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Maturation of human dendritic cells with *Saccharomyces cerevisiae* (yeast) reduces the number and function of regulatory T cells and enhances the ratio of antigen-specific effectors to regulatory T cells

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Abstract

We compared the effects of yeast-treated human dendritic cells (DCs) with CD40L-matured human DCs for the induction of effector cells and the number and functionality of $CD4^+CD25^+CD127^-$ $FoxP3^+$ regulatory T cells (Tregs). DCs were treated with yeast or CD40L and cocultured with isolated autologous $CD4^+$ T cells. $CD4^+CD25^+CD127^-$ T cells isolated from the coculture of $CD4^+$ T cells plus yeast-treated DCs (yeast coculture) had a lower expression of FoxP3 and decreased suppressive function compared to $CD4^+CD25^+CD127^-$ T cells isolated from the coculture of $CD4^+$ T cells plus CD40L-treated DCs (CD40L coculture). Also, compared to the CD40L coculture, the yeast coculture showed increases in the ratio of $CD4^+CD25^+$ activated T cells to Tregs and in the production of Th1-related cytokines (IL-2, TNF-α, IFN-γ) and IL-6. In addition, yeast-treated DCs used as antigen-presenting cells (APCs) incubated with the tumor antigen CEA enhanced the proliferation of CEA-specific $CD4^+$ T cells compared to the use of CD40L-matured DCs used as APCs. This is the first study to report on the role of yeast-treated/matured human DCs in reducing Treg frequency and functionality and in enhancing effector to Treg ratios. These results provide an additional rationale for the use of yeast as a vector in cancer vaccines.

Keywords

Saccharomyces cerevisiae; regulatory T cells; human dendritic cells

1. Introduction

In preclinical and clinical studies, numerous vectors [1–8] have been used to infect or transfect and mature human dendritic cells (DCs) to present a variety of antigens. Recombinant *Saccharomyces cerevisiae* (yeast) expressing full-length influenza matrix

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protein has been shown to be a versatile vehicle for presenting antigen to human DCs [1]. Preclinical studies have demonstrated the ability of yeast vectors encoding antigens such as chicken ovalbumin, HIV gag, HCV, and mutant ras to activate specific immune responses [9–12]. We have previously shown that heat-killed recombinant *S. cerevisiae* containing the transgene encoding carcinoembryonic antigen (yeast-CEA) can activate human DCs, resulting in increased expression of CD80, CD83, CD54, CD58, and MHC class II, and increased production of IL-12p70, TNFα, IFNγ and IL-2, compared to untreated DCs [13]. Yeast-CEA-activated DCs were used as antigen-presenting cells (APCs) to generate specific T-cell lines capable of lysing CEA^+ human tumor cells. We found a similar level of activation of a CEA-specific T-cell line between DCs treated with yeast-CEA and DCs sequentially exposed to yeast and CEA peptide. Furthermore, *in vivo* studies in CEA-transgenic mice have demonstrated that vaccination with a heat-killed recombinant yeast-CEA induces $CD4^+$ and $CD8^+$ CEA-specific immune responses, reduces tumor burden, and extends overall survival [14]. A Phase I study employing yeast-CEA is ongoing.

DCs recognize *S. cerevisiae* through dectin, mannose-fucose receptors, and toll-like receptors (TLRs) such as TLR-2 and TLR-4 [15]. TLR agonists are potent activators of innate immune responses and DC maturation; as a consequence, they promote an adaptive immune response when administered with antigens [16]. An important property of TLR activation is the capacity to enhance IL-12 production from DCs and, consequently, the ability to promote Th1-type responses, which play a key protective role in tumor immunity [17]. It has also been shown that production of IL-6 by DCs in response to TLR-4 ligation blocks the suppressive effect of T regulatory cells (Tregs), allowing activation of pathogen-specific adaptive immune responses [18].

Dendritic cells, which are potent APCs specialized to initiate immunity, have also been recognized as crucial players in the induction of T-cell tolerance [19]. The mechanism by which DCs induce tolerance is unclear, but could be either direct (induction of anergy or deletion of responding T cells) or indirect (activation of Tregs). In any case, immature DCs are capable of activating Tregs *in vitro* and *in vivo*, and fully mature DCs have tolerogenic properties [20,21]. Murine $CD4^+CD25^+$ Tregs can proliferate in the absence of added cytokines *in vitro* and *in vivo* when stimulated with antigen-loaded mature DCs [22], and human mature DCs are specialized to expand and maintain $FoxP3^+$ Tregs in T-cell cultures from healthy donors and myeloma patients [23]. Human myeloid DCs show greater activity compared with other APCs in increasing the number of functional Tregs and the expression of FoxP3 protein per cell. This expansion in Treg number and function is dependent on DC/T cell contact through CD80/CD86 membrane costimulatory molecules and the presence of endogenous IL-2. Recent studies have also demonstrated human DCs' capacity to generate antigen-specific Tregs from $CD4^+CD25^-$ T cells [24].

CD40L-matured DCs were used as a control as CD40L appears to be one of the most effective stimuli to induce DCs to produce massive amounts of bioactive IL-12. In addition, ligation of CD40L on human DCs can induce a marked upregulation of adhesion and costimulatory molecules, thus augmenting T-cell stimulatory capacity. CD40L signals also regulate DC-derived IL-7 production that, in turn, maximizes antigen-specific T-cell yields [25,26]. We show here that in the presence of yeast-treated DCs, $CD4^+CD25^+CD127^-$ T cells had decreased levels of FoxP3 expression, and that this correlated with a decrease in Treg suppressive function. High levels of IL-6 and Th1-related cytokines such as IL-2, TNF-α, and IFN-γ in the supernatant of $CD4^+$ T cells cocultured with yeast-treated DCs also suggest that *S. cerevisiae* activates human DCs to produce cytokines that alter the balance between $CD4^+$ effector cells and Tregs, resulting in an enhanced immune response to antigens. The study reported here is the first to compare the effect of yeast-treated and

CD40L-matured human DCs on the induction of $CD4^+CD25^+CD127^-$ $FoxP3^+$ human Tregs.

2. Materials and Methods

2.1 Generation of DCs

Peripheral blood mononuclear cells (PBMCs) from healthy donors and carcinoma patients were obtained from heparinized blood and separated using lymphocyte separation medium gradient (MP Biomedicals; Aurora, OH) according to the manufacturer's guidelines. DCs were generated from PBMCs as previously described [27]. Briefly, PBMCs (2×10^7) were resuspended in AIM-V medium (Invitrogen; Carlsbad, CA) and allowed to adhere in a 6-well plate for 2 h. Adherent cells were cultured for 5 days in AIM-V medium containing 100 ng/ml of recombinant human (rh) GM-CSF and 20 ng/ml of rhIL-4. The culture medium was replenished every 3 days.

2.2 Maturation of DCs with yeast or CD40L

An antigen-free *S. cerevisiae* construct (GlobeImmune; Louisville, CO) was used as a control. Human DCs were treated for 48 h at a yeast:DC ratio of 10:1 in a final volume of 3 ml of complete AIM-V medium/well. As a control, 1 μg/ml of CD40L (Axxora; San Diego, CA) was added to the DC culture 24 h prior to harvesting [13].

2.3 DC/$CD4^+$ T-cell cocultures

Mature DCs were adhered in a 6-well plate (2×10^5 or 4×10^5 DCs/well) for 2 h in AIM-V medium. The medium was then carefully removed and DCs were cocultured with purified autologous $CD4^+$ T cells in RPMI 1640 medium (Mediatech; Manassas, VA) supplemented with 10% human AB serum (Gemini Bio-Products; West Sacramento, CA). $CD4^+$ T cells were obtained using a $CD4^+$ T-cell isolation kit II (Miltenyi Biotec; Auburn, CA). $CD4^+$ T cells (2×10^6) were cocultured with DCs (2×10^5 or 4×10^5) at a $CD4^+$ T cell:DC ratio of 10:1 or 5:1 in a final volume of 4 ml/well for 5 days. In some experiments, DCs were incubated with CEA protein (AspenBio Pharma; Castle Rock, CO) at a concentration of 20 μg/ml and flu protein (USBiological; Swampscott, MA) at a concentration of 10 μg/ml.

2.4 Isolation of $CD4^+CD25^+CD127^-$ Tregs

$CD4^+CD25^+CD127^-$ Tregs were isolated from the DC/$CD4^+$ T-cell cocultures using a $CD4^+CD25^+CD127^-$ Treg isolation kit (Miltenyi Biotec), as previously described [28].

2.5 Flow cytometry analysis

Four-color flow cytometry was used to characterize the phenotype of the $CD4^+$ T-cell population to investigate the balance of $CD25^+$ activated T cells and Tregs in the presence of DCs treated with yeast or CD40L. Cells were resuspended in staining buffer (PBS containing 3% fetal bovine serum) and stained for 30 min at 4°C with FITC-conjugated anti-CD4, PE-Cy7-conjugated anti-CD25 (BD Biosciences; San Jose, CA), and PerCP-Cy5.5-conjugated anti-$CD127^-$ (eBioscience; San Diego, CA). Cells were then fixed and permeabilized using a fix/perm kit (eBioscience) according to the manufacturer's instructions, and labeled with APC-conjugated anti-FoxP3 antibody (236A/E7 clone; eBioscience) or its isotype control antibody (eBioscience) as a negative control. 1×10^5 cells were acquired and analyzed on a Becton Dickinson LSRII (BD Biosciences) using DiVa software (BD Biosciences). $CD4^+CD25^+CD127^-$ Tregs isolated from the DC/$CD4^+$ T-cell coculture were analyzed by 3-color flow cytometry using FITC-conjugated anti-CD4, PE-conjugated anti-CD25 (BD Biosciences) and APC-conjugated anti-FoxP3 antibody.

2.6 Immunosuppression assay

The immunosuppression assay was a modification of a previously described procedure [29]. Briefly, isolated $CD4^+CD25^-$ T cells (1×10^4 cells/well) were cultured alone or with $CD4^+CD25^+CD127^-$ Tregs isolated from the cocultures at a 1:1 ratio with 1 μg/ml of anti-CD3 antibody (OKT3; eBioscience) in the presence of irradiated (3,500 rad) T cell-depleted PBMCs (5×10^4 cells/well) in a 96-well flat-bottomed plate at 37°C and 5% CO_2. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products), 100 units/ml of penicillin, 100 μg/ml of streptomycin (Mediatech), and 2 mmol/l of L-glutamine (Mediatech). Proliferation was measured by 1 μCi (0.037 MBq)/well [^{3}H]thymidine (PerkinElmer; Waltham, MA) incorporation pulsed on day 4 and quantified 18 h later using a liquid scintillation counter (Wallac; Waltham, MA). All experiments were done in triplicate. Proliferation of $CD4^+CD25^-$ T cells without coculturing with Tregs constituted 100% of the proliferation.

2.7 Quantitative DNA Methylation Analysis of FoxP3 by Real-Time PCR

Quantitative DNA methylation analysis of FoxP3 was performed in collaboration with Epiontis GmbH (Berlin, Germany). Briefly, cocultures of $CD4^+$ T cells and yeast-treated DCs or CD40L-treated DCs were established using 3 different healthy donors. $CD4^+$ T cells were collected on day 5. DNA was extracted from $1–2 \times 10^6$ $CD4^+$ T cells by Puregene Core Kit A (Qiagen; Valencia, CA), according to the manufacturer's protocol. Analysis of FoxP3 Treg-specific demethylated region (TSDR) by real-time PCR was performed by Epiontis GmbH, following the protocol described by Wieczorek *et al.* [30]. Amounts of methylated and unmethylated FoxP3 DNA were estimated from calibration curves by linear regression on crossing points from the second-derivative maximum method. The proportion of unmethylated DNA was computed as the ratio of unmethylated FoxP3 TSDR-DNA and the sum of methylated and unmethylated FoxP3 TSDR-DNA. For female patients, this ratio was corrected with a factor of 2 due to the fact that 1 of the 2 TSDR alleles is methylated as a result of X inactivation.

2.8 Detection of cytokines

Supernatants were collected 48 h after DCs were exposed to yeast or CD40L, and 2 and/or 5 days after DCs were cocultured with $CD4^+$ T cells. All samples were screened for secretion of IFN-γ, IL-2, IL-8, IL-1β, GM-CSF, IL-12p70, IL-6, TNF-α, and IL-10 using a multiplex cytokine/chemokine kit (Meso Scale Discovery; Gaithersburg, MD). TGF-β1 was analyzed by ELISA kit (R&D Systems; Minneapolis, MN and BD Biosciences). Supernatants of $CD4^+$ T-cell lines stimulated for 24 h with peptide-pulsed autologous CD40L-treated DCs in IL-2-free medium were screened for secretion of IFN-γ by ELISA kit (Biosource International; Camarillo, CA).

2.9 $CD4^+$ T-cell proliferation assay

This assay was performed using samples from patients with CEA- or MUC1-expressing metastatic cancers post-vaccination with PANVAC, a poxviral-based vaccine engineered to express the transgenes for CEA and MUC1 and a triad of human T-cell costimulatory molecules (designated TRICOM) composed of B7.1, ICAM-1 and LFA-3. In a study conducted at the National Cancer Institute, patients received recombinant vaccinia (PANVAC-V) as a prime and recombinant fowlpox (PANVAC-F) as multiple booster vaccinations [31]. DCs from these patient samples (1×10^4/well) were cultured for 5 days with GM-CSF and IL-4 and treated with yeast (yeast:DCs = 10:1) for 48 h or with CD40L (1 μg/ml) for 24 h. $CD4^+$ T cells were isolated using Miltenyi columns by negative selection and incubated with DCs (CD4:DCs = 10:1; 1×10^5/well) for 7 days, using PHA (2 μg/ml) as positive control and myoglobulin (20 μg/ml) as negative control (Sigma-Aldrich; St. Louis,

MO). CEA protein (AspenBio Pharma) was used at 20 μg/ml. On day 7, [^{3}H]thymidine was added to the culture, incubated for 6 h, and read with a Wallac Trilux B-scintillation counter (fold increase = CD4 proliferation with CEA protein/CD4 proliferation with or without myoglobulin protein). The experiment was performed in triplicate.

2.10 Generation of CEA-specific CD4$^+$ T-cell lines

CD4$^+$ T-cell lines were generated from PBMCs of a vaccinated gastro-esophageal (GE) junction cancer patient. The class II CEA peptide used in this study has been previously described [32]. This CD4$^+$ T-cell epitope was selected from the amino acid sequence of CEA using the algorithm tables from 3 HLA-DR alleles (*DRB1*0101*, *DRB1*0401*, and *DRB1*0701*), as previously described [33]. The CEA peptide selected (YACFVSNLATGRNNS; 653–667; designated p653) was synthesized by Bio-Synthesis, Inc. (Lewisville, TX), with purity > 95%. HIV class II peptide (WIILGLNKIVRMYSPTSI; 133–150) was used as a negative control (Bio-Synthesis). CEA-specific CD4$^+$ cell lines were generated following a previously described protocol [34]. Briefly, yeast-treated and CD40L-treated DCs were pulsed with 10 μg/ml $CEA_{653–667}$ peptide for 2 h at incubator. The peptide-pulsed DCs were then mixed with 3×10^4 autologous 37°C in a 5% CO_2 isolated CD4$^+$ T cells at a CD4:DC ratio of 3:1. Culture medium consisted of RPMI 1640 supplemented with 5% human AB serum. Seven days later, CD4$^+$ T cells were isolated from the cultures and stimulated with autologous yeast-treated DCs or CD40L-treated DCs and pulsed with CEA peptide (at 10 μg/ml) for 2 h. Two days after this second stimulation with peptide, rhIL-2 was added to each well at a final concentration of 10 IU/ml.

2.11 Tetramer staining

PE-labeled $CEA_{653–667}$/DRB1-0401 tetramer was prepared by the NIH/NIAID MHC Tetramer Core Facility (Atlanta, GA) and PE-labeled hCLIP/DRB1-0401 tetramer was used as a negative control. The staining was performed as previously described [35]. Briefly, cells were stained with tetramer for 3 h at 37°C, washed and stained with CD4-FITC for 30 min at 4°C. Cells (1×10^4) were acquired and analyzed on an LSRII cytometer (BD Biosciences).

2.12 Statistical analysis

Statistical significance was calculated by VassarStats software (Poughkeepsie, NY) using a 2-tailed paired Student's *t* test. $P < 0.05$ was considered statistically significant.

3. Results

We first compared the effect of yeast- and CD40L-treated human DCs on CD4$^+$ T-cells. DCs from three healthy donors were treated with yeast for 48 h at a yeast:DC ratio of 10:1, or with CD40L for 24 h. Isolated autologous CD4$^+$ T cells were cocultured with the yeast or CD40L-treated DCs at CD4$^+$ T cell:DC ratios of 10:1 and 5:1 for 5 days. Cultures were then analyzed by flow cytometry for percent of Tregs, with Tregs defined as CD4$^+$CD25$^+$CD127$^-$ FoxP3$^+$-expressing T cells. As shown in Table 1, CD4$^+$ T cells cocultured with yeast-treated DCs at a ratio of 5:1 generated higher levels of CD4$^+$CD25$^+$ T cells than the coculture at a ratio of 10:1 (19.3% and 11.6%, respectively), without a concomitant increase in Tregs. This resulted in a higher ratio of activated CD4$^+$ T cells to Tregs in the coculture (3.8:1 and 2.4:1 at effector T cell:Treg ratios of 5:1 and 10:1, respectively). Effector T cells were defined as CD4$^+$CD25$^+$ T cells minus CD4$^+$CD25$^+$CD127$^-$ FoxP3$^+$ T cells. In contrast, levels of Tregs increased in the coculture of CD4$^+$ T cells and CD40L-treated DCs, resulting in a decrease in the ratio of effector T cells to Tregs (0.91:1 and 1.50:1 at CD4$^+$ T cell:DC ratios of 5:1 and 10:1, respectively). Data on 2 additional healthy donors are presented in Supplementary Tables 1A and 1B.

To confirm that the difference in the effector T cell:Treg ratio in the cocultures was not influenced by the lack of signal 1 in the coculture of $CD4^+$ T cells and CD40L-treated DCs, an additional healthy donor sample was analyzed with CEA protein added to the coculture (Table 2). CEA was used as an antigen as we have previously found that some healthy donors respond to CEA [36]. The absolute number of $CD4^+$ T cells and the percentage of $CD4^+CD25^+$ T cells in the $CD4^+$ T-cell population were again higher in the coculture of $CD4^+$ T cells and yeast-treated DCs than in the coculture of $CD4^+$ T cells and CD40L-treated DCs, with and without CEA protein. As shown in Table 2, the frequency of Tregs in $CD4^+CD25^+$ T cells was higher when $CD4^+$ T cells were cocultured with CD40L-treated DCs compared to yeast-treated DCs with and without CEA. This also translated to comparatively higher ratios of effector T cells:Tregs in the coculture of $CD4^+$ T cells and yeast-treated DCs. Data on 2 additional healthy donors are presented in Supplementary Tables 2A and 2B.

We next investigated the production of cytokines and chemokines in the coculture of $CD4^+$ T cells with yeast- or CD40L-treated DCs. Results showed higher levels of IFN-γ, TNF-α, GM-CSF, IL-2, IL-8, and IL-6 produced in the yeast cocultures, with and without CEA protein (Table 3). This suggested that a strong Th1 response was elicited by concomitant yeast and antigen. Low levels of immunosuppressive cytokines such as IL-10 and TGF-β were detected in cocultures with both yeast- and CD40L-treated DCs (data not shown). Similar results were observed in additional coculture experiments using samples from 5 healthy donors. Data on 2 additional healthy donors are presented in Supplementary Tables 3A and 3B.

We next evaluated the phenotype and functionality of $CD4^+CD25^+CD127^-$ Tregs isolated from the cocultures of $CD4^+$ T cells and yeast-treated or CD40L-treated DCs from 5 healthy donors. Tregs were isolated using $CD4^+CD25^+CD127^-$ Miltenyi columns, and FoxP3 expression was analyzed by flow cytometry gating on $CD4^+CD25^{high}$ cells (Fig. 1). The expression of FoxP3 in $CD4^+CD25^{high}$ Tregs generated from the coculture of $CD4^+$ T cells and yeast-treated DCs was significantly decreased compared to $CD4^+CD25^{high}$ Tregs generated from the coculture of $CD4^+$ T cells and CD40L-treated DCs (33.4% and 61.2%, respectively). Additional analysis showed that Tregs isolated from both cocultures were $CD127^-$. Similar results as shown in Fig. 1 were obtained from additional experiments using PBMC from 4 different healthy donors. The functional activity of Tregs isolated from the cocultures was determined by suppression assays using autologous $CD4^+CD25^-$ T cells as effector cells and autologous irradiated T cell-depleted PBMCs as APCs (Fig. 2). Tregs isolated from the coculture of $CD4^+$ T cells and yeast-treated DCs also exhibited a lower suppressive effect on $CD4^+CD25^-$ effector proliferation than Tregs isolated from the coculture of $CD4^+$ T cells with CD40L-treated DCs.

Previous studies have demonstrated the possibility that FoxP3 expression could be induced in human naïve $CD4^+$ T cells after cell activation. This transient expression of FoxP3 does not confer any suppressive competence to activated T cells [37]. Recently, Olek *et al.* have shown that stable expression of the FoxP3 gene in natural Tregs requires DNA demethylation at a highly conserved region of the human FoxP3 gene (Treg-specific demethylated region, TSDR). DNA demethylation was not detected in the FoxP3 gene transiently expressed in activated effector cells [30,38,39]. We therefore analyzed the DNA demethylation in the FoxP3 locus of $CD4^+$ T cells isolated from the yeast- or CD40L-cocultures. In preliminary data using three healthy donors, we found decreased FoxP3 TSDR demethylation levels (% of demethylated FoxP3) in the $CD4^+$ T cells isolated from the yeast coculture compared to those from the CD40L coculture (4.9 vs 7.3, 4.5 vs 5.9, and 5.3 vs 6.0%, respectively). The CD40L cocultures had higher levels of demethylated Tregs. Data from the DNA demethylation analyses were thus similar to results obtained from the

yeast coculture, which had a higher ratio of activated $CD4^+$ T cells to Tregs than the CD40L coculture.

Because the mechanism by which DCs are activated plays a key role in antigen-specific T-cell activity, we further evaluated the effect of yeast-treated DCs on the antigen-specific $CD4^+$ T-cell response. $CD4^+$ T cells isolated from carcinoma patients pre- and post-vaccination with rV-, rF-CEA-MUC1-TRICOM, i.e., PANVAC-V/F were cocultured with irradiated yeast-treated DCs or CD40L-treated DCs with or without CEA protein for 7 days. The CEA-specific response was measured by proliferation assay [31]. As seen in Fig. 3, the CEA-specific $CD4^+$ T-cell response in a patient with carcinoma was higher both pre- and post-vaccination when $CD4^+$ T cells were cocultured with yeast-treated DCs compared to CD40L-treated DCs, with or without the addition of CEA protein. The fold increases of CEA-specific CD4 proliferation (cpm of CD4 response to CEA/cpm of CD4 response to myoglobulin control) were higher in the coculture of $CD4^+$ T cells and yeast-treated DCs compared to the coculture of $CD4^+$ T cells and CD40L-treated DCs. Similar results were observed with T cells isolated from 2 additional cancer patients who had been vaccinated with PANVAC-V/F.

To further evaluate the ability of yeast-treated DCs to generate antigen-specific T-cell responses, we performed studies to establish a CEA-specific CD4 T-cell line *in vitro* using yeast-treated DCs as APCs and a class II CEA peptide (p653). PBMCs from a colorectal carcinoma patient were used in the study. Three CD4 T-cell lines were generated and designated as T-A, T-B, and T-C. T-A was generated by stimulating $CD4^+$ T cells with yeast-treated DCs pulsed with peptide p653. T-B was generated by stimulating $CD4^+$ T cells with yeast-treated DCs only. T-C was generated by stimulating $CD4^+$ T cells with CD40L-treated DCs pulsed with peptide p653. The specificity of the T-cell lines was determined by a PE-labeled $CEA_{653–667}$/DRB1-0401 tetramer. As shown in Table 4, a higher level of CEA tetramer binding T cells was detected in the T-A cell line compared to the T-C cell line. No positive CEA-specific T cells were detected in the T-B cell line. These results suggest that yeast-treated DCs can present CEA peptide and activate CEA-specific $CD4^+$ T cells at a higher level than CD40L-matured DCs. These T-cells were further evaluated by analysis of IFN-γ production by the T-A, T-B, and T-C T-cell lines after stimulation with peptide p653 at IVS-3 (Table 4). IFN-γ production by T-A was higher than by T-C after stimulation with autologous CD40L-matured DCs pulsed with peptide p653. As a negative control, production of IFN-γ from T-A and T-C was < 15.6 pg/ml when stimulated with autologous CD40L-matured DCs pulsed with HIV peptide or no peptide.

4. Discussion

Numerous studies have demonstrated that yeast strains such as *Candida albicans*, *Malassezia furfur*, and *S. cerevisiae* can be phagocytosed and processed for antigen presentation by human DCs [15,40,41]. The cell wall components of *S. cerevisiae*, including β-1,3-D-glucan and mannan, act like pathogen-associated molecular patterns (PAMPs) and interact primarily with TLR-2, TLR-4, mannose receptor, and dectin-1 on DCs. This triggers an innate “danger” signal on DCs and the production of several cytokines (IL-12, IFN-g, IL-2 and TNF-α), which generates a strong Th1 immune response that enhances the proliferation and cytotoxicity of CTLs [10,13,42,43]. We have also demonstrated that human DCs treated with yeast-CEA produce higher levels of IL-7 compared to DCs treated with CD40L (unpublished data). At the same time, yeast can efficiently mature human DCs, leading to increased levels of CD80, CD83, CD54, CD58, and MHC class I and II [10]. We have previously demonstrated that a heat-killed recombinant yeast construct expressing CEA (yeast-CEA) can be successfully processed by human DCs to activate a CEA-specific CD8 T-cell response. Gene expression profiles of human DCs treated with yeast-CEA

showed increased expression of numerous genes involved in the production of chemokines and cytokines and their receptors, and genes related to antigen uptake, antigen presentation and signal transduction [13]. A previous murine study from our laboratory found that vaccination with yeast-CEA elicited both a $CD4^+$ and a $CD8^+$ T-cell response. In this study the frequency of Tregs was not significantly altered, but the net effect was an increase in the ratios of $CD4^+$ effector cells to Tregs and $CD8^+$ effector cells to Tregs, thus enhancing the anti-tumor response. Indeed, yeast-CEA vaccinated CEA-transgenic mice displayed reduced tumor burden and increased overall survival compared to mock-treated or control-yeast treated mice in both pulmonary metastasis and pancreatic tumor models [14].

The studies reported here investigated for the first time the effect of yeast-treated DCs on the $CD4^+$ T-cell population, especially on the ratio of $CD4^+$ effector cells to Tregs. Several studies have shown that antigen-loaded mature DCs can induce proliferation of Tregs *in vitro* and *in vivo* [19,22]. It has been demonstrated that human myeloid mature DCs (mDCs) are superior APCs for maintaining and expanding $CD4^+CD25^{high}FoxP3^+$ Tregs *in vitro* in healthy donors and patients with myeloma [23]. One of the major challenges of tumor immunotherapy and the use of cancer vaccines is the development of strategies to overcome immune suppression. Evidence that Tregs may play an important role in the suppression of antitumor immunity comes from several clinical investigations showing that a higher frequency of Tregs in tumor correlates with poor clinical outcome [44–46]. We recently demonstrated that the suppressive function of Tregs was enhanced in prostate cancer patients compared to healthy donors [29]. It has also been reported that Tregs can suppress the proliferation of naïve $CD4^+$ T cells and inhibit IL-2 secretion of $CD4^+$ effector cells upon activation by tumor-specific antigen [47].

In this study, we demonstrated that the coculture of human $CD4^+$ T cells and yeast-treated autologous DCs can enhance the $CD4^+$ effector to Treg ratio compared to the coculture of $CD4^+$ T cells and CD40L-treated autologous DCs, with or without signal 1. These results suggest that yeast-treated human DCs can enhance $CD4^+$ T-cell immune responses partly due to an increase in the $CD4^+$ effector to Treg ratio. We also observed that the percentage of $CD4^+CD25^+CD127^-$ $FoxP3^+$ Tregs in $CD4^+CD25^+$ T cells decreased in the coculture of $CD4^+$ T cells and yeast-treated DCs compared to the coculture of $CD4^+$ T cells and CD40L-treated DCs, with or without signal 1. These data were further confirmed by the quantitative analysis of DNA demethylation in the FoxP3 locus of the $CD4^+$ T cells collected from the yeast or CD40L cocultures. To ascertain that the decrease in $CD4^+CD25^+CD127^-$ $FoxP3^+$ Tregs was not influenced by the increase in $CD4^+CD25^+$ effector cells, we analyzed the phenotype of isolated $CD4^+CD25^+CD127^-$ T cells and found that Tregs generated from the coculture of $CD4^+$ T cells and yeast-treated DCs had lower levels of FoxP3 expression compared to Tregs generated from the coculture of $CD4^+$ T cells and CD40L-treated DCs. FoxP3 is the most reliable molecular marker for natural Tregs, and it has been demonstrated that FoxP3 expression correlates with Treg function [28]. High-level expression of FoxP3 is sufficient to confer suppressive activity to normal non-Treg cells [48]. An evaluation of the functionality of Tregs generated from the 2 cocultures used here showed that Tregs generated from the coculture of $CD4^+$ T cells and yeast-treated DCs exerted a lower suppressor function compared to Tregs from the coculture of $CD4^+$ T cells and CD40L-treated DCs. These results suggest that yeast-induced maturation of DCs leads to decreased FoxP3 expression in Tregs and, consequently, to decreased suppressive function of Tregs, facilitating $CD4^+$ T-cell immune responses.

In this study, we analyzed the production of cytokines and chemokines in 2 separate cocultures. High levels of IFN-γ, TNF-α, IL-8, GM-CSF, and IL-2 were detected in the coculture of $CD4^+$ T cells and yeast-treated DCs, which mediated a high level of Th1 immune response. Higher levels of IL-6 were also detected in the coculture of $CD4^+$ T cells

and yeast-treated DCs than in the coculture of $CD4^+$ T cells and CD40L-treated DCs. It has been recently demonstrated that TLR-4 activation on DCs can block the suppressor function of Tregs, due in part to the production of IL-6 [18]. IL-6 induces FoxP3 mRNA down-regulation in Tregs, and acts synergistically with IL-1 to down-regulate FoxP3 expression via a pathway dependent on transcription factor STAT3 [49]. This observation suggests that the decrease in FoxP3 expression and Treg functional activity generated from the coculture of $CD4^+$ T cells and yeast-treated DCs may also be due in part to the presence of IL-6. The addition of anti-IL-6 and anti-IL-6 receptor antibody did not increase the Treg frequency (unpublished data). These results are consistent with findings showing that TLR-4 activation can enhance production of IL-12 and IL-6 by DCs and consequently promote the differentiation of the IFN-γ-secreting Th1 subtype of $CD4^+$ T cells, which in turn can lead to an enhanced CTL response against tumors [50].

To determine whether DCs treated with yeast could increase the antigen-specific $CD4^+$ T-cell response compared to DCs treated with CD40L, we investigated CEA-specific $CD4^+$ T-cell proliferation in cancer patients. The results showed that yeast-treated DCs pulsed with the tumor antigen CEA induced a higher level of CEA-specific $CD4^+$ T-cell immune response compared to CD40L-treated DCs used as APCs. To confirm these data, we generated a CEA-specific $CD4^+$ T-cell line using yeast-treated DCs. We performed a class II tetramer binding assay to identify antigen-specific T-cells, because we did not have enough PBMC to make autologous DCs to be used as APC in a class II ELISPOT assay. IFN-γ and tetramer binding results on $CD4^+$ T-cell lines suggest that yeast-treated DCs can efficiently generate CEA-specific $CD4^+$ T-cell immune responses. These findings also demonstrate that yeast-treated human DCs can decrease the expression of FoxP3 and the suppressive function of $CD4^+CD25^+CD127^-$ Tregs, and thus provide an additional rationale for the clinical evaluation of recombinant yeast constructs such as yeast-CEA in cancer vaccine immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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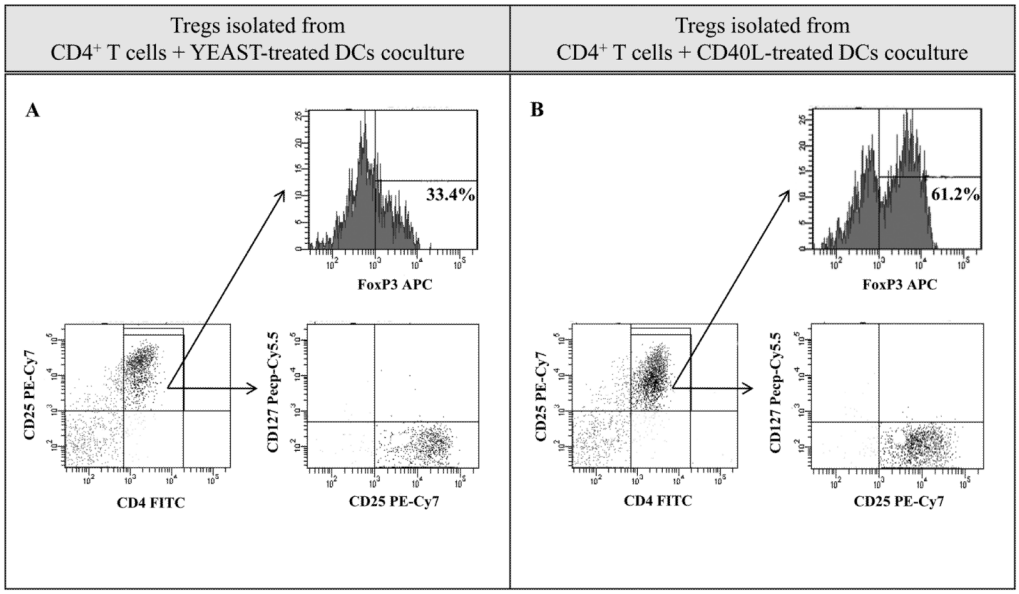

Fig. 1.

$CD4^+CD25^+CD127^-$ Tregs isolated from the coculture of $CD4^+$ T cells and yeast-treated DCs vs. CD40L-treated DCs showed decreased FoxP3 expression. $CD4^+$ T cells plus yeast-treated DCs (A), and $CD4^+$ T cells plus CD40L-treated DCs (B), were cocultured for 5 days. On day 5, $CD4^+CD25^+CD127^-$ Tregs isolated from the cocultures were analyzed by FACS. Cells were stained with CD4 FITC, CD25 PE, and FoxP3 APC antibodies and acquired on an LSRII. The numbers in the upper right of the graphs represent the % of $FoxP3^+$ cells in $CD4^+CD25^{high}$ Tregs. The figure is representative of 3 experiments on 3 healthy donors.

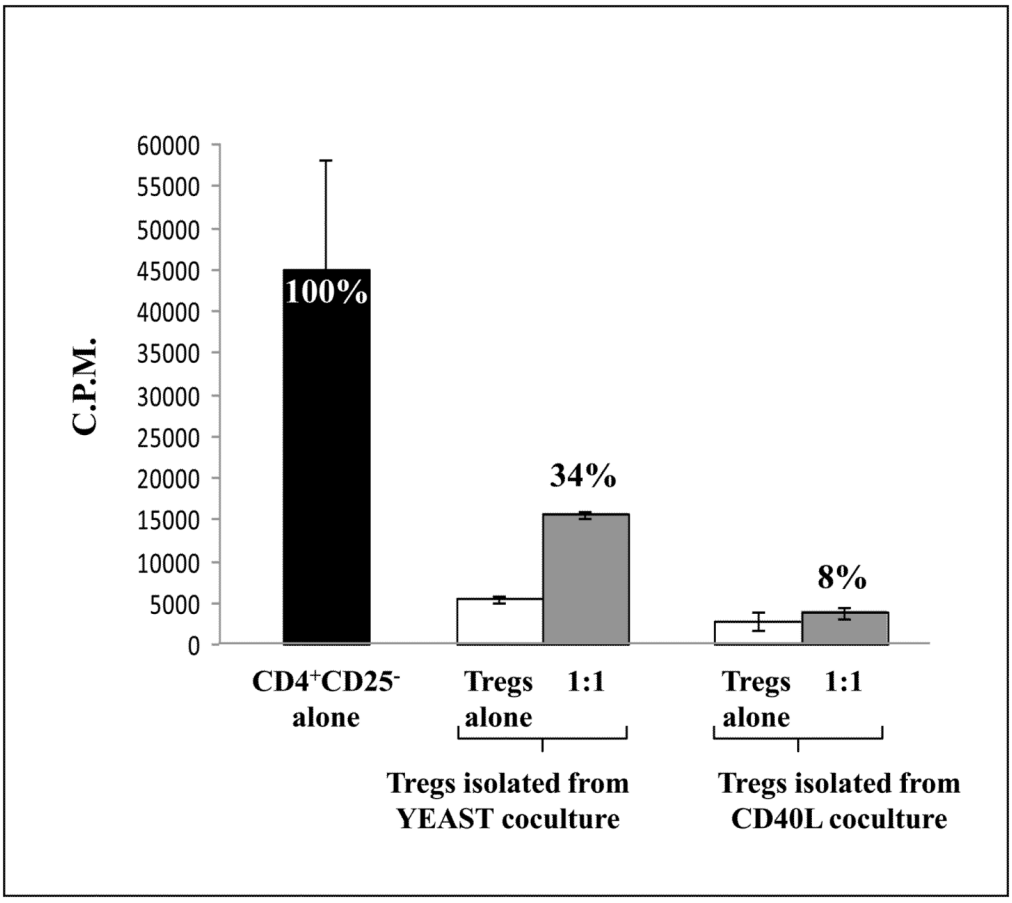

Fig. 2.

Tregs generated from yeast coculture exhibit decreased suppressive function on $CD4^+CD25^-$ effector cells compared to Tregs generated from CD40L coculture. A representative histogram shows the suppression of $CD4^+CD25^-$ T-cell proliferation by $CD4^+CD25^+CD127^-$ Tregs in a healthy donor. $CD4^+CD25^+CD127^-$ Tregs generated from yeast and CD40L cocultures were isolated by Miltenyi columns and rested for 24 h. Tregs were then cultured alone or cocultured at a 1:1 ratio with autologous $CD4^+CD25^-$ T cells, upon stimulation with anti-CD3 and irradiated autologous T-depleted PBMCs. Experiments were performed in triplicate and results are expressed as the mean ± SD (cpm). Percentages indicate the level of proliferation.

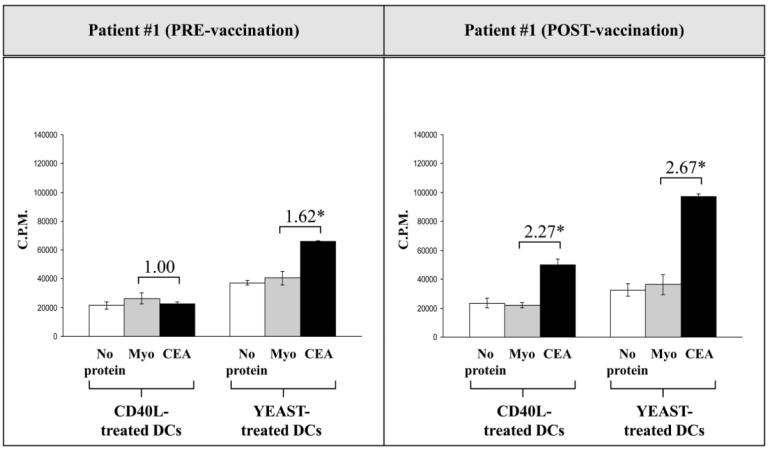

Fig. 3.

CEA-specific $CD4^+$ T-cell proliferation increased in a colorectal cancer patient pre- and post-vaccination when $CD4^+$ T cells were stimulated with DCs treated with yeast and incubated with CEA protein. DCs from a colorectal cancer patient were isolated pre- and post-vaccination and cultured for 5 days with GM-CSF and IL-4. DCs were then treated with yeast (yeast:DCs = 10:1) for 48 h or with CD40L (1 μg/ml) for 24 h. $CD4^+$ T cells were isolated using Miltenyi columns by negative selection and incubated with DCs ($CD4^+$ T cells:DCs = 10:1) for 7 days, using 2 μg/ml PHA as a positive control and 20 μg/ml myoglobulin as a negative control. CEA protein was used at 20 μg/ml. On day 7, ^3H was added to the culture, incubated for 6 h, and read with a Wallac Trilux B-scintillation counter. The numbers above the CEA columns represent the fold increase of CD4 proliferation with CEA protein and myoglobulin protein. The experiment was performed in triplicate and the results are expressed as the mean ± SD (cpm).

Table 1

$CD4^+CD25^+$:Treg ratio in coculture of $CD4^+$ T cells plus autologous DCs treated with yeast or with CD40L (HD#1)

Coculture (ratio)	Total number of cells/well	$CD4^+$ (% in total number of cells)	$CD4^+CD25^+$ (% in $CD4^+$)	Tregs		Ratio effector cells:Tregs
				(% in $CD4^+$)	(% in $CD4^+CD25^+$)	
$CD4^+$: CD40L- treated DCs (10:1)	2.65×10^6	2.41×10^6 (91.2%)	2.12×10^5 (8.8%)	0.86×10^5 (3.5%)	40.3%	1.5:1
$CD4^+$: YEAST- treated DCs (10:1)	3.50×10^6	3.10×10^6 (88.8%)	3.59×10^5 (11.6%)	1.10×10^5 (4.4%)	30.6%	2.4:1
$CD4^+$: CD40L- treated DCs (5:1)	2.81×10^6	2.50×10^6 (89.3%)	2.12×10^5 (8.5%)	1.10×10^5 (3.4%)	51.8%	0.9:1
$CD4^+$: YEAST- treated DCs (5:1)	4.12×10^6	3.53×10^6 (85.7%)	6.81×10^5 (19.3%)	1.41×10^5 (4.0%)	20.7%	3.8:1

Tregs = $CD4^+CD25^+CD127^-FoxP3^+$

Effector cells = $CD4^+CD25^+$

DCs were treated with yeast at a yeast:DC ratio of 10:1 for 48 h or with CD40L (1 μg/ml) for 24 h. Isolated autologous $CD4^+$ T cells were cocultured with yeast-treated DCs or CD40L-treated DCs at 2 ratios (10:1 or 5:1). FACS analysis was performed on day 5.

Ratio of effector cells:Tregs = ([CD4+CD25+] - [CD4+CD25+CD127-FoxP3+Tregs]/[CD4+CD25+CD127-FoxP3+Tregs]).

HD = healthy donor.

Table 2

$CD4^+CD25^+$:Treg ratio in coculture of $CD4^+$ T cells plus autologous DCs treated with yeast or CD40L and with or without CEA protein (HD#1)

Coculture (ratio)	Total number of cells/well	$CD4^+$ (% in total number of cells)	$CD4^+CD25^+$ (% in $CD4^+$)	Tregs		Ratio effector cells:Tregs
				(% in $CD4^+$)	(% in $CD4^+CD25^+$)	
$CD4^+$: CD40L-treated DCs (5:1)	3.46×10^6	3.1×10^6 (89.7%)	5.6×10^5 (18.2%)	1.9×10^5 (6.4%)	33.9%	1.8:1
$CD4^+$: YEAST-treated DCs (5:1)	4.11×10^6	3.74×10^6 (91.1%)	9.8×10^5 (26.3%)	2.3×10^5 (6.3%)	23.4%	3.1:1
$CD4^+$: CD40L-treated DCs (5:1) + CEA	3.84×10^6	3.43×10^6 (89.5%)	8.3×10^5 (24.3%)	2.4×10^5 (7.2%)	28.9%	2.4:1
$CD4^+$: YEAST-treated DCs (5:1) + CEA	4.53×10^6	4.17×10^6 (92.0%)	13.7×10^5 (32.9%)	2.9×10^5 (7.1%)	21.1%	3.6:1

Tregs = $CD4^+CD25^+CD127^-FoxP3^+$

Effector cells = $CD4^+CD25^+$

DCs were treated with yeast at a yeast:DC ratio of 10:1 for 48 h or with CD40L (1 μg/ml) for 24 h. Isolated autologous $CD4^+$ T cells were cocultured with yeast-treated DCs or CD40L-treated DCs at a ratio of 5:1 with or without CEA protein. FACS analysis was performed on day 5.

Ratio of effector cells:Tregs = ([CD4+CD25+] - [CD4+CD25+CD127-FoxP3+Tregs]/[CD4+CD25+CD127-FoxP3+Tregs]).

HD = healthy donor.

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Table 3

Cytokine production from coculture of $CD4^+$ T cells and DCs (HD#1)

Coculture (ratio)	IL-2 (pg/ml)	IL-8 (pg/ml)	GM-CSF (pg/ml)	IFN-γ (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
$CD4^+$: CD40L-treated DCs (5:1)	11	5,871	62	682	0	28
$CD4^+$: YEAST-treated DCs (5:1)	154	7,976	218	1,583	76	139
$CD4^+$: CD40L-treated DCs (5:1) + CEA	9	8,990	81	1,857	187	39
$CD4^+$: YEAST-treated DCs (5:1) + CEA	79	10,106	322	4,785	288	194

DCs were treated with yeast at a yeast:DC ratio of 10:1 for 48 h or with CD40L (1 μg/ml) for 24 h. Isolated autologous $CD4^+$ T cells were cocultured with yeast-treated DCs or CD40L-treated DCs at a ratio of 5:1 with or without CEA protein. After 5 days, culture supernatants were collected and screened for cytokine and chemokine production.

HD = healthy donor.

Table 4

Tetramer binding and IFN-γ production from CEA-specific $CD4^+$ T cells

T-cell line	Treatment of DCs	Tetramer Binding		IFN-γ Production		
		Control tetramer hCLIP (% of positive cells)	CEA class II tetramer (% of positive cells)	HIV peptide	No peptide	CEA peptide
T-A	Yeast + CEA peptide	0.2	8.5	< 15.6	< 15.6	892 pg/ml
T-B	Yeast	0.2	0.4	< 15.6	< 15.6	63 pg/ml
T-C	CD40L + CEA peptide	0.2	6.2	< 15.6	< 15.6	659 pg/ml

T-A: CEA-specific $CD4^+$ T-cell line generated using DCs treated with yeast and pulsed with class II CEA peptide p653

T-B: CEA-specific $CD4^+$ T-cell line generated using DCs treated with yeast without CEA peptide

T-C: CEA-specific $CD4^+$ T-cell line generated using DCs treated with CD40L and pulsed with class II CEA peptide p653

For the analysis of IFN-γ production, $CD4^+$ T-cell lines at IVS3 were stimulated for 24 h with autologous CD40L-matured DCs pulsed with CEA peptide in IL-2-free medium. HIV peptide or no peptide were used as negative controls. Supernatants were screened for secretion of IFN-γ by ELISA

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