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A Novel Th Cell Epitope of Candida albicans Mediates Protection from Fungal Infection

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Fungal pathogens are a frequent cause of opportunistic infections. They live as commensals in healthy individuals but can cause disease when the immune status of the host is altered. T lymphocytes play a critical role in pathogen control. However, specific Ags determining the activation and function of antifungal T cells remain largely unknown. By using an immunoproteomic approach, we have identified for the first time, to our knowledge, a natural T cell epitope from Candida albicans. Isolation and sequencing of MHC class II-bound ligands from infected dendritic cells revealed a peptide that was recognized by a major population of all Candida-specific Th cells isolated from infected mice. Importantly, human Th cells also responded to stimulation with the peptide in an HLA-dependent manner but without restriction to any particular HLA class II allele. Immunization of mice with the peptide resulted in a population of epitope-specific Th cells that reacted not only with C. albicans but also with other clinically highly relevant species of Candida including the distantly related Candida glabrata. The extent of the reaction to different Candida species correlated with their degree of phylogenetic relationship to C. albicans. Finally, we show that the newly identified peptide acts as an efficient vaccine when used in combination with an adjuvant inducing IL-17A secretion from peptide-specific T cells. Immunized mice were protected from fatal candidiasis. Together, these results uncover a new immune determinant of the host response against Candida spp. that could be exploited for the development of antifungal vaccines and immunotherapies. The Journal of Immunology, 2012, 188: 5636–5643.

Oppportunistic fungi have emerged as increasingly frequent causes of infections. Although some fungi live as commensals in healthy individuals, they cause severe disease when host defenses are breached. Candida spp. are among the clinically most relevant fungal agents and account for a wide range of diseases (1). Whereas Candida albicans remains the most frequently isolated species, non-albicans species of Candida also act as serious health hazards and are thus gaining increasing attention (2). The spectrum of these non-albicans species is large, and some are only distantly related to C. albicans, such as for instance Candida glabrata, which is a closer relative to the pathogenic Saccharomyces cerevisiae in terms of its codon usage and its monomorphic lifestyle. Nevertheless, C. glabrata is an increasingly frequent cause of fungal infections, and because of its inherent drug tolerance, it is one of the most dreaded fungal species, along with C. albicans (3).

Invasive candidiasis ranks among the four most frequent nosocomial infections and is most often a consequence of general immunosuppression, prolonged treatment with antibiotics, or breaches in anatomical barriers (e.g., surgery or central venous catheter) (3). It is associated with a mortality of >30% and a high morbidity in those who survive. Other forms of candidiasis are less severe, including superficial infections of the skin, the oropharyngeal mucosa, and the vagina (4–6). These diseases are often associated with defects in cellular immunity but they can also occur in individuals that have no obviously weakened immune system. Although mucocutaneous Candida infections are not themselves life-threatening, they constrict the patient’s quality of life severely. The increasing incidence of resistance to antifungal drugs and an inherent drug tolerance of some fungi cause major obstacles to an efficient treatment. This situation evokes an urgent need for the development of novel therapeutic approaches against fungal infections and has renewed the interest in the development of vaccination strategies against mycoses, including passive vaccination with Abs as well as induction of cell-mediated immunity by active immunization (7, 8). However, a thorough knowledge of the immune response that clears or controls infecting fungi is essential for the development of efficacious immunotherapies and protective vaccines.

Despite the well-accepted notion that Th cells are a crucial element in the development of optimal protective immunity against fungal diseases (1, 9), important basic principles of T cell activation remain unknown. First and foremost, the antigenic determinants recognized by fungus-specific T cells have not yet been mapped. The cognate interaction between T cells and APCs is a prerequisite for their activation, clonal expansion, and differentiation into effector cells. The identification of fungal Ags is
thus key to understanding antifungal T cell immunity. Detailed knowledge on the presented antigenic peptides would greatly enhance the possibility to detect, enumerate, and characterize fungus-specific T cells and would constitute important progress in the design and development of potential antifungal vaccines.

We have thus set out to characterize natural T cell epitopes of *C. albicans*. By using an immunoproteinomic approach, we have identified a naturally processed and MHC class II (MHCII)-bound peptide that is recognized by up to a fourth of all *C. albicans*-specific Th cells. This high frequency is remarkable, given the large number of peptides that can possibly be generated from the complex fungal proteome. The novel antigenic peptide is derived from a cell wall-associated adhesin, which critically contributes to fungal pathogenicity. It is functionally conserved in many non-albicans *Candida* species of high clinical importance, including close relatives of *C. albicans* such as *Candida dubliniensis* and even very distant species of the genus such as *C. glabrata*. The biological relevance of the new epitope is underlined by the finding that epitope-specific T cells are not restricted to mice but are also present in the human T cell repertoire. Memory Th cells from all tested individuals responded to peptide stimulation. Finally, we could show that vaccination of mice with the identified peptide epitope mediates T cell-dependent protection from candidiasis. These data offer new insights into basic mechanisms of antifungal immunity and provide a promising candidate for specific therapeutic applications.

Materials and Methods

**Mice, cells, and fungal strains**

C57BL/6 mice were purchased from Janvier Elevage, and JHT mice (10) were bred at our animal facility. All mice were used at 6–12 wk of age. All mouse experiments were conducted in strict accordance with the guidelines of the Swiss Animal Protection Law and were approved by the Veterinary Office of the Canton of Zurich, Switzerland (license number 184/2009).

The mouse DC<sup>1940</sup> cell line, a CD8<sup>+</sup>-like dendritic cell line derived from a CD11c<sup>-/−</sup> transgenic mouse (11), was grown in IMDM with GlutaMAX I supplemented with 1% FCS, 50 mM HEPES, and 1% penicillin and streptomycin (all from Invitrogen).

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The *C. albicans* laboratory strain CS5314 was used in all experiments unless otherwise indicated. Clinical isolates of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *Candida krusei*, and *Candida tropicalis* were obtained from O. Petrinin (Cantonal Institute of Microbiology, Bellinzona, Switzerland). All *Candida* strains were inoculated in YPD medium at a density of OD<sub>600</sub> 0.1 and grown for 15 h at 30°C. For heat inactivation, 10<sup>6</sup> yeast cells per milliliter were boiled for at least 60 min.

**Epitope identification**

DC<sup>1940</sup> cells (9 × 10<sup>6</sup>) were infected with *C. albicans* at a multiplicity of infection of 3. 1A<sup>-/−</sup>-bound peptides were isolated 4 h postinfection as previously described (12). Briefly, infected cells were lysed in PBS containing 1.2% (w/v) CHAPS and complete protease inhibitor (Roche). Cells were centrifuged and the soluble fraction containing the MHC–peptide complex was applied to an affinity column consisting of an anti-MHCII Ab (clone M5-114; BioXCell) coupled to CNBr-activated Sepharose 4B (GE Healthcare). Peptide-loaded MHCII molecules were eluted with 0.2% trifluoroacetic acid, and the peptides were then separated from the MHCII molecules by size exclusion with Amicon filter tubes (10 kDa; Millipore). Purified peptides were separated on an in-house-made reverse-phase tip column (7 μm × 80 mm) packed with C<sub>D</sub> material (3 μm, 200 Å, AQ, Bischoff) attached to an Eksigent nano-HPLC system (Eksigent Technologies). The column was equilibrated with 97% solvent A (A: 1% acetonitrile, 0.2% formic acid in water) and 3% solvent B (B: 80% acetonitrile, 0.2% formic acid in water). Peptides were eluted using the gradient 0–50 min, 3–40% B, 50–60 min, 50–97% B, at a flow rate of 0.2 μl/min. High accuracy mass spectra were acquired with an LTQ-Orbitrap XL (Thermo Scientific) in the mass range of 300–2000 m/z. Up to five or up to three of the most intense ions were selected for data-dependent tandem mass spectrometry (MS/MS) acquisition in the linear ion trap or in the Orbitrap, respectively. Only doubly and triply charged ions were chosen for fragmentation using collision-induced fragmentation. Target ions already selected for MS/MS were dynamically excluded for 60 s. After data collection, peak lists were generated using Mascot Distiller software 2.3.2 (Matrix Science).

All MS/MS data were analyzed using Mascot 2.3 (Matrix Science). MS spectra were searched against the Swiss-Prot database (release January 2010) that was concatenated with an in-house–built contaminant database and concatenated with the decayed Swiss-Prot database. Precursor ion mass tolerance was set to 7 ppm, and the fragment ion mass tolerance was set to 0.6 Da. Oxidation of methionine was specified as a variable modification.

Scaffold (version Scaffold_3.00.08; Proteome Software) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at >99.0% probability at a FDR of 2.0% by the Peptide Prophet algorithm (13). Peptide identifications were accepted if they could be established at >99.0% probability. Protein probabilities were assigned by the Protein Prophet algorithm (14).

For peptide affinity predictions, the Immune Epitope Database (IEDB) Analysis Resource (http://tools.immuneepitope.org) was used. pALs and pATG peptides were synthesized at EMC Microcollections. The mannoprotein 65 (MP65) peptide pool was from JPT Peptide Technologies, and the p41 peptide (15) was a kind gift from N. Khanna (Basel, Switzerland).

**Infections**

Mice were infected with 2.5 × 10<sup>6</sup> CFU *C. albicans* sublingually as described (16) without immunosuppression. In some experiments, mice were infected twice via the same route and with the same dose with an interval of at least 3 wk between the two infections. In vaccination experiments, mice were infected intravenously with 3 × 10<sup>6</sup> CFU *C. albicans* and monitored for morbidity over a period of maximally 28 d. Mice were euthanized when they showed severe signs of pain or distress or lost 15% of their body weight.

**Immunizations**

Mice were immunized s.c. with 200 μg pALs in IFA (Sigma) mixed with either 1 mg curdian (Wako) or 5 μg CpG1668 (Mycrosynth). T cell responses in the draining lymph nodes were analyzed on day 7 after immunization. Alternatively, mice were challenged with *C. albicans* 3 wk after immunization. For immunization with clinical isolates of *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. krusei*, and *C. glabrata*, mice were injected in the footpads with 5 × 10<sup>5</sup> heat-inactivated yeast cells.

**Analysis of mouse T cell responses**

Cervical lymph node from sublingually infected mice were removed at the peak of the T cell response, which was on day 10 after primary infection or on day 4 after secondary infection (A.G. and S.L.L., unpublished observations). Draining lymph node from immunized mice were removed on day 7 after immunization. Lymph node cells were restimulated in vitro for 6 h with DC<sup>1940</sup> cells that were pulsed with pALs (5 μg/ml) or 2.5 × 10<sup>7</sup>/ml heat-inactivated *C. albicans* or left unpulsed. Brefeldin A (10 μg/ml; AppliChem) was added for the last 4 h. In some experiments, an anti-MHCII Ab (clone M5-114, 10 μg/ml) was added. Cells were stained in ice-cold PBS supplemented with 2 mM EDTA, 10% FCS, and 0.02% sodium azide with anti-CD4 (clone RM4-5) and anti-CD3e (clone 145-2C11), fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences), and then resuspended in Perm/Wash buffer (BD Biosciences) containing anti-IL-17A (clone TC11-18H10.1) and anti–IFN-γ (clone XMG1.2) Abs. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo software (Tree Star). For cytokine quantification by ELISA, a fixed number of cervical lymph node cells was restimulated for 4 h with peptide (5 μg/ml unless indicated otherwise) or 2.5 × 10<sup>5</sup>/ml heat-inactivated *C. albicans* (or *C. dubliniensis, C. tropicalis, C. krusei, and C. glabrata*, as indicated) without adding DC<sup>1940</sup> cells, and cytokines were quantified by sandwich ELISA. For blocking experiments, an MHCII-specific Ab (clone M5-114) was added at 10 μg/ml.

**Human T cell responses**

Experiments with human blood samples were approved by the Ethics Committee of the University Hospital of Zurich and were performed and analyzed anonymously. PBMCs were purified from the blood of healthy donors via a Ficoll gradient and stimulated for 10 d with 2.5 × 10<sup>5</sup>/ml heat-inactivated *C. albicans* and IL-2. For testing their Ag specificity, the cells were then restimulated overnight with fresh autologous monocytes that had been pulsed with pALs (5 μg/ml) or 2.5 × 10<sup>5</sup>/ml heat-inactivated *C. albicans* or were left untreated. IL-2 and IL-17A cytokine
secretion was determined by ELISPOT assay according to the manufacturer’s instructions (Mabtech AB). ELISPOT plates (MAB S45; Milipore) were analyzed on an AID ELISPOT reader Version 3.4. For blocking experiments, an MHCII-specific Ab (clone TÜ39) was added at 2.5 μg/ml.

**HLA typing**

HLA typing was done by PCR at the interdisciplinary HLA typing laboratory of the University Hospital of Zürich.

**Statistical analysis**

Statistical significance was determined by a two-tailed unpaired *t* test using GraphPad Prism (GraphPad Software, La Jolla, CA) with *p* < 0.05, **p** < 0.01, and ***p*** < 0.001.

For morbidity experiments, statistical significance was determined by a log rank test using GraphPad Prism with *p* < 0.05.

**Results**

**Identification of I-^A^b-bound peptide epitopes from C. albicans**

To identify naturally processed Candida-derived peptide epitopes in an unbiased manner, we used an immunoproteomic approach. DC^1940^ cells were infected in vitro with *C. albicans*, and peptide-loaded MHCII complexes were isolated by affinity chromatography. MHC-bound peptides were then eluted and sequenced using liquid chromatography coupled to MS/MS (Fig. 1A). The identified sequences were annotated by comparison with the *C. albicans* genome database. Four *C. albicans*-derived peptides could be identified, three of which displayed largely overlapping sequences differing in length by one or two amino acids at the C-terminal end (Fig. 1B). They were derived from a homologous region of the two closely related proteins Als1 and Als3. A high degree of sequence similarity was also found with all other members of the Als family (Supplemental Table I). The longest of the three identified Als1/Als3-derived peptides covering the amino acid residues 236–253 was chosen for further analysis and is referred to henceforth as pALS. According to the IEDB T cell epitope prediction tool [SMM align method (17)], the affinity of pALS binding to I-^A^b is predicted to be moderate with I_C50 = 264 nM. The fourth identified peptide was derived from the autophagy related protein 11, and we thus named it pATG (Fig. 1B). It is only 9 aa in length, and its binding affinity to I-^A^b was predicted to be low with I_C50 = 2375 nM.

**A large fraction of Candida-specific T cells recognizes pALS**

To validate the identified MHCII ligands as bona fide T cell epitopes, we used an experimental model of candidiasis. Oropharyngeal infection of mice with *C. albicans* was found to induce a robust T cell response. A significant proportion of the endogenous CD4^+^ T cells isolated from the cervical lymph nodes of infected mice responded to the fungus by producing IL-17A, whereas cells from uninfected mice did not show any response. This was apparent by intracellular cytokine staining (Fig. 2A, 2B) and by measuring secreted IL-17A in the supernatant after restimulation with heat-killed organisms (Fig. 2C). The population of Candida-specific Th17 cells was expanded 10-fold and produced increased levels of IL-17A after reinfection with the fungus (Fig. 2A–C and data not shown). Notably, no IFN-γ or IL-4 production could be detected after either primary or secondary infection (Fig. 2A and data not shown).

We then asked whether the Candida-responsive T cell population contained any cells specific for the identified pALS peptide. When restimulating cervical lymph node cells from infected mice, we found that 15–25% of the *C. albicans*-specific CD4^+^ T cells recognized pALS (Fig. 2A, 2B). This was equally true after primary and secondary infection (0.06% pALS-responsive versus 0.3% *C. albicans*-responsive T cells in primary infected mice; 0.6% pALS-responsive versus 3% *C. albicans*-responsive T cells in secondary infected mice). The strong T cell stimulatory activity of pALS was also reflected by the amount of secreted cytokine that could be detected in the supernatant of peptide-stimulated T cells (Fig. 2C). The activity of the peptide was dependent on its presentation by MHCII because cytokine production in response to pALS was blocked when an anti-MHCII Ab was added to the restimulation cultures (Fig. 2D, 2E). In conclusion, these data show that the newly identified pALS peptide is a bona fide T cell epitope derived from *C. albicans* that is recognized, at least in C57BL/6 mice, by a prominent fraction of Th cells.

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**FIGURE 1.** Identification of I-^A^b-bound peptide epitopes from *C. albicans*. (A) Representative MS/MS spectrum for one of the identified peptides. (B) Sequence, source protein, amino acid positions, and predicted binding affinity (according to the SMM align method of the IEDB prediction tool) for the four identified MHCII ligands. Boldface letters indicate the core sequence of each peptide predicted to bind the MHCII binding groove.
To test whether our second candidate of isolated peptides also functioned as a T cell epitope, we restimulated cervical lymph node cells from infected mice with pATG. Only a very small fraction of *C. albicans*-specific CD4+ T cells recognized this peptide, and cytokine production in response to pATG was detectable only in T cells isolated from mice that had undergone two rounds of infection (Fig. 3). In the same experiments, we also included a peptide pool of MP65, which was previously reported to act as a fungus-derived Ag (18, 19), and an *Aspergillus fumigatus*-derived peptide (p41) that has been proposed to cross-stimulate *Candida*-specific T cells (15). Similar to pATG, MP65 induced IL-17A production from *Candida*-specific T cells after secondary infection, but the response was much lower than the one induced by pALS (Fig. 3), and it was below the detection limit in T cells from mice that were infected only once (Fig. 3B). We could not detect any cytokine production from *C. albicans*-specific T cells restimulated with the p41 peptide (Fig. 3). In summary, pALS-specific T cells primed in response to *C. albicans* outnumbered by far any other previously proposed T cell Ag specificities.

*pALS is conserved in different Candida ssp.*

To investigate whether the pALS epitope is conserved in different non-*albicans* species of *Candida*, we immunized mice with pALS mixed with curdlan, a fungal β-glucan that acts as a very potent

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**FIGURE 2.** pALS is a CD4+ T cell epitope in C57BL/6 mice. (A–C) Mice were infected sublingually with *C. albicans* either once (1° infection) or for two consecutive rounds (2° infection). Cervical lymph nodes were restimulated in vitro with heat-inactivated *C. albicans* (C.a.) or pALS peptide or left untreated (unstim). Cytokine production was analyzed by intracellular staining and flow cytometry (A, B) or by ELISA (C). Numbers in (A) indicate percent IL-17A+ cells among CD3+CD4+ T cells. (D and E) Cervical lymph node cells from 2° infected mice were restimulated with pALS as described for (A)–(C), but in presence or absence of anti-MHCII Ab. Cytokine production was measured by intracellular staining and flow cytometry (D) or by ELISA (E). Data shown are representative of at least three (A, C) or two (D, E) independent experiments. In (B), each symbol represents one mouse, and the data are representative of three independent experiments. In (C) and (E), each bar represents the mean and SD of triplicate restimulations with pooled lymph nodes from at least three mice per group. *p < 0.05, **p < 0.01, ***p < 0.001. nd, Not detected.

**FIGURE 3.** Comparison of different *Candida*-derived Ag candidates for their T cell stimulatory capacity. (A) Cervical lymph node cells from *C. albicans*-infected mice (2° infection) were restimulated with 5 μg/ml pALS, pATG, MP65, or p41, and IL-17A production was analyzed by intracellular cytokine staining followed by flow cytometry. Numbers indicate percent IL-17A+ cells among CD3+CD4+ T cells. (B) Cervical lymph node cells from primary and secondary infected mice were restimulated with the indicated range of concentrations of pALS, pATG, MP65, or p41, and IL-17A secretion in the supernatant was measured by ELISA (note the logarithmic scale). Data shown are representative of two independent experiments. Each symbol in (B) represents the mean and SD of duplicate restimulations. *p < 0.05, **p < 0.01, ***p < 0.001.
adjunct for priming Th17-biased CD4+ T cell responses (20, 21), and probed the reactivity of the induced T cells with different Candida species. Surprisingly, pALS-specific T cells responded to all tested Candida by secreting IL-17A, whereas no response was detected in T cells isolated from unimmunized control mice (Fig. 4A). The strength of the response correlated with the degree of conservation of the peptide sequence in the Als orthologs (Supplemental Table I) and in turn with the phylogenetic relationship between the species. Whereas the response of pALS-specific T cells to C. dublinsiensis and C. tropicalis, two close relatives of C. albicans, was only slightly reduced compared with the maximal response triggered by the peptide itself, C. krusei and C. glabrata stimulated pALS-specific T cells less effectively. Nevertheless, the epitope was sufficiently well conserved throughout all tested species to elicit IL-17A from pALS-specific T cells. The fact that the pALS epitope was conserved in even distantly related species of Candida became also obvious when we immunized mice with heat-inactivated preparations of the different Candida species and then probed the activated T cells from these mice for their reactivity to pALS (Fig. 4B). Again, the response was strongest with the species most closely related to C. albicans (from which pALS was isolated) and declined in descending order according to the degree of relationship. The conservation of the identified peptide pALS in different species of Candida, of which some are only distantly related to C. albicans, strongly underlines its significance as an important T cell epitope.

pALS is recognized by human memory T cells

We next wondered whether pALS also served as an epitope for human T cells. Candida-specific memory T cells can be found in the blood of most healthy individuals (22), who have been exposed to the commensal fungus during their lifetimes. Notably, those T cells belong primarily to the Th17 subset (22). To interrogate the repertoire of human Candida-specific T cells, we stimulated PBMCs isolated from healthy volunteers with the pALS peptide. To increase the sensitivity of the assay, we polyclonally expanded C. albicans-specific PBMCs before exposing them to the peptide and quantifying IL-2– and IL-17A–secreting cells as readout for their ability to recognize the epitope. To our surprise, we found that all tested donors that reacted to C. albicans did also respond to pALS (Fig. 5A). As expected, the number of detected spot-forming cells varied between donors. An anti-HLA class II Ab that was added to the cultures inhibited the response completely in all donors (Fig. 5B). Despite the limited number of individuals tested, it seemed that certain HLA alleles were associated with increased peptide responsiveness. All individuals displaying a strong response to pALS carried a DRB3* and/or DQB1*03 allele (data not shown). In support of our experimental data, the predicted affinity of both alleles for binding to pALS was intermediate compared with that of other HLA alleles, which showed lower affinities. This initial observation will be corroborated by the analysis of a larger group of individuals. In conclusion, these data show that the newly identified C. albicans-derived T cell epitope was recognized in association with not only mouse MHC but also human HLA class II molecules and that the response was not restricted to individuals with one specific HLA allele.

Immunization of mice with pALS mediates protection from candidiasis

Finally, we sought to evaluate the potential of pALS as a vaccine candidate and thus tested whether T cells specific for pALS bear any protective capacity against candidiasis. For this, mice were immunized with the peptide mixed with curdlan. Three weeks later, the mice were challenged with a high dose of C. albicans. Because oropharyngeal candidiasis is a self-limiting infection in immunocompetent mice, which resolves within a few days and independently of lymphocytes (data not shown), the mice were infected via the i.v. route in this experiment. Whereas the majority of the control mice displayed a high degree of morbidity as a consequence of infection, we found that immunization with pALS greatly increased the number of mice that were protected from fatal systemic candidiasis (Fig. 6A). Immunization with

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**FIGURE 4.** The pALS epitope is conserved in non-albicans Candida species. (A) Mice were immunized s.c. with pALS and curdlan inIFA (+pALS) or with curdlan in IFA alone (~pALS). Draining brachial lymph nodes were removed on day 7 and restimulated with pALS or heat-inactivated conidia of two to three different clinical isolates of C. albicans (C.a.), C. dublinsiensis (C.d.), C. tropicalis (C.t.), C. krusei (C.k.), or C. glabrata (C.g.). IL-17A secretion was quantified by ELISA. Each symbol represents the mean of duplicate restimulations with an individual clinical isolate. Data shown are representative of two independent experiments. (B) Mice were immunized with heat-inactivated conidia of C. albicans, C. dublinsiensis, C. tropicalis, C. krusei, or C. glabrata (one clinical isolate each) 7 d before draining popliteal lymph nodes were removed and restimulated with pALS or heat-inactivated conidia of the respective strains used for immunization. IL-17A secretion was quantified by ELISA. The results are depicted as relative amount of cytokine produced in response to pALS restimulation in comparison with the amount of cytokine produced in response to restimulation with the respective immunization strain. The response to restimulation with the immunization strain was set to 100% for each sample. Each bar represents the mean and SD of triplicate restimulations. Data shown are representative of three independent experiments.

**FIGURE 5.** pALS is recognized as an epitope by human T cells. (A) PBMCs from healthy volunteers were stimulated with pALS or with heat-inactivated C. albicans (C.a.) or left untreated (unstim). IL-2– and IL-17–producing spot-forming cells (SFC) were detected by ELISPOT assay. (B) PBMCs from healthy volunteers were stimulated with pALS as in (A), but in presence or absence of an anti-MHCII Ab. Each symbol represents data from one healthy donor. In (B), the plotted values were subtracted of the background of medium stimulation.
that pALS-specific Th17 cells can protect mice from candidiasis. In conclusion, these data show whereas curdlan favored a Th17 response (Fig. 6C, 6E; Supplementary Fig. 1; and data not shown). The choice of adjuvant for vaccination appeared to be critical because mice that were immunized with pALS and curdlan in IFA alone or with curdlan alone or together with an unrelated lymphocytic choriomeningitis virus-derived Th cell epitope was not sufficient for protection (Fig. 6A and data not shown) despite the fact that curdlan can induce anti-β-glucan Abs (21). Moreover, the antifungal effects of the pALS vaccine did not require B cells, because vaccination of B cell-deficient mice led to similarly prolonged survival (Fig. 6B), although with slightly altered kinetics, and a minor contribution of B cells can thus not be excluded. The enhanced survival of pALS plus curdlan immunized mice correlated with the presence of pALS-specific IL-17A–producing CD4+ T cells, which additionally recognized C. albicans as demonstrated by their ability to respond to restimulation with the heat-killed organism (Fig. 6C). The choice of adjuvant for vaccination appeared to be critical because mice that were immunized with pALS and the TLR9 agonist CpG were not protected from candidiasis despite the fact that Candida-specific CD4+ T cells were primed to a similar extent in this setting (Fig. 6D, 6E). However, T cells primed in presence of CpG differentiated into IFN-γ-secreting Th1 cells, which lacked the ability to produce IL-17, whereas curdlan favored a Th17 response (Fig. 6C, 6E; Supplementary Fig. 1; and data not shown). In conclusion, these data show that pALS-specific Th17 cells can protect mice from candidiasis.

Discussion

T cell-mediated immunity plays a crucial role in host protection from fungal infections. Whereas major progress has been made recently in understanding the immune mechanisms mediating fungal control, the Ag specificities of antifungal T cells have remained elusive. We have been able for the first time, to our knowledge, to isolate a naturally processed peptide epitope from C. albicans, which is immunologically highly relevant. This newly identified peptide is recognized by almost every fourth Candida-specific Th cell isolated from infected mice. We were surprised to find such a high abundance of a single Ag specificity among the entire population of Candida-responsive CD4+ T cells, given the high complexity of the Candida proteome and hence the expected large number of potential MHCI-binding ligands. Consistent with the selective induction of Th17 immunity during oropharyngeal candidiasis, pALS-specific T cells isolated from the cervical lymph nodes of orally infected mice secreted IL-17A, which is immunologically highly relevant. This newly identified peptide was identified on the basis of its capacity to bind to I-Ab in mice did also serve as an epitope for human Th cells. Although the extent of the reaction varied between different HLA haplotypes, cells from all individuals tested were able to mount a pALS-specific response. Only a few nonviral pathogen-derived Th cell epitopes have to date been identified experimentally (25,
26). Our data show that the unbiased approach of purifying and sequencing MHCIIBound peptides from infected Ag-specific cells is an efficient and direct method to isolate highly relevant pathogen-derived epitopes. In contrast to peptide binding algorithms, which still display a high failure rate because of the scantily confined motifs for MHCIIBinding, this method directly analyzes candidate T cell epitopes that are processed and presented by infected Ag-specific cells.

The peptide that we isolated is derived from a conserved region of two closely related members of the agglutinin-like sequence (ALS) gene family (27), which mediate adherence to and invasion of host cells (27, 28). The capacity to invade host tissues is an important virulence determinant of C. albicans. Mutant strains lacking Als1 or Als3 display reduced although not completely abolished pathogenicity (27, 28). Functional redundancy of different Als proteins seems to apply not only to their function as adhesins/invasins but also to their role as Ag source. pALS-specific T cells responded equally well to restimulation with Als1- or Als3-deficient strains compared with the response to wild-type C. albicans (data not shown), indicating that different Als proteins can compensate for each other in providing the epitope fragment. The high degree of sequence conservation of the pALS peptide in all members of the ALS family underlines the significance of this epitope and suggests that it may have been selected through evolution. Strikingly, even distant relatives of C. albicans encode sequence homologs bearing epitopes that can be recognized by pALS-specific T cells. By mounting a T cell response directed against a virulence factor, the host avoids the escape of the fungus from immune recognition. This phenomenon of T cell epities being derived from virulence factors, which are unlikely to acquire mutations, has also been observed for other pathogens. A prominent example is made by influenza A virus (29). The majority of all published CD4+ T cell epitopes of this virus is derived from hemagglutinin A, which is responsible for targeting host cells for infection and which is thus strongly associated with viral pathogenicity (30). Listeriolysin O from Listeria monocytogenes is another example of a virulence factor that is at the same time source protein for an MHCIIBound T cell epitope (31).

In addition to the Als proteins, the 65-kDa mannoprotein MP65 from Candida also act as a Th cell Ag as previously reported (18, 19) and confirmed in this study, although the latter only activates a minor proportion of Candida-specific T cells, and the precise range of amino acids binding to MHCIIB have not been determined in case of MP65. Nevertheless, the common feature of these two Candida-derived T cell Ags is their localization to the fungal cell wall. This makes them easily accessible to host proteases for processing and MHCIIBinding and may give them a selective advantage over Ags hidden in the cytoplasm for serving as a source of T cell epitopes. This feature may also apply to other yet undefined T cell epitopes from Candida and even more generally for other classes of microbes bearing a thick and rigid cell wall.

Importantly, when the pALS peptide was used as vaccine, this resulted in protection of mice from experimental candidiasis. The beneficial effect of immunization was peptide-specific and dependent on MHCIIMediated presentation. In addition, the choice of adjuvant strongly affected the anti-candidal effect of the vaccine. Whereas induction of IL-17A secretion by peptide-specific T cells correlated with host protection, Th1 type cytokines did not. This is in line with the association of IL-17 immunity with fungal diseases (6, 32). Our data suggested thus that pALS-specific T cells promoted fungal control and reduced disease severity during candidiasis. Together with the finding that pALS recognition by Th cells is not restricted to the mouse, this makes the epitope an attractive candidate for prophylactic therapy against candidiasis. In this respect, it is interesting to note that the 432-aa N-terminal part of Als3 (Als3pN) is currently used as a vaccine candidate in preclinical and clinical studies (33). The protective effects of this vaccine were suggested to depend on Th1 and Th17 cells (34), but the Ag specificity of these T cells remained unclear. Our data may now provide the missing link. The effect of the Als3p-N vaccine is likely to rely on the fact that this protein fragment comprises the amino acid stretch that defines the pALS epitope.

Our data on the identification of a naturally processed and presented fungal T cell epitope in mouse and human is thus not only a major advancement in understanding basic mechanisms of antifungal immunology. Its high degree of conservation in multiple clinically highly relevant Candida species and its capacity to induce protection from disease make it a promising candidate for specific therapeutic applications.

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Disclosures

The authors have no financial conflicts of interest.

References


### Supplementary Table 1: Conservation of the pALS epitope

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>Accession no.</th>
<th>aa position</th>
<th>IC50 (nM)</th>
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<td>Als1 (C. albicans)</td>
<td>KGLNDWNYPVSSESFSYT</td>
<td>P46590</td>
<td>236-253</td>
<td>264</td>
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<td>264</td>
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<td>411</td>
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1. No sequence data available C. krusei
2. Grey underlined letters indicate sequence deviation from pALS
3. Swiss-Prot annotations
4. Predicted value for affinity to I-A<sup>b</sup>. Peptides with IC<sub>50</sub> values <50 nM are considered high affinity; <500 nM intermediate affinity; and <5000 nM low affinity
**Vaccination with pALS mediates protection from candidiasis.**

**(A)** Mice were immunized subcutaneously with pALS and curdlan in IFA (curdlan + pALS) or with curdlan in IFA alone. Seven days after immunization, the draining lymph nodes were isolated and re-stimulated with pALS (5 μg/ml) or 2.5x10⁵/ml heat-inactivated C. albicans, or left untreated (unstim), and cytokine secretion was analyzed by ELISA.

**(B)** Mice were immunized subcutaneously with pALS and CpG in IFA (CpG + pALS) or with CpG in IFA alone and cytokine production by draining lymph node T cells was analyzed as in (A).

Each bar represents the mean and standard deviation of triplicate re-stimulations with pooled lymph nodes from at least three mice per group. Data shown are representative of at least two independent experiments. nd, not detected.