Direct Binding of the pH-Regulated Protein 1 (Pra1) from *Candida albicans* Inhibits Cytokine Secretion by Mouse CD4+ T Cells

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Opportunistic infections with the saprophytic yeast *Candida albicans* are a major cause of morbidity in immunocompromised patients. While the interaction of cells and molecules of innate immunity with *C. albicans* has been studied to great depth, comparatively little is known about the modulation of adaptive immunity by *C. albicans*. In particular, direct interaction of proteins secreted by *C. albicans* with CD4+ T cells has not been studied in detail. In a first screening approach, we identified the pH-regulated antigen 1 (Pra1) as a molecule capable of directly binding to mouse CD4+ T cells in vitro. Binding of Pra1 to the T cell surface was enhanced by extracellular Zn ions which Pra1 is known to scavenge from the host in order to supply the fungus with Zn ions. In vitro stimulation assays using highly purified mouse CD4+ T cells showed that Pra1 increased proliferation of CD4+ T cells in the presence of plate-bound anti-CD3 monoclonal antibody. In contrast, secretion of effector cytokines such as IFNγ and TNF by CD4+ T cells upon anti-CD3/anti-CD28 mAb as well as cognate antigen stimulation was reduced in the presence of Pra1. By secreting Pra1, *C. albicans* thus, directly modulates and partially controls CD4+ T cell responses as shown in our in vitro assays.

Keywords: *Candida albicans*, ph-regulated antigen 1 (Pra1), CD4+ T cells, immune evasion, cytokine secretion

INTRODUCTION

*Candida albicans* is a commensal on human skin and mucosal surfaces. In situations of immunosuppression, *C. albicans* may, however, become pathogenic. Prominent examples of *C. albicans*-induced pathologies are mucosal or skin candidiasis as well as *C. albicans* septicemia in ICU and/or HIV/AIDS patients (Klein et al., 1984; Sangeorzan et al., 1994; Leroy et al., 2009). In the latter cohorts, loss of CD4+ T cells is the hallmark of immunodeficiency. This highlights the importance of CD4+ T cells for controlling *C. albicans* infections in humans.
To allow commensalism, *C. albicans* has evolved a number of evasion strategies to protect itself from attack by the host’s immune system (Zipfel et al., 2011). Immune evasion might be beneficial during commensal growth as it avoids potentially harmful inflammation and adaptive immune responses. The very same mechanisms might, however, contribute to *C. albicans* pathogenicity once epithelial barriers are disturbed. Research into the factors driving *C. albicans* pathogenicity led to the discovery of the pH-regulated antigen 1 (Pra1) as a multifaceted immune evasion protein (Zipfel et al., 2011). Pra1 interferes with innate immunity including the complement cascade on different levels thereby efficiently protecting the fungus from complement attack. Moreover, Pra1 scavenges zinc from the host, thus, ensuring sufficient supply of the fungus with this bivalent cation (Citiulo et al., 2012). For both functions, complement inhibition and zinc scavenging, Pra1 is first secreted, interacts with complement proteins or zinc in solution and then the complex of Pra1 and its binding partner are recruited back to the *C. albicans* surface (Zipfel et al., 2011; Citiulo et al., 2012).

As Pra1 is secreted by *C. albicans* we hypothesized that this fungal protein might also be capable of bypassing fungal sensing by DCs (Romani, 2011) and of directly interacting with CD4+ T cells, thus, modulating T cell function in its favor. Having established that recombinantly expressed Pra1 binds to mouse T cells, we, thus, analyzed the impact of Pra1 on T cell activation, expansion and effector cytokine secretion. Our data suggest that *C. albicans* directly modulates anti-fungal immunity through secreting T cell-binding proteins like Pra1.

**MATERIALS AND METHODS**

**Mice**

Wild-type C57BL/6j mice and OT-II C57BL/6j mice (Barnden et al., 1998) were bred in the animal facility of the Institute for Virology and Immunobiology, University of Würzburg. CD55−/− C57BL/6 mice (Sun et al., 1999) were obtained from the University of Cardiff and also bred in our animal facility. Crry−/− C57BL/6 (Ruseva et al., 2009) and CD59a−/− C57BL/6 mice (Holt et al., 2001) were bred at Cardiff University. All mice were kept in a specified pathogen free conventionally housed environment and used for experiments between 6 and 21 weeks of age.

**Antibodies and Flow Cytometry**

The following antibodies and reagents were used to stain mouse cells: anti-CD4 Alexa Fluor 647 (clone RM4-5), anti-IFNy Alexa Fluor 488 (clone XMG1.2), Streptavidin-PerCP (all BioLegend, San Diego, CA, USA), anti-CD25 biotin (clone 7D4, BD Pharmingen, Franklin Lakes, NJ, USA) anti-CD55 unconjugated (RIKO-3, Biolegend), anti-CD11b FITC (clone M1/70), anti-B220 Alexa Fluor 647 (clone RA3-6B2) (all BD Pharmingen), anti-CD3 PerCp (clone 145-2C11, BioLegend).

For staining of Pra1 a polyclonal antibody was raised in rabbits by immunization with purified recombinant Pra1. Aspf2-antiserum was generated by immunization of mice with purified recombinant Aspf2. Secondary polyclonal antibodies for staining of primary antibodies were goat anti-mouse-Ig FITC and donkey anti-rabbit-Ig PE (Jackson ImmunoResearch, West Grove, PA, USA). Flow cytometry was performed on a FACSCalibur or LSR II flow cytometer using either CellQuest or DIVA software (BD Bioscience, Franklin Lakes, NJ, USA). We used FlowJo (TreeStar) to further analyze FACS data.

**Protein Expression and Purification**

Recombinant Pra1wt and Aspf2 were expressed in *Pichia pastoris* and isolated via the His-tag (Luo et al., 2009; Bacher et al., 2014). For protein overexpression and purification, the *pra1* gene encoding a protein lacking the C-terminal 61- amino acid was amplified from the pPICZaB-Pra1wt clone using the sequence specific forward primers ACTGAATTCTGAGGACATCGCAGTTTGAAGAAGCG CGGCACGTTACGTTACC and reverse primer ACTCTTG ACTGGCGCACCTTGGCACGGAATTC, containing the restriction sites EcoRI and KpnI. The PCR product and plasmid pPICZaB were enzymatically digested, ligated, and sub-cloned into pPICZaB (Invitrogen, Karlsruhe, Germany). The resulting plasmid pPICZaB-Pra1ΔC61 was transfected and overexpressed in *Pichia pastoris* X33 (EasySelect™Pichia Expression Kit, Invitrogen, Karlsruhe, Germany). The Pra1ΔC61 was purified as described (Luo et al., 2009).

**Organ Processing and FACS Stainings**

Single cell suspensions were generated by mashing cervical, axillary, inguinal and mesenteric lymph nodes or spleens through a cell strainer (Falcon, Pittsburg, PA, USA). Single cell suspensions of splenocytes were then subjected to red cell lysis by hypotonic shock. Lymph node and red cell-lysed spleen cells were then resuspended in buffered salt solution (BSS) containing 0.1% (w/v) bovine serum albumin (BSA). Total lymph node or spleen cells were incubated with Pra1 (10 µg/ml) or Aspf2 (10 µg/ml) in PBS at 37°C for 30 or 45 min. For investigation of the influence of zinc on Pra1 binding, ZnCl2 (1, 10, or 100 µM) was added while incubating cells together with Pra1. After washing bound Pra1 or Aspf2 were detected with a polyclonal anti-Pra1- (rabbit) or anti-Aspf2- (mouse) antiserum followed by PE anti-rabbit Ig polyclonal antibody (donkey; Dianova) or FITC anti-rabbit Ig polyclonal antibody (donkey; Dianova). For further stainings the samples were blocked with normal rabbit serum (1:500) or normal mouse Ig (20 µg/ml, Sigma) followed by incubation with anti-CD4 mAb (Alexa Fluor 647) alone or together with anti-CD3 mAb (PerCP). For Kv1.3 detection, ShK-F6CA (0.3 µg/ml; Bachem AG, Bübendorf, Switzerland) was incubated together with mAb against cell surface proteins for 30 min at room temperature (Beeton et al., 2003).

**Polyclonal Stimulations in Vitro**

To test for co-stimulation lymph node cells from WT mice were first enriched for CD4+ T cells (MagniSort Mouse CD4 T cell Enrichment Kit, eBioscience, Santa Clara, CA, USA or CD4+ T cell isolation kit, Miltenyi) resulting in at least 93% pure CD4+ T cells. Afterwards the cells were stained with anti-CD4-Alexa Fluor 647 and CD4+ T cells sorted using the FACS Aria III (BD) cell sorter (100% purity). For analysis of cell proliferation cells
were incubated for 5 min at RT with 5 μM Vybrant CFSE SE Cell Tracer Kit (CFSE, Life Technologies, Carlsbad, CA, USA). Anti-CD3-mAb (2.5 μg/ml, clone 145-2C11, BioLegend) was bound to 96-flat bottom-plates (Greiner, Kremsmuenster, Austria) after incubation on the plate o/n at 4°C dissolved in 0.1 M NaHCO₃-buffer (pH 9). After coating of the plate, non-specific binding was blocked by incubation with normal mouse immunoglobulin (20 μg/ml in BSS/0.1% BSA (w/v), Sigma Aldrich, St. Louis, MO, USA) at 37°C for 30 min. 1 × 10⁵ CFSE-labeled CD4⁺ T cells were added per well and anit-CD28 mAb E18 (Exbio) (Dennehy et al., 2006) (1 and 10 μg/ml) or Pra1 (0.1 μg/ml – 100 ng/ml) were added in solution. For the cultures, we used complete RPMI 1640 medium supplemented with 1 mM sodium pyruvate, non-essential amino acids MEM (0.05–2 mM), 100 U/ml penicillin and 100 μg/ml streptomycin, 2 mM L-glutamine (all Gibco) and 10% (v/v) heat-inactivated fetal calf serum. After 3 days, CD4 and CD25 were stained and expression of both markers, together with CFSE dilution, was analyzed by flow cytometry. To determine cytokine secretion, magnetically purified CD4⁺ T cells were cultured with plate-bound anti-CD3 mAb in the presence of soluble Pra1 (1–100 ng/ml) or anti-CD28 mAb (clone E18) according to the manufacturer's instructions.

**Cytokine Detection in Culture Supernatants**

Concentrations of the indicated cytokines were determined in culture supernatants using LEGENGplex™ (Biolegend) according to the manufacturer's instructions.

**Statistics**

Summary graphs were generated and statistical testing was done using Excel © 14.4.1 (Microsoft) and Prism 4.0c © (GraphPad). P < 0.05 was considered statistically significant.

**Ethics Statement**

Stadt Würzburg (City of Würzburg) and UK Home Office (PPL 30/3038) approved breeding of the mice used in this study and the animals were culled by Annex IV approved techniques in accordance with Directive 2010/63/EU.

**RESULTS**

**Pra1 Directly Binds to Mouse CD4⁺ T Cells in a Zinc-Dependent Manner**

As Pra1 expression of *C. albicans* is induced upon contact with human cells and as it has already been shown to strongly modulate innate immunity (Zipfel et al., 2011), we studied direct binding of Pra1 to mouse CD4⁺ T cells *in vitro*. We used recombinantly expressed Pra1 purified from *Pichia pastoris* for staining and found that Pra1 bound to all splenocytes in a dose-dependent manner (*Figure 1A*, left histogram). Among total splenocytes CD11b⁺ CD3⁻ monocyctic cells bound Pra1 particularly well (*Figure 1A*, middle left histogram), which was expected as complement receptor 3 (CR3, Mac1, CD11b/CD18) had been identified as a cellular receptor for Pra1 on mouse leukocytes (Soloviev et al., 2007, 2011). Splenic B (*Figure 1A*, middle right histogram) and T cells (*Figure 1A*, right), i.e., CD4⁺ and CD8⁺ T cells (*Figure 1B*), also clearly bound Pra1, albeit to a lesser extent than the monocyctic cells.

As Pra1 binds zinc (Citiulo et al., 2012) we tested whether zinc influences Pra1 binding to mouse CD4⁺ T cells. Zn²⁺ which is found in serum at a concentration of 10 μM (Feske et al., 2012) and beyond increased Pra1 binding to mouse CD4⁺ T cells (*Figures 2A–C*) with plateau levels of binding reached after 30 min of incubation (*Figure 2D*). Moreover, a *pra1* deletion mutant encoding a Pra1 protein lacking the putative zinc-binding...
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FIGURE 1 | Candida albicans Pra1 binds to monocytes, B and T cells. (A) Total mouse splenocytes were incubated with Pra1 in the absence of exogenously added zinc. Pra1 binding was detected indirectly as described in Section “Materials and Methods”. Pra1 bound to all splenocytes (left histogram), CD11b+CD3− monocytes (middle left histogram), B220highCD3− B cells (middle right histogram) and T cells (right histogram) dose-dependently. (B) Total mouse lymph node cells were gated for CD4+ and CD8+ T cells as depicted and Pra1 binding to these subpopulations determined (in the absence of exogenously added zinc). (A,B) One of at least two experiments with similar result is shown.

domain (Pra1 Δ238–299) (Citiulo et al., 2012) showed almost no binding to mouse CD4+ T cells (Figure 2E).

The zinc binding capacity of Pra1 is shared by its homolog in A. fumigatus, i.e., the Aspf2 protein (Citiulo et al., 2012). We, therefore, used recombinantly expressed (P. pastoris) and purified Aspf2 and tested whether Aspf2 also directly binds to mouse CD4+ T cells. Aspf2, in contrast to Pra1, however, did not bind to the mouse T cells even when ZnCl2 was added to the buffer (Figure 3). Thus, Pra1, but not Aspf2, directly binds to mouse CD4+ T cells and Pra1 binding is enhanced in the presence of extracellular zinc.

Complement Regulatory Proteins Expressed by Mouse CD4+ T Cells Do Not Interact with Pra1

So far, only complement receptor 3 (CR3, Mac1, CD11b/CD18) has been identified as a cellular receptor for Pra1 on mouse leukocytes (Soloviev et al., 2007, 2011). As the staining pattern of Pra1 showed that Pra1 binds similarly well to all mouse CD4+ T cells (Figure 1B) we hypothesized that a complement regulatory protein expressed by all mouse T cells might be the receptor for Pra1. Therefore, we analyzed Pra1 binding to CD4+ T cells of CD55−/− mice in more detail as CD55, Crry, and CD59a are the three complement-regulatory proteins expressed by mouse T cells (Miwa and Song, 2001). While CD4+ T cells of CD55−/− mice were clearly devoid of CD55 expression at the cell surface (Figure 4A) binding of Pra1 was not reduced in the absence of CD55 (Figure 4B). Moreover, addition of zinc also increased binding of Pra1 to mouse CD4+ T cells of CD55−/− mice (Figure 4B). Apart from CD55−/− mice we also studied binding of Pra1 to CD4+ T cells of Crry−/− and CD59a−/− mice, which was also not reduced (Figure 4C). Therefore, Pra1 does not seem to interact with any of the three complement regulatory proteins expressed on the surface of mouse CD4+ T cells.

Pra1 Binding Co-stimulates Mouse CD4+ T Cells

To gain further insight into the functional consequences of Pra1 binding to CD4+ T cells, we first studied its impact on T cell activation and proliferation in vitro. To avoid confounding effects
through the interaction of Pra1 with CD11b/CD18 expressed by monocyctic cells in our cultures, we FACS-sorted mouse CD4\(^+\) T cells which lack CD11b/CD18 to more than 99% purity. Stimulation of these highly pure CD4\(^+\) T cells by plate-bound anti-CD3 mAb and titrated amounts of Pra1 led to a dose-dependent increase in proliferation and CD25 expression similar to what we observed by adding an anti-CD28 mAb (Dennehy et al., 2006) (Figures 5A,B). Moreover, Pra1 truly induced a co-stimulatory signal in the T cells as in the absence of CD3 stimulation Pra1 did not activate the cells (Figure 5C). The same effect was observed for the anti-CD28 mAb (Figure 5C).

Binding of Pra1 to mouse CD4\(^+\) T cells, thus, enhanced T cell activation and proliferation, which comprise the first steps of the adaptive immune response.

Cytokine Secretion by In Vivo Generated Mouse CD4\(^+\) Memory T Cells is Inhibited in the Presence of Pra1

While the activation of naïve T cells and clonal expansion mark the beginning of the CD4\(^+\) T cell response, secretion of cytokines such as IFN\(\gamma\) characterize its effector and
memory phase. We, therefore, analyzed cytokines in the supernatants of purified CD4⁺ T cells, containing in vivo generated memory T cells, stimulated via plate-bound anti-CD3 mAb and soluble Pra1 or anti-CD28 mAb (Dennhly et al., 2006) (Figure 6A). In contrast to its co-stimulatory effect on T cell activation and proliferation Pra1 suppressed secretion of both Th1 and Th2 cytokines (Figure 6A). Only IL-17 secretion appeared not to be affected, while secretion of IL-10 was below the detection limit in these experiments. Seemingly at odds with our observation concerning expression of the IL-2 receptor α-chain, CD25 (Figure 5), IL-2 concentrations were also reduced in the presence of Pra1. We assume that this reflects increased IL-2 consumption through increased receptor expression rather than reduced IL-2 production (Malek, 2008) uniting these two findings. To further test the capacity of Pra1 to inhibit cytokine secretion we added Pra1 to purified CD4⁺ T cells which we co-stimulated with anti-CD3/anti-CD28 mAb-coated Dynabeads® (Figure 6B). Even under these conditions, which more faithfully mimic T cell-antigen presenting cell interactions than stimulation via plate-bound antibodies, Pra1 reduced cytokine, i.e., IFNγ, secretion by the CD4⁺ T cells (Figure 6B). The same was true for the supernatant of cultured C. albicans containing the whole array of secreted fungal proteins (Figure 6B).

Apart from binding to CD4⁺ T cells, Pra1 interacts with CD11b/CD18 integrin (Mac1) expressed by monocyte and granulocytic cells (Soloviev et al., 2011). To test whether Pra1 also suppresses IFNγ secretion in the presence of Mac1-expressing antigen-presenting cells (APCs) we stimulated total splenocytes from T cell receptor-transgenic OT-II mice with 1 μM OVA-peptide 323–339 in the presence of 100 or 1 ng/ml Pra1 (Figure 6C). Also under these conditions Pra1 inhibited IFNγ secretion by the OT-II CD4⁺ T cells.

Both in the presence of recombinant Pra1 as well as C. albicans supernatant, secretion of cytokines by mouse CD4⁺ T cells was, thus, reduced.

**FIGURE 3** The Pra1 homolog of A. fumigatus, Aspf2, does not bind to mouse CD4⁺ T cells. Representative staining of Aspf2 on gated CD4⁺ T cells derived from the spleen as performed before with Pra1. Aspf2- and Pra1-staining on cells from the same mouse with Pra1- and Aspf2- (black) and background staining (gray). The bar diagram shows relative MFI of the Aspf2-signal with and without ZnCl₂ (relative MFI(Aspf2) = MFI(Aspf2)/MFI(background); means ± SD; n = 3).

**Depending on the Strength of the TCR Signal Pra1 Also Reduces Secretion of IFNγ by In Vitro Generated Th1 Cells**

T cells isolated from healthy mice producing effector cytokines are by definition mostly resting memory T cells. During acute invasive C. albicans infection or C. albicans-induced inflammation the fungus, however, mainly encounters effector T cells. Therefore, we first deliberately generated OT-II Th1 effector cells during a five-day culture in vitro followed by a two-day resting phase and subsequent re-stimulation of the Th1 cells in the presence of APCs and different concentrations of peptide antigen and Pra1 (Figure 7A). Addition of Pra1 to Th1 cells stimulated with 0.1 μM OVA peptide reduced IFNγ secretion into the supernatant (Figure 7A, middle), while this was not the case at 1 μM OVA peptide (Figure 7A, right). Analyzing intracellular IFNγ expression by the Th1 cells after PMA/ionomycin re-stimulation, further, showed that the reduced secretion of IFNγ into the culture supernatant in the presence of Pra1 was not due to a per se lower capacity of the Th1 cells to produce IFNγ. Without OVAp re-stimulation the expression of IFNγ by the Th1 cells was, however, reduced suggesting that Pra1 increases the threshold for stimulation-induced cytokine secretion by CD4⁺ T cells. Incubation of Th1 cells with Pra1 showed, in comparison to OT-II CD4⁺ T cells cultured under Th0 conditions in parallel, that Th1 cells bind Pra1 better than Th0 cells (Figures 7C,D) suggesting that differentiated effector memory Th1 cells are a primary target of Pra1. In autoreactive pathogenic T cells Kv1.3 has been shown to be the main voltage-gated potassium channel and blocking the channel with the ShK peptide inhibits the autoreactive pathogenic T cells in animal models of autoimmunity in vivo (Beeton et al., 2001) and in cell cultures of human T cells in vitro (Wulff et al., 2003). Using a fluorescently labeled ShK peptide we observed that the Th1 cells expressed more Kv1.3 channels than Th0 cells and that Pra1 binding and Kv1.3 expression were positively correlated in Th1 cells (Figure 7E). Pra1, thus, preferentially bound to effector/memory Th1 cells inhibiting IFNγ secretion provided TCR stimulation did not surpass a certain threshold.

**DISCUSSION**

In this study, we describe the direct interaction of the secreted C. albicans protein Pra1 with mouse CD4⁺ T cells. Binding of Pra1 to the CD4⁺ T cells was enhanced by extracellular Zn²⁺. Moreover, Pra1 binding inhibited cytokine secretion from CD4⁺ T cells in vitro thus constituting a novel immune evasion mechanism for C. albicans.

In line with its known capacity to scavenge Zn²⁺ ions (Citiulo et al., 2012) Pra1 bound more efficiently to mouse CD4⁺ T cells in the presence of extracellular zinc than in its absence (Figure 1). This activity was in contrast to what we observed for Aspf2, the zinc-binding Pra1-homolog of A. fumigatus (Citiulo et al., 2012). Aspf2 did not bind to mouse CD4⁺ T cells – either in the presence or absence of Zn²⁺ (Figure 3). As both proteins carry a HIS-tag, which, of course, by itself is capable of binding Zn²⁺ (Evers...
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FIGURE 4 | Comparison of Pra1 binding to CD4+ T cells of wild-type and CD55−/− mice. (A) CD55 staining of lymphocytes from WT (black) and CD55−/− mice (gray). As a staining control, WT lymphocytes were incubated without the anti-CD55 primary mAb (dashed dark gray). (B) Binding of Pra1 to lymph node CD4+ T cells from WT and CD55−/− mice. Gray: Staining control without primary mAb. Left bar diagram show relative MFI of Pra1 signal in WT and CD55−/− cells (relative MFI(Pra1) = MFI(Pra1)/MFI(background); means ± SD; n = 3; two-sided t-test). Right diagram depicts MFI of Pra1 staining of CD55−/− CD4+ T cells in the presence of different concentrations of ZnCl2 (mean, upper and lower quartile, field of values, n = 3). (C) Comparison of Pra1 binding to splenic CD4+ T cells of WT, Crry−/− and CD59a−/− mice (n = 3-8 mice/group; means ± SD; two-sided t-test; *p < 0.05).

et al., 2008), the enhanced binding of Pra1 to mouse CD4+ T cells after addition of ZnCl2 was not merely mediated by the HIS-tag. Moreover, even under conditions where we did not add ZnCl2 during the staining procedure we detected a positive signal for Pra1 binding (Figures 1, 2). This data implies that the Pra1 binding to the surface of the CD4+ T cells is not strictly zinc-dependent and/or that free zinc present in preparations of lymph node cells and splenocytes might be sufficient to allow for Pra1 binding.

The molecular basis for the enhanced Pra1 binding mediated by ZnCl2 is so far not clear. We envisage that Zn2+ binding might induce a conformational change in Pra1 as has been described for many other Zn2+-binding proteins (Ebert and Altman, 2008). Such a structural change has, however, not yet been described for Pra1.

While the receptor for Pra1 on the surface of mouse CD4+ T cells is still elusive, CR3 (CD11b/CD18, Mac-1) expressed by neutrophils and monocyctic cells has been shown to bind Pra1.
FIGURE 5 | Pra1 co-stimulates mouse CD4+ T cells. (A) CFSE dilution and CD25 expression by mouse CD4+ T cells after a 3 day culture in the presence or absence of plate-bound αCD3 mAb and Pra1 (1 ng/ml) or αCD28 mAb (1 µg/ml) added in solution. (B) Summary graph depicting a dose-dependent increase in co-stimulation in the presence of plate-bound αCD3 mAb and Pra1 (0% = frequency CFSElow CD25+ with αCD3 mAb only; 100% = frequency CFSElow CD25+ with αCD3 + 10 µg/ml αCD28 mAb). (C) Direct stimulatory activity of Pra1 in the absence of plate-bound αCD3 (0% = frequency CFSElow CD25+ medium only; 100% = frequency CFSElow CD25+ with plate-bound αCD3 mAb). (B,C): Means ± SD (n = 3 individual experiments). Two-sided t-test: *p < 0.05.

and that this binding is important to protect mice after systemic C. albicans infection (Soloviev et al., 2011). On mouse CD4+ T cells it is, however, not a complement regulatory protein that interacts with Pra1 (Figure 4). Therefore, it is unlikely that modulation of complement activation, which has been shown to crucially contribute to T cell stimulation and differentiation (Arbore and Kemper, 2016), accounts for the effects of Pra1 on mouse CD4+ T cells. Analysis of Kv1.3 expression in parallel to Pra1 binding to in vitro polarized CD4+ Th1 cells, however, showed that cells with the highest capacity to bind Pra1 also expressed high levels of Kv1.3 (Figure 7). While we do not, yet, know whether Kv1.3 is a receptor for Pra1 it may not be the only molecule Pra1 interacts with on the T cell surface. Kv1.3 expression cannot be detected on resting T cells by FACS using the ShK-F6CA peptide (Beeton et al., 2003), while Pra1 binding to resting T cells is detectable by flow cytometry as detailed in this study. Functionally, Pra1 might interfere with K+ currents through Kv1.3 by direct binding to the channel or by binding in the vicinity of Kv1.3 and ‘delivering’ Zn2+ ions. Extracellular Zn2+ binds to Kv1.3 inhibiting the transport of K+ ions through the channel (Teisseyre and Mozrzymas, 2002, 2006).

Apart from directly interacting with CD4+ T cells, Pra1 could also modulate T cell responses by binding to APCs via interaction with CD11b/CD18 (Soloviev et al., 2011) or via the still unknown Pra1 receptor also expressed on T cells. Therefore, it was important to study the effects on cytokine secretion by CD4+ T cells in the presence of APCs. Irrespective of whether APCs were present in our assays Pra1 inhibited cytokine secretion...
by the CD4\(^+\) T cells (Figures 6, 7) suggesting that the direct interaction of Pra1 with the CD4\(^+\) T cells was also the crucial event in the cultures containing APCs.

Recombinant Pra1 and supernatant of C. albicans cultures inhibited IFN\(\gamma\) release from CD4\(^+\) T cells (Figure 6). While we do not know to what extent Pra1 contributes to the overall inhibitory effect of the C. albicans supernatant this observation highlights that C. albicans, through its secretome, modulates CD4\(^+\) T cell responses. Further experimentation is required to delineate whether Pra1 is the only C. albicans protein mediating these effects or, more likely, whether other secreted fungal proteins also contribute to effector T cell inhibition.

Apart from Th1 cells, Th17 cells also crucially contribute to anti-fungal immunity either through direct effects or by

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**FIGURE 6** Modulation of cytokine secretion by Pra1 upon polyclonal and antigen-specific CD4\(^+\) T cell stimulation. (A) The left panel shows the absolute amount of cytokines secreted by purified WT CD4\(^+\) T cells after three days of stimulation with plated-bound anti-CD3-mAb (2.5 \(\mu\)g/ml) either alone or together with Pra1 or anti-CD28 mAb (clone E18, 10 \(\mu\)g/ml) added in solution. The right column shows the amounts of secreted cytokines normalized to the ‘anti-CD3 mAb only’ cultures (=100%; first bar in the left panel). Means ± SD of \(n=5\) individual experiments are shown. (B) IFN\(\gamma\) secretion upon stimulation of purified CD4\(^+\) T cells with anti-CD3/anti-CD28 mAb-coated Dynabeads\(^\text{R}\). Pra1 was added at 1 ng/ml and the C. albicans supernatant was diluted 1:25. Means ± SD of triplicate cultures are shown. The experiment was repeated with similar result. (C) Lymph node cells from OT-II mice were stimulated with 1 \(\mu\)M OVA\(\beta\) in the absence or presence of Pra1 as indicated before supernatants were harvested on day three and IFN\(\gamma\) concentrations determined (means ± SD of triplicate cultures; experiment was repeated with similar result). (A–C) Two-sided t-test. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\).
supporting Th1 versus Th2 cell differentiation (Romani, 2011; Zelante et al., 2016). In contrast to other cytokines, IL-17 release from CD4+ T cells was not reduced in the presence of Pra1 (Figure 6). This might have to do with the degree of TCR signal strength required to induce optimal cytokine release from different CD4+ T helper cell subpopulations. For Th1 cells we observed that strong TCR stimulation overcame Pra1-induced suppression of cytokine release (Figure 7). As maximal IL-17 release, in contrast to IFNγ release, has been reported to require low TCR stimulation (Purvis et al., 2010) further experimentation is required to determine whether, indeed, Pra1 differentially regulates cytokine release from Th1 and Th17 cells.

In summary, our data identify Pra1 as an inhibitor of mouse CD4+ effector T cell function in vitro, thus, mediating evasion of C. albicans from potentially harmful CD4+ T cell responses. While subversion of the CD4+ T cell response during commensalism might be of mutual benefit for C. albicans and the host, during invasive infection/sepsis blocking protective CD4+
T cell immunity might worsen clinical outcome. Therefore, the findings of our study suggest that therapeutic targeting of soluble Pra1 might enhance CD4+ T cell responses protecting the host from invasive *C. albicans* infections.

**AUTHOR CONTRIBUTIONS**

AB designed research studies, conducted experiments, acquired and analyzed data, and wrote the paper. PD provided reagents, designed research studies, and interpreted data. SW conducted experiments, acquired and analyzed data. TRH provided reagents, designed research studies, and interpreted data. WS provided reagents and interpreted data. PH provided reagents and designed research studies. AB provided reagents, designed research studies, and interpreted data. TH designed research studies and analyzed and interpreted data. PZ provided findings of our study suggest that therapeutic targeting of soluble *C. albicans* Pra1 might enhance CD4+ T cell responses protecting the host from invasive *C. albicans* infections.

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