 10 min to remove cellular debris. Clarified urine was concentrated 50-fold on a Centricon Plus-20 5000-molecular weight-cutoff filter (Millipore) at 4000 *g* at 4°C for 30 min. Urine from 4 healthy adults was treated identically. Retentates from each sample were denatured under reducing conditions and electrophoresed on a 7.5% SDS-PAGE gel. As an additional control, recombinant Pep_{263} was added to an aliquot from the retentate of 1 healthy donor. Proteins were transferred to a nitrocellulose membrane (Bio-Rad), blocked with TBST (50 mmol/L of Tris-HCl, 150 mmol/L of NaCl [pH 7.5], and 0.1% of Tween 20) that contained 5% skimmed milk powder (TBS-MT), and incubated at 4°C for 16 h with mAb 163.5 (stock concentration, 8.3 mg/mL) at a dilution of 1:500 in TBS-MT. The blot was washed with TBST and incubated at 25°C for 2 h with HRP-conjugated sheep anti-mouse IgG (GE Healthcare Bio-Science) at a dilution of 1:5000 in TBS-MT. After another wash with TBST, the membrane was developed with SuperSignal West Femto (Pierce) and exposed to Kodax Blue XB-1 film for 1 min.

Human subjects

Isolation of PBMCs from adults was approved by the Yale Human Investigations Committee (HIC #1047). Collection of urine from the infant received an exemption because the specimen would otherwise have been discarded.

Statistical analysis

Statistical comparisons were performed using an unpaired Student *t* test; a *P* value of <.05 was considered statistically significant.

RESULTS

Int1-dependent T cell activation, Vβ expansion, and IFN-γ secretion induced by C. albicans *blastospores*

Coculture of human PBMCs with Int1-positive *C. albicans* blastospores for 3.5 days induced an average of 15 foci of T cell blasts, each of which had a diameter of ≥5 μm (figure 2*A*); Int1-negative mutants missing amino acids 434−1664 [14, 17] or 21−1664 induced on average 1 foci of T cell blasts per well, which was equivalent to the induction rate in medium alone (figure 2A).

Unless otherwise stated, cells were harvested on the fourth day of coculture for analysis by flow cytometry. Both Int1-positive strains and their respective reintegrants activated T lymphocytes, as measured by up-regulation of the IL-2 receptor CD25; Int1-negative isogenic strains were significantly less active ($P < .002$). A mutant with 2 disrupted copies of *EFG1* (which encodes a transcription factor linked to enhanced filamentous growth [18]) was not impaired (figure 2*B*). The pattern was identical with a second activation marker, CD69 (figure 2*B*).

Populations of $CD3^+$ cells (T lymphocytes) that expressed CD25 (30.1% of cells) or CD69 (25.8% of cells) in response to Int1-positive *C. albicans* contained both $CD4^+$ cells and $CD8^+$ cells, in addition to $CD4^+CD8^+$ cells (table 2); a similar pattern was noted with SEB (table 2).

In coculture with PBMCs from 5 adult donors, the Int1-positive strain was associated with up-regulation of the IL-2 receptor in all donors; isogenic Int1-negative mutants were inactive. These effects were statistically significant on days 4−7 ($P < .02$) (figure 2*C*).

A monoclonal antibody against the HLA-DR α-subunit had no effect on T lymphocyte activation in response to phytohemagglutinin but partially inhibited T cell activation induced by toxic shock toxin (TSST-1) or by Int1-positive *C. albicans* blastospores ($P < .04$). Blockade of HLA-DR did not affect the negligible T cell response induced by isogenic Int1-negative mutants (figure 2D).

In the absence of antigen-presenting cells, neither SEB, the Int1-positive strain, or the isogenic Int1-negative mutant induced T cell activation (figure 2*E*). In the presence of adherent antigen-presenting cells, T lymphocytes were activated by SEB and the Int1-positive strain. Paraformaldehyde fixation of antigen-presenting cells did not inhibit T cell activation induced by SEB or by Int1-positive blastospores. Int1-negative blastospores were inactive under all conditions.

The expansion of T lymphocyte populations bearing specific Vβ subsets is a hallmark of superantigens, although there is considerable donor-to-donor variation in the combination of subsets expanded [26]. For example, SEB expands T lymphocytes expressing Vβ subsets 3, 12, 14, 15, 17, or 20 [26,27]. We defined "expansion" as a response to Int1-positive cells that was 5 times the response to Int1-negative cells. All donors expanded Vβ subsets 2, 3, or 14 in Int1-dependent fashion (figure 3). One donor also expanded Vβ7.1 (figure 3). In no case did we observe expansion of Vβ subsets by Int1-negative cells. In 3 experiments involving a donor with expansion of Vβ2 and Vβ14 in response to Int1-positive *C. albicans*, a mean (±SD) of 80.0% ± 6.6% cells expressing Vβ2 and 77.8% ± 6.7% expressing Vβ14 also expressed CD25; in response to SEB, 79.3% ± 6.4% of cells expressing Vβ14 also expressed CD25. Thus, >75% of Vβ2 and Vβ14 T cells were activated in response to Int1-positive *C. albicans*.

Expansion of Vβ subsets is accompanied by release of proinflammatory cytokines [28,29]. *C. albicans* blastospores elicited TNF-α, IL-6, and IFN-γ, with the latter cytokine elicited in an Int1-dependent fashion (table 3); neither IL-4 nor IL-10 was detected, which was consistent with observations reported elsewhere [30]. Staining for intracellular IFN-γ showed that 97.1% of IFN-γ was produced by $CD25^+$ cells in response to Int1-positive *C. albicans*, a result equivalent to what was observed with SEB.

Characterization of a monoclonal antibody to the amino terminus of Int1

Linear peptides spanning amino acids 239−278 of Int1 were used to generate murine mAb. Only mAb 163.5 recognized Pep_{263}, a construct encompassing the first 263 amino acids of Int1 (figure 4).

mAb 163.5 significantly inhibited activation of T lymphocytes ($P < .035$) (figure 5*A*) and expansion of Vβ subsets 2 ($P < .02$) and 14 ($P < .02$) (figure 5*B*) induced by Int1-positive *C. albicans*, compared with the activity of an irrelevant IgG1 control. Neither mAb 163.5 nor the isotype control inhibited T cell activation or expansion of the Vβ14 subset triggered by SEB (figure 5*A* and 5*B*). Therefore, mAb 163.5 was specific for Int1.

mAb 163.5 also inhibited IFN-γ production by Int1-positive *C. albicans* by 50% (mean IFN-γ level [±SD], 22.89 ± 12.59 ng/mL vs. 11.26 ± 6.01 ng/mL in the presence of mAb 163.5; $P = .002$). Control mAb also showed an inhibitory effect of 18% (mean IFN-γ level [±SD], 22.89 ± 12.59 ng/mL vs. 18.75 ± 11.63 ng/mL in the presence of control mAb), but this effect was not statistically significantly different from the level of IFN-γ produced by Int1-positive *C. albicans* alone.

Localization of superantigen-like activity to the amino terminus of Int1p

Because of the inhibitory effects of mAb 163.5, a construct encompassing the first 263 amino acids of Int1 (Pep_{263}) was expressed and purified. In the presence of Int1-negative blastospores, soluble Pep_{263} was associated with blastogenic foci of T lymphocytes (data not shown), expansion of Vβ subsets 2 and 14, and release of IFN-γ (figure 5*C* and 5*D*). mAb 163.5 significantly inhibited the expansion of Vβ subsets 2 ($P = .02$) and 14 ($P = .01$) and the release of IFN-γ ($P < .01$) in response to Pep_{263} (figure 5*C* and 5*D*), compared with the effects of an irrelevant IgG1 isotype control.

Structural requirements for the function of soluble Pep_{263}

Mutation of key histidine residues in classical superantigens aborts their potency [31–33]. Although expression of native Pep_{263} ending in $_{260}KLKH_{263}$ activated T lymphocytes and expanded Vβ subsets 2 and 14 as previously shown, truncation constructs ending in K_{262}, L_{261}, or K_{260} were no more active than medium alone. Substitution of A, D, or R for H_{263} did not restore the construct's ability to activate T lymphocytes or to expand Vβ subsets 2 and 14.

Requirement for Int1-negative blastospores in the binding of Pep_{263} to T lymphocytes and MHC class II molecules

Int1-negative strains were unable to activate T lymphocytes; expand Vβ subsets 2, 3, or 14; or elicit significant amounts of IFN-γ (figures 2, 3, and 5); nevertheless, the combination of soluble Pep_{263} and Int1-negative blastospores mediated these effects (figure 5*C* and 5*D*). Control experiments determined that heat-killed Int1-negative blastospores or live *Saccharomyces cerevisiae* could not substitute for replicating Int1-negative blastospores (figure 6). We hypothesized that Int1-negative blastospores were required for the interaction of Pep_{263} with T lymphocytes or antigen-presenting cells.

By FACS analysis, biotinylated Pep_{263} bound to a mean (±SD) of 5.8% ± 0.3% of $CD3^+$ T lymphocytes in the presence of Int1-negative blastospores but only to 1.2% ± 0.5% of T lymphocytes in their absence. Pep_{263} bound equivalently to $CD4^+$ and $CD8^+$ T lymphocytes but preferentially to $CD4^+CD8^+$ cells in a ratio of 1:1:6.

Approximately 10%−11% of each PBMC preparation was positive for mannosylated bovine serum albumin as a marker for the mannose receptor (MR) on antigen-presenting cells; 90.3% of MR-positive cells were also positive for HLA-DR (figure 7). When biotinylated Pep_{263} was added, 96.9% of MR-positive cells also stained with streptavidin-PE, but the detection of HLA-DR was reduced more than 5-fold (from 90.3% of cells to 15.9% of cells). Thus, in the presence of Int1-negative blastospores, binding of Pep_{263} to antigen-presenting cells interfered with the detection of a class II antigen.

Detection of Pep_{263} in a clinical specimen

Western immunoblotting with mAb 163.5 identified bands at 44 and 22 kDa in urine from a 43-day-old premature infant with catheter-associated *C. albicans* fungemia (figure 8); these bands corresponded to 2 fragments of purified Pep_{263}; similar bands were not detected in uninfected urine. These results show that Pep_{263} was generated in vivo.

DISCUSSION

Superantigens elicit their effects by bridging the α or β subunit of the MHC class II locus and T lymphocytes bearing certain Vβ sequences at the T cell receptor [26,34]. Although superantigens are not processed within the antigen-presenting cell or presented in the peptide groove, participation of MHC class II and costimulatory molecules such as B7−1 is required [35]. By obviating the mechanisms that control the well-modulated production of

proinflammatory cytokines from antigen-specific T lymphocytes upon presentation of microbial antigens in the peptide groove, most bacterial superantigens recruit up to 10,000-fold more T lymphocytes of both $CD4^+$ and $CD8^+$ phenotypes, thereby triggering excessive release of cytokines such as IFN-γ, TNF-α, or IL-6.

Results reported here indicate that several effects associated with superantigens—activation of T lymphocytes independently of antigen processing and presentation, expansion of T cell populations expressing specific Vβ subsets, and elicitation of IFN-γ—are associated with a polypeptide (Pep_{263}) derived from the amino terminus of the *C. albicans* cell wall protein Int1. As with classic superantigens, such as TSST-1 or staphylococcal enterotoxin A, the C-terminal histidine residue of Pep_{263} is essential for T lymphocyte activation and Vβ expansion [32,33, 36]. Soluble Pep_{263} binds to T lymphocytes and to MHC class II molecules; however, because Pep_{263} requires the presence of Int1-negative blastospores for full activity, its effects might best be called “superantigen like.”

These activities amplify the putative roles of Int1 in adhesion, morphogenesis, and virulence derived from earlier mutational analyses [13,14]. Localization of Int1 to the septin ring at the junction of the mother-daughter dyad in budding yeast cells, a key marker for polarity [37], may also provide a critical orientation that enables Pep_{263} to enhance virulence. Although elegant experiments have indicated the importance of mannosylation as a signal for Toll-like receptors and mannose receptors [38–40], Int1 appears not to be extensively modified by mannnosylation, despite several N-glycosylation sites in the sequence.

mAb 163.5, directed against a linear epitope of Pep_{263}, combats these effects, whether induced by Int1-positive *C. albicans* blastospores or by soluble Pep_{263}. Inhibitory mechanisms may include interference with the exposure of Pep_{263}, its degradation, or its ability to link α or β subunits of MHC class II molecules to T lymphocytes expressing the relevant Vβ subsets. As proof of specificity, mAb 163.5 does not inhibit T cell activation, Vβ expansion, or IFN-γ release in response to SEB.

Two separate lines of evidence formed the critical underpinnings for these studies. First, a surprising identity between the extensively characterized superantigen MAM and the amino terminus of Int1 (figure 1) is much stronger than the identity of MAM with other superantigens, such as SEB, SEC-1, mouse mammary tumor virus, and HIV-1 gp160 [15].

Second, in vitro stimulation of human PBMCs with an extract prepared from boiled *C. albicans* blastospores led to a selective increase in gene transcripts for Vβ 5.1 and 5.2 [12]. Forty-eight hours after injection of a *C. albicans* filtrate into the skin, infiltrating T lymphocytes bearing Vβ 5.1 and 8.1 subsets were detected. As noted by Walsh et al. [12], these results raised the tantalizing prospect of a *C. albicans*–derived superantigen-like moiety; however, the reactive species was not further characterized.

There are at least 2 possible explanations for the differences between our results and those of Walsh et al. [12]. First, they used PCR for detection of Vβ transcripts, whereas we used flow cytometry to measure the binding of specific antibodies to expressed Vβ sequences. Second, they boiled their reagents, which may have significantly altered the predominant cell surface proteins of *C. albicans*. Expression of Pep_{263} in *S. cerevisiae* and purification by affinity chromatography should be less damaging to glycosylation and native conformation.

Although Pep_{263} induces T cell activation, specific Vβ expansion, and production of IFN-γ, the requirement for the Int1-negative mutant (figure 5*C* and 5*D*) does not fit the paradigm of a bacterial superantigen. Because heat-killed Int1-negative blastospores and live *S. cerevisiae* are ineffective (figure 6), some *C. albicans*–dependent, active process must be involved. Our experiments have not ruled out a role for Int1-negative blastospores in modifying

the amino terminus of Pep_{263} or in contributing non–Int1-derived peptides to optimize its presentation, much as $SEA_{126–131}$ peptides are required for optimal presentation of TSST-1 [41]. Mutant-induced proteolysis of the KLKH sequence at the carboxyl terminus of Pep_{263} seems unlikely, because removal of H_{263} nullifies activity. Interestingly, SEC-1, SEC-2, and SEC-3 have a KLKN sequence within the region that determines Vβ subset specificity [42]. Further experiments are underway to differentiate among these possibilities.

Although fulminant death from candidemia occurs in but a minority of patients, at least 2 potential clinical interventions are suggested by these studies. In addition to candidal mannans and β-glucans, which serve as potent elicitors of TNF-α [43,44], the production of IFN-γ in response to Int1 and the possible contributions of Toll-like receptors, costimulatory molecules, and HLA alleles may permit modulation of the host response [38,45]. In this regard, human immunodeficiency virus–infected subjects receiving recombinant IFN-γ showed reduced candidal infections and improved 3-year survival in a phase III trial [46]. As a first step to therapeutic neutralization or augmentation, a case-control study correlating HLA-DR haplotype, IFN-γ production, and outcome of candidemia could help to identify patients with overabundant or insufficient IFN-γ. Such approaches have proven extraordinarily informative in identifying genetic risk for superantigen-mediated morbidity during group A streptococcal infection [28,47].

A humanized form of mAb 163.5 may also confer therapeutic benefits through its ability to modulate Int1-dependent T cell activation, Vβ expansion, and IFN-γ production. The identification of a *C. albicans* protein responsible for superantigen-like effects and the development of a monoclonal antibody that inhibits these activities in vitro suggest potential strategies for risk assessment, prevention, and therapy of *C. albicans* bloodstream infection in susceptible populations.

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APPENDIX

SUPPLEMENTARY MATERIALS AND METHODS

PCR-mediated disruption of INT1

Disruption of both alleles of *INT1* at nucleotide 64 of the open reading frame was performed in *C. albicans* strain BWP17. Table 4 shows the PCR primers used to amplify the DNA constructs for gene disruption. For gene disruption, PCR mixtures contained 1 mL of miniprep template DNA (pGEM-URA3 and pRS-ARG4Δ*Spe*1), 2 μL of a 10-mmol/L solution of deoxynucleoside triphosphates, 2 mL of a 10-mmol/L stock of each (forward and reverse) primer, and 0.75 mL of a 100 mmol/L solution of $MgSO_4$; water was inserted until the mixture reached a volume of 50 mL, after which 0.5 mL of a 1 U/mL solution of Vent enzyme (New England BioLabs) was added. The PCR cycling conditions were as follows: 94°C for 5 min followed by 30 cycles of 94°C for 45 s, 50°C for 1 min and 72°C for 3 min with a final extension step of 72°C for 8 min. Strain BWP17 was grown to mid-exponential phase (~0.5 at A_{600} nm) in 50 mL YPD at 30°C. After collection by centrifugation, the cells were washed in 5 mL of LATE buffer (0.1 mol/L of lithium acetate, 10 mmol/L of Tris · HCl [pH 8.0], and 1 mmol/L of EDTA) at room temperature. After centrifugation, the cells were resuspended in 0.5 mL of LATE. One hundred microliters of cells were mixed with 45 mL of each PCR product and 5

mL of carrier DNA (Sigma calf thymus DNA at 10 mg/mL). The transformation mixture was incubated at room temperature for 30 min, mixed with 0.7 mL of PLATE buffer (40% of PEG 3350, 0.1 mol/L of lithium acetate, 10 mmol/L of Tris-HCl [pH 8.0], and 1 mmol/L of EDTA), and incubated at room temperature overnight. The transformation mixture was heated to 42° C for 1 h and centrifuged for 3 min at 2000 *g*. The pelleted cells were gently washed twice in ~0.4 mL of YPD without resuspension. The cells were suspended in 0.2 mL of YPD and spread on CSM agar without arginine and uridine (1.7 g/L of yeast nitrogen base without amino acids, 5 g/L of ammonium sulfate, 2% glucose, and 0.72 g/L of Bio 101 CSM dropout mix minus arginine and uridine). The plates were supplemented with 50 mL of uridine at 50 μg/mL in the first round of disruption, since the *ARG4*-mediated disruptions were done first. A rapid extraction protocol provided template DNA for verifying genotypes of resultant Arg-positive and Arg-positive/Ura-positive colonies [20]. Genotyping PCRs confirmed *INT1* gene disruptions in strain VBIDM2 (figure 9), using 1.5 mL of DNA with locus-specific detection primers (table 4) and the cycling conditions described earlier. The primer combination of INT1U, 5Det-INT1, and 3Det-INT1 was used to detect wild-type and disrupted *INT1* alleles.

Construction of NH_2-tagged 6XHis-Pep_{263} expression vector

pBluescript SK phagemid (Stratagene) was used to assemble the *GAL1* promoter and the sequence coding for the first 263 amino acids of Int1 tagged with 6 histidines at either the amino or carboxy terminus, followed by the *MATα* transcription termination sequence. Plasmid pCG01 [14] containing the *GAL1:10* promoter and the entire *INT1* open reading frame served as a template for PCR amplification with primers DDC11F and DDC11R to yield product PCR1. The *GAL1* promotor flanked by a 5' *Eco*RI site and a 3' *Bam*HI site followed by 13 base pairs of the *INT1* 5' untranslated region (UTR) ending in sequential *Sph*I and *Hin*dIII sites was excised from PCR1 and cloned into pBSIISK as an *Eco*RI/*Hin*dIII fragment to generate pBS · PCR1. Primers DDC15F and DDC15R generated PCR2, which was cloned into the *Sph*I/*Hin*dIII sites of pBS · PCR1 to generate pBS · PCR1,2, thereby placing the signal methionine codon followed by the 6XHIS-Pep_{263} coding sequence and stop codon immediately downstream of the *GAL1* promoter and 5' *INT1* UTR.

The *MATα* transcription termination sequence was PCR amplified from vector pYD1 (Invitrogen), using primers DDC13F and DDC1R. The *Hin*dIII/*Sal*I fragment was cloned into pBS · PCR1,2 to yield pBS · PCR1,2,3. The sequence of the entire *Eco*RI/*Sal*I insert was confirmed using T3 and T7 primers (HHMI/Keck Foundation Biotechnology Resource Laboratory).

Site-directed mutagenesis of Pep_{263} codon 251

The CUG codon is translated as serine in *Candida albicans* and as leucine in *S. cerevisiae* [21,22]. *INT1* contains 6 CUG codons, one of which lies within the first 789 nucleotides of *INT1*. To correct for the difference in the product of the CUG codon between *C. albicans* and *S. cerevisiae*, 1 round of site-directed mutagenesis was performed using the Quickchange XL site-directed mutagenesis kit (Stratagene) to convert the *C. albicans* CTG serine codon at amino acid 251 to TCG, such that expression in *S. cerevisiae* substituted a serine residue for leucine at this position. Briefly, gel-purified mutagenesis primers DCMUT2F and DCMUT2R were used to PCR amplify pBS · PCR1,2,3, according to the manufacturer's instructions. The manufacturer's recommended temperatures were used for denaturation and annealing. After 18 cycles, 10 U of restriction enzyme DPN1 were added to the PCR reaction to digest the methylated template DNA. XL-10 Gold competent cells were transformed with digest. DNA preparations from ampicillin-resistant colonies were sequenced with T3 and T7 primers to confirm the codon change. Expression vector p6XH-Pep_{263} was created by cloning the 1600-bp *Eco*RI/*Sal* I fragment derived from pBS · PCR1,2,3 with TCG at codon 251 into Yep357

(ATCC 37732), a yeast shuttle vector containing the *URA3* selectable marker and a 2-μm replicon to maintain high copy number in *S. cerevisiae*.

Construction of COOH-tagged Pep_{263}-6XHis expression vector

The 452-bp GAL1 promoter excised from pBS · PCR1,2,3 by *Eco*RI/ *Bam*HI digestion was cloned into the corresponding restriction sites of Yep357. p6XH-Pep_{263} served as template for PCR amplification of the 5' UTR of *INT1* followed by the sequence encoding Pep_{263}-6XHis, using primers DDC43F and DDC29R. The *Bam*HI/*Hin*d III digest of this PCR product along with the *Hin*dIII/*Sal*I digest of the *MATα* transcription termination PCR product were cloned into the *Bam*HI/*Sal* I sites of pBSIISK. Sequence was confirmed using the T3 and T7 primers. This *Bam*HI/*Sal*I fragment was cloned into the corresponding restriction sites of Yep357 that contained the *GAL1* promoter to yield expression vector pPep_{263}-6XH.

Expression of Pep_{263}-6XHis

Protease-deficient *S. cerevisiae* strain BJ3501 (table 3) was transformed with expression vector p6XH-Pep_{263} or pPep_{263}-6XH by of use lithium acetate and was plated on complete medium containing 2% glucose minus uridine. Transformants were grown to saturation in liquid medium containing 2% raffinose and 0.01% glucose and subcultured at an OD_{600} of 0.1 in CM minus uridine with 2% raffinose and 2% galactose. Expression of 6XHis-Pep_{263} or Pep_{263}-6XHis was induced by galactose for 18 h at 30° C with shaking at 250 rpm. Yeast cells were washed twice with dH_2O and resuspended at 200 mg wet cell paste per mL of Y-Per dialyzable yeast protein extraction reagent (Pierce). Cells were lysed for 4 h at 30°C with shaking at 250 rpm. Lysates collected after centrifugation of cell debris were filtered through a 0.45-μm cellulose membrane to remove all particulates and were stored at −80°C, pending purification.

Purification of Pep_{263}

6XHis-tagged Pep_{263} was purified by metal chelate affinity chromatography, using HiTrap chelating HP columns (Amersham Biosciences) charged with $NiSO_4$. Lysates diluted with an equal volume of 2× column buffer (40 mmol/L of phosphate, 1 mol/L of NaCl, and 40 mmol/L imidazole [pH 7.4]) were applied to Ni^{2+} columns equilibrated with 1× column buffer. Columns were washed extensively with the same buffer before elution of Pep_{263} with column buffer that contained 300 mmol/L of imidazole. Pep_{263} eluates were equilibrated with PBS (pH 7.4 [Invitrogen]), using 5000-molecular weight-cutoff centrifugal filter devices (Amicon). Protein concentration was determined using the 2-D Quant kit (Amersham). Pep_{263} preparations were run on 12% SDS-PAGE gels and either stained with the SilverXpress kit (Invitrogen) to access purity or transferred to nitrocellulose for Western blotting with $PentHis^{HRP}$ mAb (Qiagen) at 1:2000 or with multiple mAbs (including mAb 163.5) at 10 μg/mL followed by anti-mouse IgG^{HRP} at 1:5000 (Amersham). Blots were developed with Supersignal West Pico chemiluminescent substrate, according to the manufacturers' instructions.

Generation and purification of Pep_{263} truncation constructs

Vector p6XH-Pep_{263} served as template for PCR amplification of the 5' *INT1* UTR and coding sequences of Pep_{262}, Pep_{261}, and Pep_{260}, using primer DDC44F paired with primers DDC35R, DDC34R, and DDC33R, respectively. Constructs substituting the histidine codon at amino acid 263 with the codons for alanine, arginine, or aspartic acid were generated using pPep_{263}-6XH as a template and primer DDC43F paired with primers DDC36R, DDC37R, and DDC40R, respectively. *Bam*HI/*Hin*dIII digests of PCR products were cloned upstream of the *MATα* transcription termination sequence inserted into pBSIISK as a *Bam*HI/*Sal*I fragment. Following sequence confirmation, *Bam*HI/*Sal*I digests of each construct were cloned

downstream of the *GAL1* promoter previously inserted into Yep357, yielding expression vectors p6XH-Pep_{262}, p6XH-Pep_{261}, p6XH-Pep_{260}, pPep_{263}A-6XH, pPep_{263}R-6XH, and pPep_{263}D-6XH. Truncation constructs were purified after expression in *S. cerevisiae*, using metal chelate affinity chromatography, as described above.

Biotinylation of Pep_{263}

One milligram of purified Pep_{263} was incubated with a 10-fold molar excess of biotin in 1 mL of PBS (pH 7.4) for 2 h at 4°C, according to the instructions of the Sulfo-NHS-LC-biotinylation kit (Pierce). Biotinylated Pep_{263} was desalted on a Zeba column by centrifugation at 1000 *g* for 2 min. Biotinylated Pep_{263} was confirmed as functionally active by its ability to increase expression of the IL-2 receptor and to expand the T lymphocytes expressing V*β*2 and 14 subsets, as described above. Biotinylated Pep_{263} was analyzed by Western immunblotting with a 1:5000 dilution of HRP-conjugated streptavidin-PE.

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Figure 1.
Identity between the amino terminus of the *Candida albicans* cell wall protein Int1 and the major histocompatibility complex class II binding site of *Mycoplasma arthritidis* superantigen MAM.

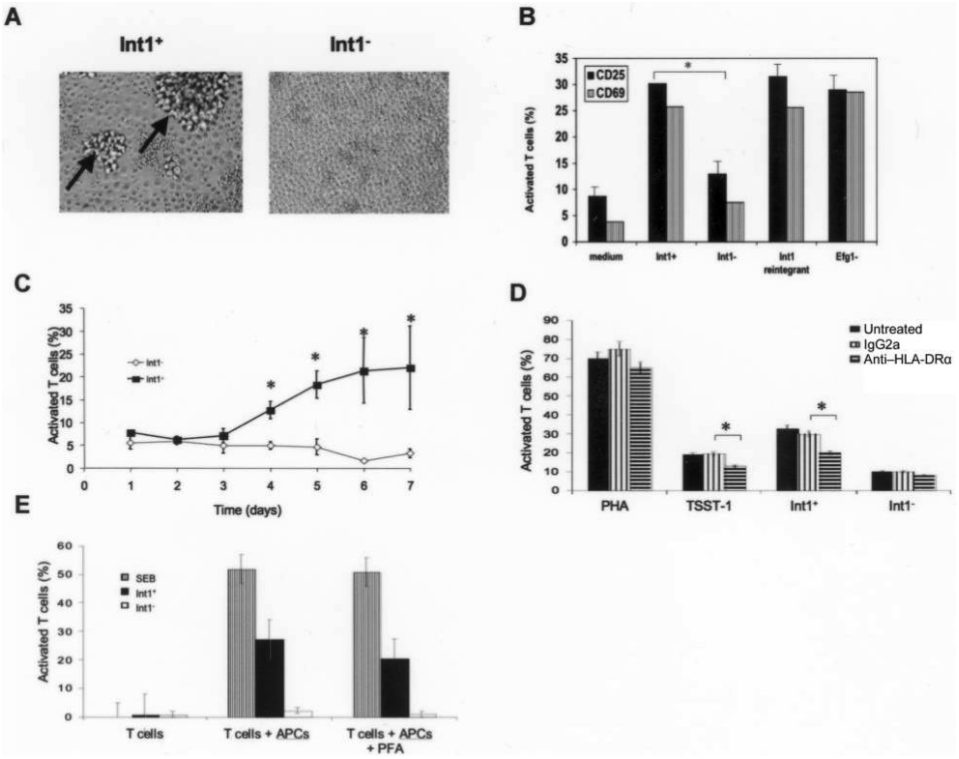

Figure 2.

A, Foci of peripheral blood mononuclear cells *(arrows)* on the fourth day of coculture with Int1-positive ($Int1^+$) *Candida albicans* blastospores or Int1-negative ($Int1^-$) *C. albicans* blastospores. *B*, Peripheral blood mononuclear cell positivity for CD25 or CD69 after coculture with $Int1^+$ *C. albicans* strain (DAY286), $Int1^-$ *C. albicans* strain (VBIDM2), $Int1^+$ reintegrant (VBIDM6−2R), or the Efg1 null mutant. **P* < .002 (*n* = 6 experiments). *C*, Activation of T cells from 5 healthy donors in response to $Int1^+$ (CAF2) or Int1-negative (CAG3) *C. albicans* blastospores. Data are mean values ± 2 SDs. **P* < .02. *D*, Effects of no treatment, IgG2a control antibody, or anti–HLA-DR*α* antibody on T cell activation evoked by phytohemagglutinin (PHA), toxic shock toxin (TSST-1), $Int1^+$ *C. albicans* blastospores (CAF2), or Int1-negative *C. albicans* blastospores (CAG3). Data are mean values ±2 SDs. **P* < .04 (*n* = 3 experiments). *E*, T cell activation induced by stimulation of T lymphocytes alone, T lymphocytes plus antigen-presenting cells, or T lymphocytes plus paraformaldehyde-fixed antigen-presenting cells after stimulation with staphylococcal enterotoxin B (SEB), $Int1^+$ *C. albicans*, or Int1-negative *C. albicans* (*n* = 2 experiments).

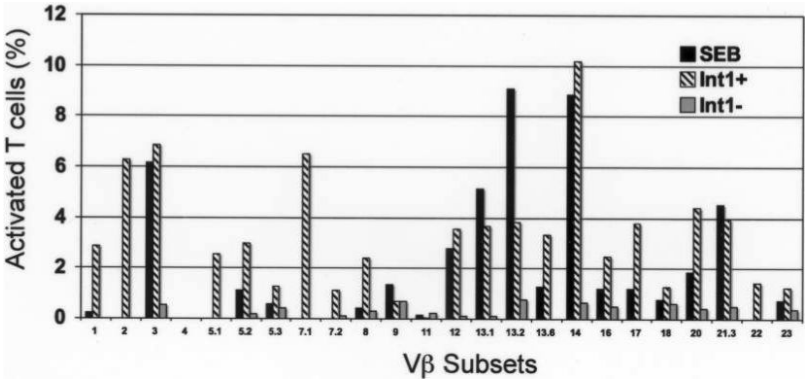

Figure 3.

Expansion of V*β* subsets from a representative donor in response to staphylococcal enterotoxin B (SEB), Int1-positive (Int1^{+}) *Candida albicans* blastospores (CAF2), or Int1-negative (Int1^{-}) *C. albicans* blastospores (CAG3).

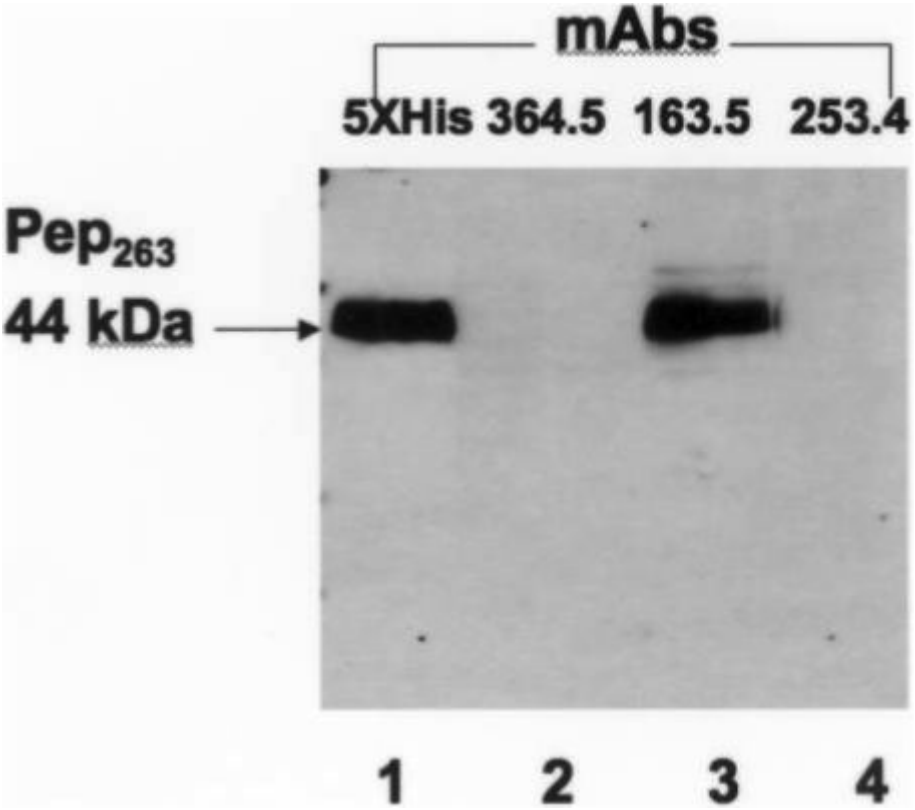

Figure 4.
Recognition of purified Pep_{263} by monoclonal antibodies (mAbs). His-tagged Pep_{263} was identified by a penta-His mAb.

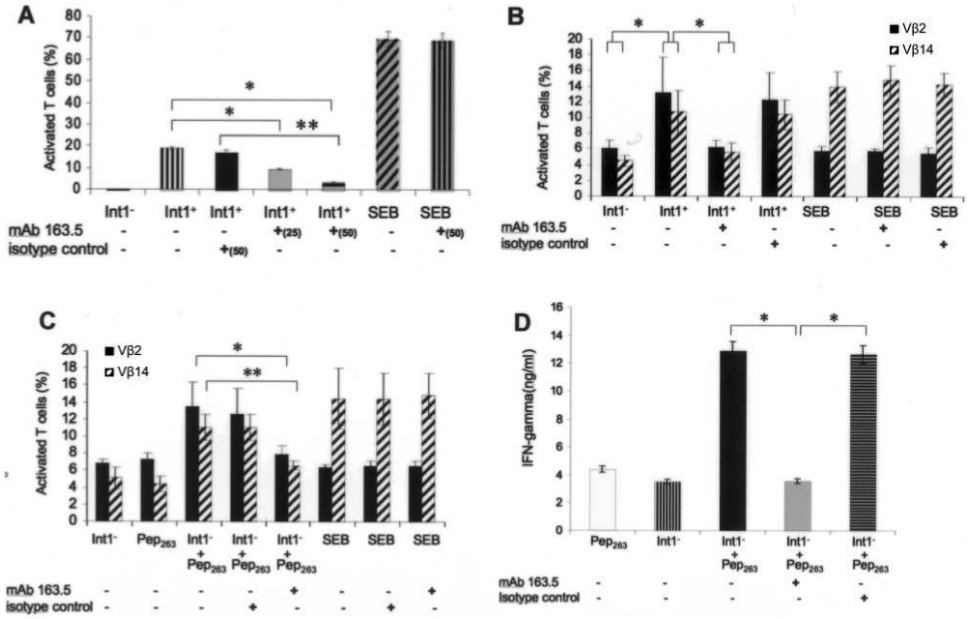

Figure 5.

A and *B*, Inhibitory effects of monoclonal antibody (mAb) 163.5 or IgG1 isotype control mAb on T lymphocyte activation (*A; *P* < .002 and ***P* < .035) or expansion of V*β* subsets 2 and 14 (*B; *P* < .02) induced by Int1-negative (Int1^{-}) *C. albicans* blastospores (VBIDM2) or Int1-positive (Int1^{+}) *C. albicans* blastospores (DAY286). Staphylococcal enterotoxin B (SEB) served as control. Data are mean values ±2 SDs (*n* = 5 experiments). *C* and *D*, Inhibitory effects of mAb 163.5 or IgG1 isotype control mAb on expansion of V*β* subsets 2 and 14 (*C; *P* = .02 for V*β*2 and ***P* = .01 for V*β*14) and secretion of interferon-*γ* (IFN-*γ*) (*D; *P* < .01) induced by soluble Pep_{263} in the absence or presence of Int1^{-} *C. albicans* blastospores (VBIDM2). Data are mean values ±2 SDs (*n* = 6 experiments).

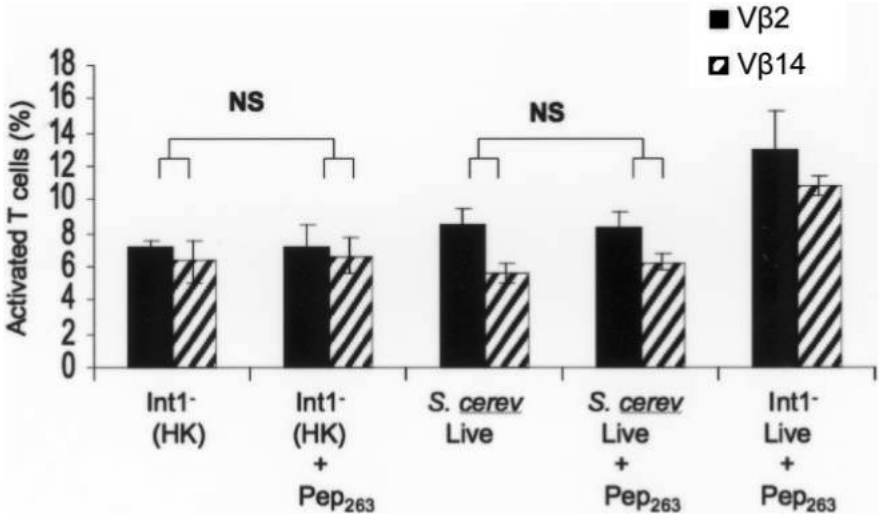

Figure 6.

Expansion of T lymphocytes bearing V*β* subset 2 or 14 by replicating Int1-negative *Candida albicans* or a heat-killed Int1-negative *C. albicans* mutant (VBIDM2) and by replicating *Saccharomyces cerevisiae*, in the absence or presence of Pep_{263}.

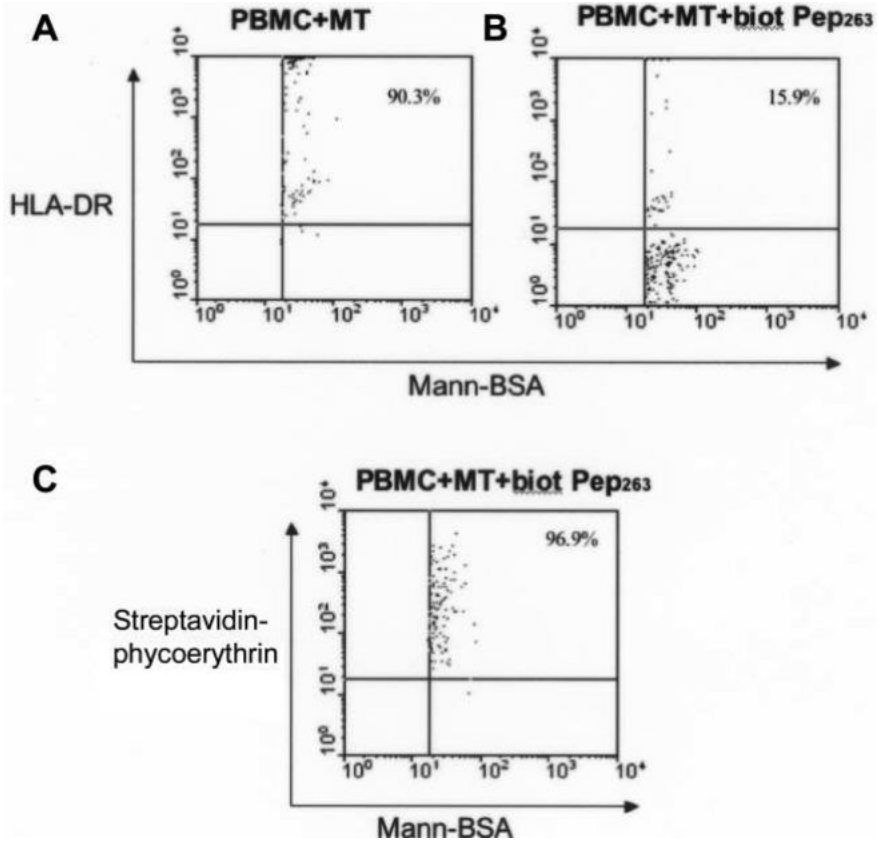

Figure 7.

A, Binding of HLA-DR antibody to antigen-presenting cells and Int1-negative blastospores (MT) in the absence of biotinylated (biot) Pep_{263}. *B*, Binding of HLA-DR antibody to antigen-presenting cells and MT in the presence of biot Pep_{263}. *C*, Binding of Pep_{263} to antigen-presenting cells. Mann-BSA, mannosylated bovine serum albumin; PBMC, peripheral blood mononuclear cell.

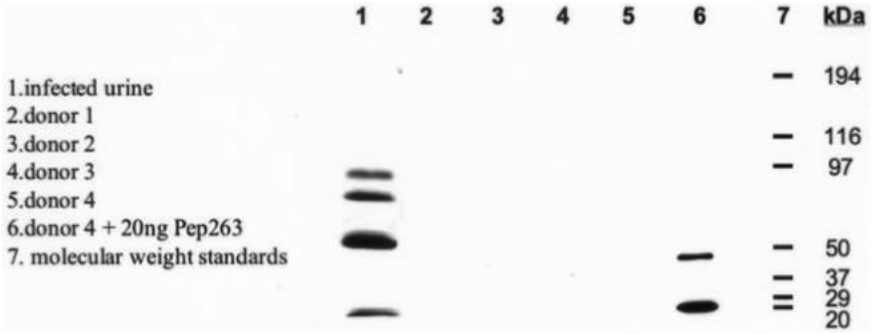

Figure 8.

Western blotting with monoclonal antibody 163.5 for detection of Pep_{263}. *Lane 1*, Urine specimen from a patient with *Candida albicans* fungemia. *Lanes 2–5*, Urine specimens from uninfected control donors. *Lane 6*, Urine specimen from an uninfected control donor plus 20 ng of purified Pep_{263}. *Lane 7*, Molecular weight standards.

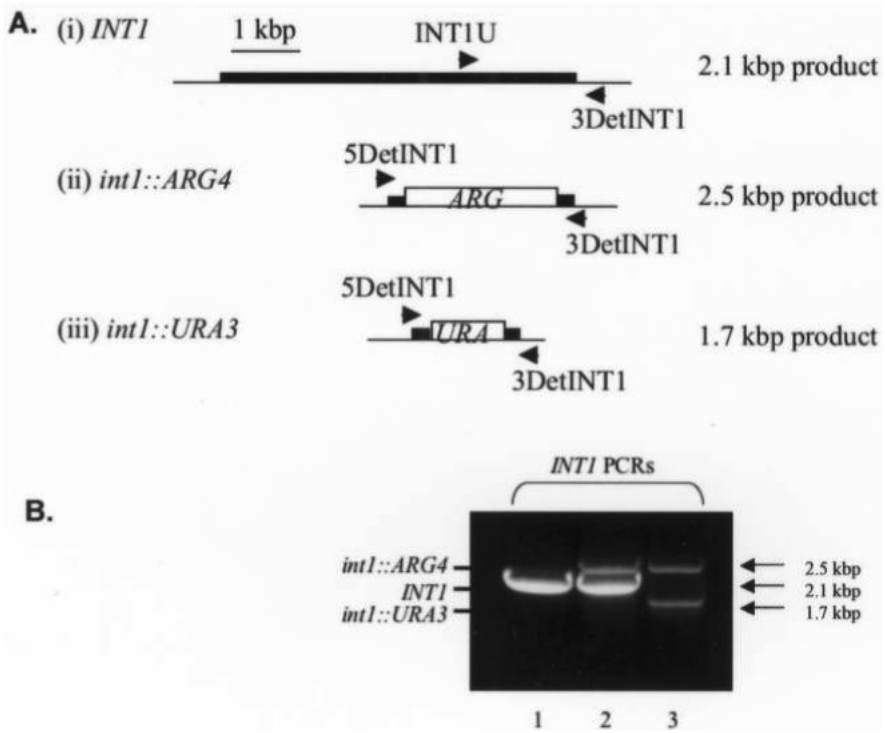

Figure 9.

A, Strategy for polymerase chain reaction genotyping of *INT1* alleles by use of forward primers INT1U and 5det-INT1 and reverse primer 3det-INT1. *B*, Genomic DNA from wild-type BWP17 *(lane 1)*, VBIDM1 *(INT1/int1::ARG4; lane 2)*, and VBIDM2 *(int1::ARG4/ int1::URA3; lane 3)*.

Table 1

Characteristics of *Saccharomyces cerevisiae* and *Candida albicans* strains used in this study.

Organism, strain	Genotype	Int1 status	Reference
S. cerevisiae			
BJ3501	Matα pep4::HIS3 prb1 his3^{-} ura3 can1 gal2	Not available	...
C. albicans			
CAF2	As SC5314 (wild-type), except *URA3/ura3::λimm434*	Positive	[17]
CAI-4[a]	As CAF2, except *ura3::λimm434/ura3::λimm434*	Positive	[17]
CAG3	As CAI-4, except *int1::hisG/int1::hisG-URA3-hisG*	Negative	[14]
Efg1 null	As CAI-4, except *efg1::hisG/efg1::hisG-URA3-hisG*	Positive	[18]
BWP17[a]	ura3::λimm434/ura3::λimm434 his1::hisG/ his1::hisG arg4::hisG/arg4::hisG	Positive	[16]
BWP17wt	As BWP17, except *arg4::hisG::ARG4::URA3/ his1::hisG::HIS1*	Positive	[16]
DAY286	As BWP17, except *ARG4::URA3::arg4::hisG/arg4::hisG*	Positive	[19]
VBIDM2	As BWP17, except *int1::ARG4/int1::URA3*	Negative	Present study
VBIDM6−2	As VBIDM2, except *int1::ARG4/int1::URA3/his1::hisG/ HIS1*	Negative	Present study
VBIDM6−2R	As VBIDM6−2, except *his1::hisG::HIS1-INT1*	Positive	Present study

[a] Used for strain construction but for experiments.

Table 2

Rates of T cell responses to Int1-positive *Candida albicans* and staphylococcal enterotoxin B (SEB).

Stimulus	Percentage of CD25$^+$ T cells, by protein(s) expressed			Percentage of CD69$^+$ T cells, by protein(s) expressed		
	CD4$^+$	CD8$^+$	CD4$^+$CD8$^+$	CD4$^+$	CD8$^+$	CD4$^+$CD8$^+$
C. albicans	42.4	27.9	26.3	36.9	35.0	12.5
SEB[a]	47.4	27.7	24.5	49.0	31.0	15.1

[a] Responses were assayed on the fourth day of coculture.

Table 3

Cytokine responses to staphylococcal enterotoxin B (SEB) and to *Candida albicans* blastospores with or without Int1.

Stimulus	Cytokine, mean level ± SD, ng/mL				
	IFN-γ	TNF-α	IL-6	IL-4	IL-10
C. albicans					
Int1 positive	15.7 ± 5.3[a]	1.8 ± 0.5[b]	30.7 ± 21.7[b]	0	0
Int1 negative	4.0 ± 0.5	2.8 ± 1.9	29.9 ± 17.7	0	0
SEB	66.4 ± 7.7	1.8 ± 0.2	5.3 ± 1.4	0	0

NOTE. Responses were assayed on the fourth day of coculture. INF-γ, interferon-γ; IL-4, interleukin-4; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor—α.

[a] $P < .01$, compared with Int1-negative strains.

[b] P = not significant, compared with Int1-negative strains.

Table 4

Supplementary primers used in this study.

Primer	Sequence, 5′-3′
Targeted gene disruption of *INT1*	
INT1FKO	ATGAACTCAACTCCAAGTAAATTATTACCGATAGATAAACATTCTCATTTACAATT ACAGCCTGTTTTCCCAGTCACGACGTT
INT1RKO	TTGAAGCATCAAATTTAGCCATGGTTGACGTCTGAACCGATTTCTATAGATAATTT CTTGTAAATTGTGGAATTGTGAGCGGATA
Detection of targeted *INT1*	
5Det-INT1	TTCTCCATCTATCCATTCCTC
3Det-INT1	CAAAATGGGCATATATTTGCC
INT1U	GTGCGGGTTCTAAACCAA
Construction of Pep_{263} expression vectors	
DDC11F	ATAGAATTCACGGATTAGAAGCCGCCGAGCGGGTGAGAGC
DDC11R	AGCAAGCTTGATATCGATCGCATGCTTCTTTTAACAATGGATC
DDC15F	ATAGCATGCACCACCATCACCATCACATGAACTCAACTCCAAGTAAATTATTACCG
DDC15R	AGCAAGCTTTCAGTGCTTTAATTTCATATCTGTCAATGCCTCTGGTTCCGAATTGAC
DDC13F	AGCAAGCTTGTTTAAACCCGCTGATCTGATAACAA
DDC1R	TAAGTCGACAATTCTCTTAGGATTCGATTCACATTC
DDC43F	CCCCGGATCCATTGTTAAAAGAAGCATGAACTCAACTCCAAGTAAATTATTACCG
DDC29R	CCCCAAGCTTTCAATGGTGATGGTGATGGTGCTTTAATTTCATATCTGTCAATGC
CUG to CTG mutagenesis	
DCMUT2F	CAACGAAGTCAAT**TCG**GAACCAGAG
DCMUT2R	CTCTGGTTC**CGA**ATTGACTTCGTTG
Pep_{263} COOH deletion mutants	
DDC44F	CCCCGGATCCATTGTTAAAAGAAGCATGCACCACCATCACCATCACATGAACTCA
DDC33R	CCCCAAGCTTTCATTTCATATCTGTCAATGCCTCTGGTTCCGAATTGACTTC
DDC34R	CCCCAAGCTTTCATAATTTCATTGTCAATGCCTCTGGTTCCGAATTGAC
DDC35R	CCCCAAGCTTTCACTTTAATTTCATATCTGTCAATGCCTCTGGTTCCGAATT
Amino acid 263 substitution	
DDC43F	CCCCGGATCCATTGTTAAAAGAAGCATGAACTCAACTCCAAGTAAATTATTACCG
DDC36R	CCCCAAGCTTTCAATGGTGATGGTGATGGTG**CGC**CTTTAATTTCATATCTGTCAA
DDC37R	CCCCAAGCTTTCAATGGTGATGGTGATGGTG**GCG**CTTTAATTTCATACTTGTCAA
DDC40R	CCCCAAGCTTTCAATGGTGATGGTGATGGTG**ATC**CTTTAATTTCATACTTGTCAA
Sequencing	
T3	AATTAACCCTCACTAAAGGG
T7	GTAATACGACTCACTATAGGGC

NOTE. Mutated codons are in bold font.