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Candida-Elicited Murine Th17 Cells Express High CTLA-4 Compared with Th1 Cells and Are Resistant to Costimulation Blockade

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Effector and memory T cells may cross-react with allogeneic Ags to mediate graft rejection. Whereas the costimulation properties of Th1 cells are well studied, relatively little is known about the costimulation requirements of microbe-elicited Th17 cells. The costimulation blocker CTLA-4 Ig has been ineffective in the treatment of several Th17-driven autoimmune diseases and is associated with severe acute rejection following renal transplantation, leading us to investigate whether Th17 cells play a role in CD28/CTLA-4 blockade-resistant alloreactivity. We established an Ag-specific model in which Th1 and Th17 cells were elicited via Mycobacterium tuberculosis and Candida albicans immunization, respectively. C. albicans immunization elicited a higher frequency of Th17 cells and conferred resistance to costimulation blockade following transplantation. Compared with the M. tuberculosis group, C. albicans–elicited Th17 cells contained a higher frequency of IL-17+ IFN-γ+ producers and a lower frequency of IL-10+ and IL-10+IL-17+ cells. Importantly, Th17 cells differentially regulated the CD28/CTLA-4 pathway, expressing similarly high CD28 following transplantation of solid organs or bone marrow (6, 16, 17).

The heterogeneity of T cell memory responses and their requirements for recall responses are critically important to the success of immunomodulatory therapy to prevent T cell–mediated rejection. Among pathogen-elicited T cell subsets (14, 15), further elevating the potential importance of cross-reactive T cell responses in mediating alloreactivity. Heterologous T cell responses have been directly demonstrated to be a barrier to tolerance induction strategies such as costimulation blockade, highlighting the importance of understanding phenotypic diversity among pathogen-elicited T cell subsets (14, 15).

The heterogeneity of T cell memory responses and their requirements for recall responses are critically important to the success of immunomodulatory therapy to prevent T cell–mediated rejection following transplantation of solid organs or bone marrow (6, 16, 17). The CD28/CTLA-4 costimulation blocker CTLA-4 Ig is efficacious for the treatment of rheumatoid arthritis (abatacept), and the derivative belatacept was recently approved for renal transplantation. However, belatacept has been associated with an increased incidence of acute cellular rejection early after transplantation (18), spurring efforts to identify T cell populations that mediate breakthrough allograft rejection episodes.

CD4+ memory Th cells are regarded as dependent on CD28 signals for recall responses (14, 19). However, these seminal studies were conducted using Th1 phenotype cells, and the subsequent discovery of the Th17 lineage, which can mediate both autoimmune pathology and graft-versus-host disease, has complicated this understanding. Recent studies have suggested that the costimulatory signals that mediate differentiation of naive Th0 cells into Th17 cells differ from those of Th1 cells, but exactly how they differ remains controversial. For example, reports have indicated that either CD28 or CTLA-4 can suppress Th17 differentiation (20–23), but that alternate costimulatory molecules are required to optimally differentiate Th17 cells (21, 22, 25, 26). Despite these reports, which rely heavily on the use of in vitro polarization with exogenous cytokines, little is known about the costimulation requirements of microbe-elicited effector and memory Th17 cells.

Interestingly, the CTLA-4 Ig derivative abatacept has shown mixed results in the treatment of the Th17-mediated diseases multiple sclerosis (MS) and inflammatory bowel disease (IBD) (27). In the murine MS model experimental autoimmune encephalomyelitis, a single dose of CTLA-4 Ig was unable to prevent relapsing disease, and, remarkably, repeated prophylactic CD28/CTLA-4 blockade actually exacerbated disease (28). A recent clinical trial in IBD demonstrated minimal efficacy and disease exacerbation in some treatment groups (29). A case report detailed the development of IBD in a patient treated with CTLA-4 Ig for rheumatoid arthritis (30). Given the variable efficacy of CTLA-4 Ig in Th17-mediated autoimmunity, the early severe rejection observed in renal transplant...
Recipients, and newly emerging appreciation of alternative costimulatory molecules required for optimal Th17 differentiation, we hypothesized that Th17 cells might be uniquely resistant to CD28/CTLA-4 blockade.

In this study, we investigated the phenotype of pathogen-elicited Th17 cells in an Ag-specific model of graft rejection. Th1 and Th17 cells were elicited via *Mycobacterium tuberculosis* and *Candida albicans* immunization. *C. albicans* immunization elicited a higher frequency of Th17 cells and correlated with resistance to costimulation blockade. Compared with the *M. tuberculosis* group, *C. albicans*-elicited Th17 cells had several features of more pathogenic Th17 cells, including a greater frequency of IL-17*γ*-producing, lowerCCR6 expression, and a lower frequency of IL-10/IL-17 coproducers. Strikingly, Th17 cells differentially regulated the CD28/CTLA-4 pathway, expressing significantly greater amounts of CTLA-4 compared with Th1 cells. Ex vivo blockade experiments demonstrate that Th17 cells are significantly less inhibited by CD28/CTLA-4 blockade with CTLA-4 Ig and were more sensitive to CTLA-4 co-inhibition. These data demonstrate phenotypic features of pathogen-elicited Th17 cell populations that shed new light on strategies for modulating pathologic T cell responses in transplantation and autoimmunity.

**Materials and Methods**

**Mice**

B6-Ly5.2/Cr (H2-Kb, CD45.1) and C57BL/6 (H2-Kb, CD45.2) were obtained from the National Cancer Institute. OT-I and OT-II transgenic mice (purchased from Taconic Farms) were bred to Thy1.1+ background at Emory University. Membrane-bound OVA (mOVA) mice were a gift of M. Jenkins (University of Minnesota, Minneapolis, MN) and were maintained in accordance with Emory University’s Institutional Animal Care and Use Committee guidelines. All animals were housed in specific pathogen-free animal facilities at Emory University.

**Adoptive transfers and pathogen immunization**

Spleens from Thy1.1+ OT-I and OT-II mice were processed to single-cell suspension and stained with mAbs for CD4 (RM4-5), CD8 (3B5), Thy1.1 (OX-7), Vα2 (B20.1), and Vβ5 (MR9-4) for flow cytometric analysis of T cell frequency. Cells were resuspended in PBS, and 1 × 104 OT-I and 1 × 105 OT-II were injected i.v. into naive B6 recipients. For *C. albicans* immunization, *C. albicans*-elicited Th17 cells were grown as yeast for 18 h overnight at 30°C in yeast extract/peptone/dextrose broth (Teknova) and then washed in PBS and dilated 1:50 in RPMI 1640 with 10% FBS. Transition to hyphae was induced for 4–6 h at 37°C and monitored by light microscopy. Mice were immunized with 1 × 106 hyphae in IFA (Difco Laboratories) mixed 1:1 in PBS and 100 μg OVA323-339 peptide (ISQVHAAHAEINEGR; GenScript) in each hind footpad. *M. tuberculosis* mice were immunized with CFA (Difco Laboratories) containing 1 mg/ml heat-killed *M. tuberculosis* diluted 1:1 in PBS and 100 μg OVA323-339 peptide. Immunizations were performed 24–48 h after adoptive transfer to B6 recipients.

**Skin transplantation and costimulation blockade**

Full-thickness tail and ear skins were transplanted onto the dorsal thorax of recipient mice and secured with adhesive bandages. Where indicated, mice were treated with 500 μg CTLA-4 Ig (Bristol-Myers Squibb, Princeton, NJ) and 500 μg hamster monoclonal anti-mouse CD154 (MR-1; BioXCell, West Lebanon, NH) on days 0, 2, 4, and 6 posttransplantation.

**Surface staining, intracellular staining, and flow cytometry**

Draining popliteal lymph nodes (LN) were processed to single-cell suspension. LN were restimulated with 10 μM OVA323-339 peptide for 6 h, with 10 μg/ml GolgiStop added for the final 5 h. Cells were surface stained with the following Abs: CD4 (RM4-5), CD8 (3B5), and CD28 (E18) or IgG2b. Intracellular cytokine staining was performed following the manufacturer’s instructions (BD Biosciences) with the following Abs: IFN-γ (XMG1.2), IL-17 (eBio17B7), CTLA-4 (UC10-4B9), or IgG. Flow cytometric analysis was performed on an LSRII flow cytometer and analyzed using FlowJo. Where indicated, OT-II cells were identified by FACS as CD4+CD8−B220+Thy1.1+, whereas OT-I cells were identified as CD8+CD4+B220+Thy1.1+.

**Ex vivo Th1/Th17 costimulation blockade**

CD4+ T cells from the draining popliteal LN of *C. albicans* and *M. tuberculosis* OVA–immunized mice were harvested and purified with the CD4+ T Cell Isolation Kit II (Miltenyi Biotec), according to the manufacturer’s protocol, using LS columns and autoMACS running buffer (Miltenyi Biotec). CD11c+ cells were enriched from B6-Ly5.2/Cr (H2-Kb, CD45.1) splenocytes. Splenocytes were macerated in HBSS containing 100 U/ml collagenase III. Remaining splenic fragments were then incubated in HBSS containing 400 U/ml collagenase III at 37°C for 30 min. Cells were then washed in HBSS with 10 mM EDTA. CD19+ and Thy1.2+ cells were depleted using magnetic microbeads and LD columns (Miltenyi Biotec). CD11c+ cells were positively selected using magnetic microbeads and LS columns (Miltenyi Biotec). The frequency of CD11c+ cells was assessed at >85% by FACS. Between 750,000 and 1,000,000 CD4+ T cells were cocultured with 100,000 CD11c+ cells for 4 d in the presence of 5 μM OVA323-339 peptide and 100 μg/ml of either CTLA-4 Ig, human Ig1-Fc, anti–CTLA-4 (9H10), anti–CD154 (MR-1), or hamster IgG (all from BioXcell) in RPMI 1640 with 10% FBS. After 4 d, cells were washed twice in media and restimulated for 4 h with 30 ng/ml PMA and 400 ng/ml ionomycin (Iono) in the presence of GolgiStop for the final 3 h. Intracellular cytokine staining for IFN-γ and IL-17 was assessed, as described above. Absolute cell counts were determined by adding CountBrite (Invitrogen) beads prior to PMA/Iono restimulation. In some experiments, 104 naïve OT-II spleenocytes were activated with 5 μM OVA323-339 peptide in the presence of 100 μg/ml of either CTLA-4 Ig, Ig1-Fc control, anti–CTLA-4 Ab, or IgG for 5 d and restimulated with PMA/Iono prior to intracellular cytokine staining, as described.

**OVA Ab detection**

Mice immunized with *M. tuberculosis* OVA or *C. albicans* OVA as described, grafted with a mOVA skin graft, and treated with CTLA-4 Ig and anti-CD154 were bled on day 10 posttransplant. Serum was prepared by centrifugation of blood samples at 12,000 rpm for 30 min at 4°C. Ninety-six-well plates (Nunc) were coated with 50 μl/well of 10 μg/ml OVA Ag (Sigma-Aldrich; A-2512) overnight at 4°C, washed three times with PBS with 0.5% Tween 20, and blocked at room temperature for 2 h with PBS with 0.2% Tween 20 and 10% FBS. Anti-OVA mAb clone 14 (Sigma-Aldrich; A6075) was used as a positive control and was incubated at decreasing concentrations starting at 2.5 μg/ml. Samples were plated at 1:10 dilutions in PBS to a final volume of 100 μl/well. Plates were then incubated at 37°C for 2 h. Following washing, 100 μl 27 μg/ml goat anti-mouse IgG-HRP was added and incubated at room temperature for 1.5 h. A total of 100 μl/well of tetramethylbenzidine substrate (BD 555214) was used following manufacturer instructions, and plates were read at OD450 on an ELISA plate reader.

**Histology**

Skin grafts were removed and frozen in cryomolds with OCT embedding compound (Tissue-Tek) on day 9 posttransplantation. Longitudinal sections of grafts were cut into 5-μm-thick sections with a cryostat (CM 1850; Leica Microsystems) and mounted on SuperFrost Plus microscope slides, fixed with 100% acetone, and stained with anti-mouse Mac-3 or anti-mouse Ly6g. Six mice were used for immunohistochemical detection by 3,3 diaminobenzidine peroxidation and counterstained with hematoxylin. Representative images are shown at 40X. At least 20 image fields were analyzed per group.

**Statistical analysis**

OT-II frequencies, surface marker expression, and cytokine production between *M. tuberculosis* OVA and *C. albicans* OVA groups were analyzed with unpaired Student t test (two-tailed). Cellular infiltration of skin grafts and anti-OVA Ab responses were analyzed using one-way ANOVA with Bonferroni posttest. In text, statistics are average ± SEM.

**Results**

*C. albicans* Ag immunization yields costimulation blockade–resistant graft rejection

Pathogen-elicited memory cells can cross-react with allogeneic Ag and mediate graft rejection (7, 14). Whereas the costimulation requirements of CD8+ memory have been well studied by our laboratory and others, the differential susceptibility of CD4+ Th memory subsets to immunomodulation is less well understood.
We hypothesized that different classes of microbes can yield memory that has variable potency in graft rejection. We activated Ag-specific CD4+ (OT-II) and CD8+ (OT-I) T cells using cognate OVA_{323–339} peptide in the presence of two well-studied microbes, intracellular M. tuberculosis (M. tuberculosis OVA) or the ubiquitous fungus C. albicans (Candida OVA; Fig. 1A). In this experimental design, CD4+ cells are activated and differentially polarized in the presence of pathogen, but both CD4+ and CD8+ cells can respond to OVA expressed in mOVA skin graft. Both the M. tuberculosis OVA and C. albicans OVA immunization strategies similarly expanded OVA-specific CD4+ cells compared with unimmunized controls (Fig. 1B). C. albicans and M. tuberculosis OVA immunization yielded similar frequencies of OVA-specific CD4+ T cells at day 9, but absolute numbers of C. albicans OVA-elicited cells were slightly decreased relative M. tuberculosis-elicited cells (Fig. 1B). OVA-specific CD8+ T cells were not activated by this immunization strategy and persisted at similar frequencies in both groups (Supplemental Fig. 1A). At day 14 postimmunization, OT-II populations contracted to similar frequencies and absolute numbers (Fig. 1C). When OVA-expressing skin grafts were transplanted to the M. tuberculosis OVA and C. albicans OVA groups, both groups of mice rejected mOVA skin grafts with similar kinetics in the absence of any immunosuppression (Fig. 1D).

We next investigated the susceptibility of both types of pathogen-elicited CD4+ cells to mediate graft rejection in the presence of costimulation blockade. We chose to use CTLA-4 Ig in the presence of anti-CD154 Ab because in this Ag-specific model of graft rejection CTLA-4 Ig is ineffective at providing long-term graft survival (31). In the presence of costimulation blockade, M. tuberculosis OVA–immunized mice were protected from graft rejection for >60 d posttransplantation (Fig. 1D). In contrast, the majority of C. albicans OVA–immunized mice rejected their grafts by 20 d posttransplant (Fig. 1D). These results indicate that Th polarization in the presence of C. albicans generates a population of CD4+ T cells that are resistant to costimulation blockade. By the time of grafting, the C. albicans hyphae were cleared, indicating that persistent infection does not account for the differences in rejection kinetics (Supplemental Fig. 1B). To determine the potential role of anti-OVA Ab in graft model, we measured the serum levels of anti-OVA Abs at 9 d postimmunization. We found that anti-OVA Abs were similar between M. tuberculosis and C. albicans groups (Supplemental Fig. 1C). Costimulation blockade following transplantation did not affect the levels of pre-existing anti-OVA serum Ab in either the M. tuberculosis OVA or the C. albicans OVA group (Supplemental Fig. 1C). Together, these data suggest that C. albicans immunization elicits a population of CD4+ Th cells that mediate costimulation blockade–resistant rejection, and that this result is not due to differential elicitation of anti-OVA Abs.

C. albicans immunization elicits a Th17-skewed phenotype compared with M. tuberculosis

M. tuberculosis and C. albicans each elicit Th1 and Th17 responses (32, 33). Therefore, we investigated the phenotype of Ag-specific CD4+ T cells during the peak of the effector response. Restimulation of draining LN cells on day 9 postimmunization yielded a significant population of IFN-γ+ Th1 cells in both groups. The frequencies of Th1 cells were not different between M. tuberculosis– and C. albicans–immunized mice (Fig. 2A). IL-17–producing Th17 cells were also found in both groups; however, C. albicans immunization yielded a significantly greater frequency of Th17 cells than M. tuberculosis immunization (M. tuberculosis OVA 6.69 ± 0.262%, C. albicans OVA 13.6 ± 1.46%, p = 0.0005; Fig. 2B). The frequency of Th17 cells was significantly greater in pathogen-immunized mice compared with mice immunized with OVA_{323–339} peptide alone (Fig. 2A, 2B), demonstrating that the balance of Th1 and Th17 cells in both M. tuberculosis OVA– and C. albicans OVA–immunized mice is...
due to the pathogen-induced differentiation of naive Ag-specific cells. These Ag-specific CD4^+ IL-17–producing cells were bona fide Th17 cells as they expressed high levels of the lineage-defining transcription factor RORγT (Fig. 2C) and low levels of the Th1 transcriptional factor T-bet compared with Ag-specific CD4^+ IFN-γ–producing cells (Supplemental Fig. 2A). These data suggested that a greater frequency of Th17 cells might underlie the differential susceptibility of M. tuberculosis– and C. albicans–immunized mice to graft rejection.

M. tuberculosis and C. albicans elicit similar frequencies of effector memory cells

We further investigated the phenotype of Ag-specific cells polarized by M. tuberculosis and C. albicans immunization. We found that both immunizations yielded similar frequencies of CD44\text{high} cells, indicating that cells in both of these immunization groups effectively encountered cognate Ag (M. tuberculosis OVA 84.9 ± 6.3% and C. albicans OVA 88.5 ± 6.4%; Fig. 3A). In contrast to naive Ag-specific CD4^+ T cells, M. tuberculosis and C. albicans OVA immunization both yielded a majority of CD44\text{high}CCR7\text{−} effector memory cells (T_EM) (M. tuberculosis OVA 63.9 ± 10.3% and C. albicans OVA 57.5 ± 6.7%; Fig. 3B).

M. tuberculosis and C. albicans immunization have been associated with induction of additional cytokines, such as IL-2 and TNF (34, 35). To assess the potential role of these cytokines, we compared the frequencies of IL-2 and TNF in the draining LN at the peak of the response following immunization. We found that M. tuberculosis and C. albicans immunization yielded similar frequencies of Ag-specific IL-2^+ and TNF^+ CD4^+ T cells (Fig. 3C, 3D). Both immunization strategies elicited similar frequencies of Th1 and Th17 cells that coproduced either IL-2 or TNF (Supplemental Fig. 2B, 2C). Together, these data suggest that a high frequency of Th17 cells is elicited by C. albicans immunization and correlates with costimulation blockade–resistant graft rejection.

C. albicans–elicited Th17 cells have a more pathogenic phenotype compared with M. tuberculosis–elicited Th17 cells

Th17 cells have been described that have either protective or pathogenic roles in mediating inflammation (36). Because Th17 cells were elicited by both M. tuberculosis and C. albicans, we investigated differences in the phenotype of Th17 cells from both groups. Th17 cells that coexpress IFN-γ have been described at sites of autoimmune inflammation (36). We found a greater frequency of IL-17^+IFN-γ^+ cells in the draining LN of C. albicans OVA mice compared with M. tuberculosis OVA mice (M. tuberculosis OVA 2.01 ± 0.315%, C. albicans OVA 4.15 ± 0.564%, p = 0.0042; Fig. 4A). The chemokine receptor CCR6 is associated with the production of IL-17 by CD4^+ cells (33, 37), but loss of its expression is characteristic of more pathogenic Th17 cells in multiple models (33, 38). Consistent with previous reports (33, 37), CCR6 expression was high on Th17 cells compared with Th1 cells (p < 0.001; Supplemental Fig. 3A). However, we found that CCR6 expression levels were significantly lower in C. albicans–elicited Th17 cells compared with M. tuberculosis–polarized Th17 cells (M. tuberculosis OVA 626 ± 19.46 mean fluorescence intensity (MFI), C. albicans OVA 492.3 ± 52.45 MFI, p = 0.0422; Fig. 4B). CCR6 expression on Th1 cells was not different between immunization groups (Supplemental Fig. 3B). Together, these findings suggest that C. albicans–elicited Th17 cells are skewed toward a pathogenic phenotype.

Conversely, production of the anti-inflammatory cytokine IL-10 has been associated with protective Th17 cells (32, 38). M. tuberculosis OVA yielded a significantly greater frequency of IL-10^+ CD4^+ T cells than C. albicans OVA immunization (M. tuberculosis OVA 3.51 ± 0.433%, C. albicans OVA 1.61 ± 0.345%, p = 0.0040; Fig. 4C). Additionally, M. tuberculosis OVA mice had a significantly greater frequency of Th17 cells that coproduced IL-10 (M. tuberculosis OVA 8.17 ± 1.32%, C. albicans OVA 3.31 ± 0.844%, p = 0.0059; Fig. 4D). These data demonstrate that...
M. tuberculosis–elicited Th17 cells display features that are less pathogenic and suggest that the inflammatory profile of C. albicans– and M. tuberculosis–elicited Th17 cells might contribute to costimulation blockade–resistant graft rejection. We reasoned that because a greater frequency of Th17 cells in the C. albicans OVA group is associated with CTLA-4 Ig-resistant graft rejection (Fig. 1D), the CD28/CTLA-4 pathway might be differentially expressed on Th1 and Th17 cells. We evaluated the expression of these molecules in M. tuberculosis– and C. albicans–polarized draining popliteal LN OT-II cells. We found that CD28 was constitutively expressed on endogenous CD4+ cells and Th1 and Th17 OT-II–restimulated cells from both M. tuberculosis and C. albicans OVA groups (Fig. 5A, 5B, Supplemental Fig. 3C). Th1 and Th17 cells expressed similar amounts of CD28 on the cell surface (Fig. 5B).

CTLA-4 is not expressed on the surface of resting CD4+ effector or memory cells, but is released rapidly from intracellular vesicles following TCR stimulation. Using intracellular staining, we found that endogenous CD4+ T cells express low levels of CTLA-4 (22.7 ± 1.74% CTLA-4+, 1557 ± 99.2 MFI; Fig. 5C, 5D), consistent with a low frequency of activation. Th1 cells upregulated CTLA-4 expression (61.5 ± 2.41% CTLA-4+, Fig. 5C, 5D). In contrast, a significantly greater fraction of Th17 cells was CTLA-4+ (74.8 ± 2.51% CTLA-4+, p = 0.0006; Fig. 5C), and Th17 cells also expressed a greater amount of CTLA-4 than Th1 cells (3870 ± 312 MFI Th1 versus 5665 ± 508 MFI Th17, p = 0.0044; Fig. 5D). CTLA-4 expression levels were similar between Th1 and Th17 cells elicited from M. tuberculosis or C. albicans (Supplemental Fig. 3D, 3E). These data demonstrate that Th1 and Th17 cells have dramatically different regulation of the CD28/CTLA-4 pathway and suggest that Th17 cells might be resistant to CTLA-4 Ig due to greater reliance on coinhibitory CTLA-4.

Th17 cells express greater amounts of CTLA-4 than Th1 cells

We hypothesized that greater expression of coinhibitory CTLA-4 would result in less suppression of Th17 cells compared with Th1 cells in the context of blockade of CD80/CD86 signals by CTLA-4 Ig. To investigate the differential functionality of physiologi-
cally relevant Th1 and Th17 cells, we purified *C. albicans*– and *M. tuberculosis*–polarized CD4+ T cells and restimulated them with OVA323–339 peptide in the presence of CTLA-4 Ig or IgG-Fc for 4 d before brief PMA/Iono restimulation. Data shown depict Th1 and Th7 OT-II cells from both immunization groups (*n* = 16–21/group, 3–4 experiments). (A) The frequency of CD28+ and (B) CD28 expression level on Ag-specific Th1 and Th17 cells (*p* = NS). (C) The frequency of CTLA-4+ (*p* = 0.0006) and (D) CTLA-4 expression level (*p* = 0.0044) on OT-II Th1 and Th17 cells. (E) CD11c+ DCs were purified from naive splenocytes and analyzed for MHC class II I-A<sup>b</sup>, CD80, and CD86 expression. (F) *M. tuberculosis* OVA and *C. albicans* OVA CD4+ T cells were cocultured with DCs for 4 d in the presence of CTLA-4 Ig IgG-Fc control, followed by brief PMA/Iono restimulation. (F) Representative frequencies (top number) and absolute numbers (bottom number) of OT-II Th1 and Th17 cells without PMA/Iono restimulation (*p* < 0.005, ***p* < 0.001). Endog CD4+, endogenous CD4+ T cells; Isotype, IgG isotype control Ab.

The coinhibitory function of CTLA-4 has been attributed to several cell-intrinsic functions, such as enhanced phosphatase signaling, competition with CD28 for CD80/CD86, and exclusion of CD28 from the immunological synapse (39, 40). Recently, cell-extrinsic functions have also been attributed to CTLA-4 on CD4+ and CD8+ T cells (41). We hypothesized that greater expression of coinhibitory CTLA-4 rendered Th17 cells more resistant to CD28/CTLA-4 blockade than Th1 cells owing to greater cell-intrinsic coinhibitory function. To test this hypothesis, we specifically blocked CTLA-4 function using a mAb that augments naive OT-II differentiation into Th1 cells during activation (42) (Supplemental Fig. 4B). Anti-CTLA-4 mAb very minimally augmented the number of Th1 cells that were cocultured with CD11c+ DCs (112 ± 9.6% of IgG; Fig. 6). In contrast, the number of Th17 cells was augmented to a significantly greater degree compared with Th1 cells (140 ± 9.2% of IgG, *p* = 0.0387; Fig. 6), demonstrating that Th17 cells are more sensitive to CTLA-4 cell-intrinsic coinhibition.

There was no difference in the effect of Th1 or Th17 cells between *M. tuberculosis* OVA and *C. albicans* OVA groups (Supplemental Fig. 4A).
Th17 cells are more susceptible to CTLA-4 coinhibition than Th1 cells. Draining popliteal LNs from *M. tuberculosis* OVA and *C. albicans* OVA mice were collected on day 9 postimmunization and cocultured with OVA323–339 and DCs in the presence of anti-CTLA-4 or IgG for 4 d before brief PMA/Iono restimulation. Data shown depict OT-II Th1 and Th7 cells from both immunization groups (*n* = 13–15/group, 3 experiments). (A) Representative frequencies (top number) and absolute numbers (bottom number) of OT-II Th1 and Th7 cells without PMA/Iono restimulation (first row) or with restimulation (middle/bottom rows). (B) Th1 and Th17 cell numbers from *M. tuberculosis* OVA and *C. albicans* OVA groups following anti-CTLA-4 blockade (*p* = 0.0387). Bar graphs depict average ± SEM. Statistical comparison performed using unpaired two-tailed Student *t* test, *p* < 0.05.

Fig. 4C). Together, these results suggest that higher expression of CTLA-4 on Th17 cells correlates to enhanced resistance to CTLA-4 Ig and greater sensitivity to CTLA-4 coinhibition, findings that suggest CTLA-4 functions as a potent cell-intrinsic coinhibitor on Th17 cells.

**FIGURE 6.** Th17 cells are more susceptible to CTLA-4 coinhibition than Th1 cells. Draining popliteal LNs from *M. tuberculosis* OVA and *C. albicans* OVA mice were collected on day 9 postimmunization and cocultured with OVA323–339 and DCs in the presence of anti-CD154 or IgG for 4 d before brief PMA/Iono restimulation. Data shown depict OT-II Th1 and Th7 cells from both immunization groups (*n* = 13–15/group, 3 experiments). (A) Representative frequencies (top number) and absolute numbers (bottom number) of OT-II Th1 and Th7 cells without PMA/Iono restimulation (first row) or with restimulation (middle/bottom rows). (B) Th1 and Th17 cell numbers from *M. tuberculosis* OVA and *C. albicans* OVA groups following anti-CTLA-4 blockade (*p* = 0.0387). Bar graphs depict average ± SEM. Statistical comparison performed using unpaired two-tailed Student *t* test, *p* < 0.05.

Th17 cells from *M. tuberculosis* immunization and strongly suggest that the differential expression of CTLA-4 on Th17 cells suggests that this pathway plays a role in the costimulation blockade–resistant rejection observed in *C. albicans* OVA–immunized mice. To investigate whether the CD40/CD154 pathway, which was also blocked in our skin graft experiments, played a role in costimulation blockade–resistant rejection, we first investigated the expression of CD154 on Ag-specific CD4+ T cells. CD154 is expressed on activated but not naive CD4+ T cells (Fig. 7A) (43, 44). We found that overall *M. tuberculosis* or *C. albicans* immunization resulted in similar expression of CD154 on Ag-specific Th1 and Th17 cells on day 9 (Fig. 7A, 7B). By day 14, the expression levels of CD154 were diminished, but were similar among Th1 and Th17 cells from *M. tuberculosis* OVA and *C. albicans* OVA mice (Fig. 7C).

To determine the ability of CD154 blockade to diminish CD4+ T cell responses, we treated Ag-specific CD4+ T cells from the draining LNs *M. tuberculosis* OVA and *C. albicans* OVA mice on day 9 postimmunization with DCs in the presence of anti-CD154 mAbs or control IgG. We found that the proliferation of Th1 and Th17 cells from *C. albicans* OVA and *M. tuberculosis* OVA mice was inhibited by CD154 blockade (*M. tuberculosis* Th1 44.7 ± 7.1%, *C. albicans* Th1 63.6 ± 4.4%, *M. tuberculosis* Th1 52.8 ± 11.5%, *C. albicans* Th1 31.5 ± 5.3% of IgG; Fig. 7C). Interestingly, *C. albicans* Th17 cells were more inhibited by anti-CD154 than *C. albicans* Th1 cells. Together, these results demonstrate that CD154 is not differentially expressed following *M. tuberculosis* or *C. albicans* OVA immunization and strongly suggest that the function of the CD154 pathway is not responsible for the costimulation blockade–resistant rejection observed in *C. albicans* OVA mice.

**FIGURE 7.** Th1 and Th17 cells express similar levels of CD154 and are inhibited by CD154 blockade. Draining popliteal LNs from *M. tuberculosis* OVA and *C. albicans* OVA mice were collected on day 9 or day 14 postimmunization and restimulated for 4 h with PMA/Iono (top number) or with restimulation (middle/bottom rows). (A) Representative histograms and (B) CD154 expression of naive OT-II, *M. tuberculosis* OVA, or *C. albicans* OVA LN cells on day 9 postimmunization. (C) CD154 expression of naive OT-II, *M. tuberculosis* OVA, or *C. albicans* OVA LN cells on day 14 postimmunization. (D) Representative frequencies (top number) and absolute numbers (bottom number) of OT-II Th1 and Th17 cells without PMA/Iono restimulation (first row) or with restimulation (middle/bottom rows). (E) Bar graphs depict average ± SEM. Th1 and Th17 cell numbers from *M. tuberculosis* OVA and *C. albicans* OVA groups following anti-CD154 blockade (*C. albicans* Th1/Th17, *p* = 0.0008). Statistical comparison performed using unpaired two-tailed Student *t* test, *p* < 0.05, **p** < 0.005. IgG, isotype control Ab.
Neutrophils are recruited to skin grafts in a costimulation blockade–independent manner by C. albicans–elicited T cell responses

CD4+ Th cells coordinate effector responses via recruitment of proinflammatory cells, such as inflammatory macrophages and neutrophils. We investigated the recruitment of these populations into skin grafts in M. tuberculosis– and C. albicans–immunized mice in the presence of costimulation blockade. Macrophages are associated with a Th1-type inflammation, whereas neutrophils are potently recruited to sites of inflammation by IL-17.

Macrophages were recruited into the grafts at similar frequencies between M. tuberculosis and C. albicans groups (Fig. 8A). Treatment with costimulation blockade diminished these populations in both groups (Fig. 8A), suggesting that macrophages are not involved in effecting rejection. Neutrophils were also recruited into the graft to similar degrees in M. tuberculosis and C. albicans mice (Fig. 8B). In M. tuberculosis OVA mice, however, costimulation blockade inhibited the population of neutrophils to a significant degree (p = 0.0263; Fig. 8B). C. albicans OVA mice treated with costimulation blockade did not diminish the recruitment of neutrophils (Fig. 8B). These data suggest that a Th17-type inflammation mediates costimulation blockade–resistant graft rejection in C. albicans OVA mice.

Discussion

In this study, we investigated the capacity of pathogen–elicited graft-specific CD4+ T cells to induce graft rejection. We found that, whereas Th1 frequencies were similarly elicited by M. tuberculosis and C. albicans immunization, a Th17-skewed response correlated with costimulation blockade–resistant graft rejection in C. albicans–immunized mice. Our results demonstrate that both the degree of Th17 pathogenicity and the frequency of CTLA-4 Ig-resistant Th17 cells contribute to the observed costimulation blockade resistance in C. albicans OVA mice.

Distinct Th17 phenotypes correlated with pathogenicity in models of autoimmunity (36). We found that C. albicans–elicited effector cells contained more IL-17+IFN-γ+ double producers, a finding that has been associated with expression of the IFN-γ/Th1 transcription factor T-bet and associated with pathogenic Th17 cells at sites of autoimmune inflammation (32, 45–47). Reduced expression of CCR6 in these mice is also congruent with previous reports, demonstrating that the loss of CCR6 correlates with IL-17+IFN-γ+ pathogenic Th17 cells (33, 38). M. tuberculosis–elicited Th17 cells, in contrast, displayed a more classical protective phenotype, typified by a greater proportion of IL-17 single producers, higher CCR6 expression, and greater production of the anti-inflammatory cytokine IL-10. In particular, IL-10 production by Th17 cells has been identified as a key feature of less pathogenic cells (32, 38). M. tuberculosis mice contained a greater frequency of IL-17+IL-10+ cells, a finding that has been associated with bystander suppression of CD8+ effector cells in a model of autoimmunity (32). Recent reports have demonstrated that a pathogenic Th17 phenotype is dependent on IL-23 that is induced TGF-β3 production (35, 38, 48–50). Our results extend previous reports using in vitro polarized cells by establishing that the degree of pathogenicity of microbe-elicited Th17 cells in vivo can vary depending on the specific microbe.

Importantly, this study also establishes that pathogen-elicited Th17 cells have a CD28/CTLA-4 expression profile that is distinct from Th1 cells. Th17 cells expressed significantly more CTLA-4 upon restimulation compared with Th1 cells, suggesting that Th17 cells might be more susceptible to CTLA-4 coinhibition. In ex vivo blockade experiments, we observed a modest effect of CTLA-4 mAb on Th1 responses, which may be explained by some degree of mAb cross-linking–induced negative signaling in our cultures. However, the significantly enhanced augmentation of Th17 responses relative to Th1 responses strongly suggests both a potent cell-extrinsic role for coinhibitory CTLA-4 on Th1 cells and greater sensitivity to CTLA-4 blockade, both findings that correlate with high expression of CTLA-4 on Th17 cells. Several recent studies have demonstrated a cell-extrinsic role for CTLA-4 in CD8+ and CD4+ T cells (51–55). Whereas our results demonstrate cell-extrinsic effects on Th1 and Th17 cells, we cannot rule out an additional cell-extrinsic role for CTLA-4 based on our experiments. The relative rarity of Th17 cells in healthy and inflamed tissues has been described in mouse models and human disease (24, 36, 56). High CTLA-4 expression on Th17 cells suggests another contributing mechanism to the low frequency at which these cells are found. Thus, our study modifies previous understanding of coinhibitory CTLA-4 on an inflammatory CD4+ lineage and demonstrates a novel role for CTLA-4 on Th17 cells.

Blockade of the CD28/CTLA-4 pathway has proven to be effective at the modulation of pathogenic T cell responses in the context of rheumatoid arthritis and renal transplantation (17). However, the relative resistance of Th17 cells to CD28/CTLA-4 blockade has important implications for the further development of this reagent, as Th17 cells play a role in autoimmunity and graft-versus-host disease, and have been reportedly involved in renal, lung, and liver allograft rejection (57–59). Furthermore, reports of the ineffectiveness of CTLA-4 Ig compounds in treating autoimmune diseases with Th17 components (IBD, MS, and systemic lupus erythematosus) further suggest that our findings have clinical relevance. These data imply that, conceivably, CTLA-4 Ig therapy for following renal or bone marrow transplantation could be more selectively administered to individuals for whom allo-
reactive Th17 populations are not prominent. In addition, this study provides a compelling mechanistic explanation to justify identifying new molecular targets to inhibit pathogenic Th17 cells.

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References


Supplemental Figure 1. M.Tb and Candida immunization and costimulation blockade treatment yields similar levels of CD8+ OT-I cells and anti-OVA antibodies. Mice were adoptively transferred with 10^8 OT-I and 10^8 OT-II cells and immunized with M.Tb or Candida with OVA peptide. (A) At 9 days post immunization OT-I cell frequencies were determined in the spleen (p = n.s., n = 5/group). (B) Positive control Candida hyphae cultures, foot pads from day 14 post immunized Candida OVA mice (n = 15, 3 independent experiments) or foot pads from naïve B6 mice were minced and cultured overnight and colony forming units were quantified. (C) Mice received an OVA skin graft and were untreated or treated with CTLA-4 Ig and anti-CD154, 10 days post transplant anti-OVA antibodies were measured in the blood using ELISA (p = n.s. all groups, n = 2-3/group). CFU, colony forming units.
Supplemental Figure 2. M.Tb and Candida elicited Th1 and Th17 cells express similar levels of T-bet and contain similar frequencies of IL-2 and TNF co-producers. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and restimulated for 4 h with PMA/Iono to identify Th1 and Th17 cells. (A) T-bet expression among OT-II Th1 and Th17 cells or in vitro Th1 polarized OT-II cells. (B) Frequency of IL-2* cells among OT-II Th1 (left) or Th17 (right) cells. (C) Frequency of TNF* cells among OT-II Th1 (left) or Th17 (right) cells. Bar graphs depict average ± SEM. Statistical comparison performed using unpaired two-tailed Student's t-test, *p<0.05.
Supplemental Figure 3. M.Tb and Candida elicited Th17 cells express more CCR6 and CTLA-4. Draining popliteal LNs from M.Tb OVA and Candida OVA mice were collected on day 9 post-immunization and restimulated for 4 h with PMA/Iono to identify Th1 and Th17 cells. (A) CCR6 expression on OT-II Th1 and Th17 cells populations (p < 0.0001, n = 7-10/group). (B) CCR6 expression on OT-II Th1 cells from M.Tb OVA and Candida OVA mice (p = n.s., n = 3-4/group). Frequency of (C) CD28+ cells, (D) CTLA-4+ cells, and (E) CTLA-4 expression among M.Tb OVA and Candida OVA Th1 and Th17 cells. Bar graphs depict average ± SEM. Statistical comparison performed using unpaired two-tailed Student’s t-test, *p<0.05.
Supplemental Figure 4

Supplemental Figure 4. Th1 and Th17 cells from M.Tb and Candida are similarly inhibited by CTLA-4 Ig and augmented by anti-CTLA-4. Purified CD4+ T cells from the draining popliteal LNs of M.Tb OVA and Candida OVA mice were co-cultured with CD11c+ DCs and OVA323-339 peptide for 4 days in the presence costimulation blockade or control molecules followed by brief PMA/Iono restimulation. (A) Effect of CTLA-4 Ig treatment of M.Tb OVA or Candida OVA mice relative to Ig-Fc control (9-13/group, 3 experiments). (B) Naïve OT-II splenocytes were stimulated with OVA323-339 peptide in the presence of CTLA-4 Ig, anti-CTLA-4 or control molecules for 4 days followed by brief PMA/Iono restimulation. Left, representative frequencies of OT-II Th1 cells. Right, relative effect of CTLA-4 Ig or anti-CTLA-4 on Th1 cell frequency normalized to Ig control molecule (n = 5-8/group, 3 experiments). (C) Effect of anti-CTLA-4 treatment of M.Tb OVA or Candida OVA mice relative to IgG control. Bar graphs depict average ± SEM. Statistical comparison performed using unpaired two-tailed Student’s t-test, *p<0.05.