Journal of Clinical Immunology, Vol. 26, No. 3, May 2006 (© 2006)
DOI: 10.1007/s10875-006-9020-8

Human Dendritic Cell Interactions with Whole Recombinant Yeast: Implications for HIV-1 Vaccine Development

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Received August 31, 2005; accepted March 02, 2006
Published online: 13 May 2006

Defects in number and function of dendritic cells (DCs) have been observed during HIV-1 infection, so therapeutic HIV-1 vaccine approaches that target or activate DCs may improve vaccine immunogenicity. To determine the potential of recombinant *Saccharomyces cerevisiae* yeast as an HIV-1 vaccine, we investigated interactions between yeast and human DCs. Yeast induced direct phenotypic maturation of monocyte-derived DCs (MDDCs) and enriched blood myeloid DCs (mDCs), but only indirectly matured blood plasmacytoid DCs (pDCs). Yeast-pulsed MDDCs and blood mDCs produced inflammatory cytokines and stimulated strong allo-reactive T cell proliferation. Both blood DC subsets internalized yeast, and when pulsed with yeast recombinant for HIV-1 Gag protein, both stimulated *in vitro* expansion of Gag-specific $CD8^+$ memory T cells. These results suggest that *S. cerevisiae* yeast have potent adjuvant effects on human DCs. Furthermore, recombinant yeast-derived antigens are processed by human blood DCs for MHC class-I cross-presentation. These DC-targeting characteristics of yeast suggest that it may be an effective vaccine vector for induction of HIV-1-specific cellular immune responses.

KEY WORDS Dendritic cells; vaccination; cytokines; yeast.

INTRODUCTION

Dendritic cells (DCs) are potent antigen—presenting cells (APCs) derived from bone marrow precursors that serve as activators of naïve $CD4^+$ and $CD8^+$ T lymphocytes and are likely to play a critical role in the induction of immune responses to vaccine antigens (Ags) (1). They are the only APC capable of efficiently processing exogenous Ag for presentation to naïve precursor cytotoxic T lymphocytes (CTL) via the major histocompatibility complex (MHC) class-I pathway (2). DCs take up Ag in the periphery and depending on the Ag, upregulate MHC and co-stimulatory molecules (e.g., CD80, CD86, CD40) in a process known as maturation (3). As part of the maturation process, DCs downregulate antigen acquisition and become primed to synthesize cytokines that enable them to become strong inducers of immunity. In addition, mature DCs can present MHC class-II-restricted antigenic epitopes to $CD4^+$ T cells and initiate help for CTL via interactions between CD40 on DCs and CD40L on the T cell (4, 5). Activated DCs secrete interleukin-12 (IL-12) and other Th1-biasing cytokines that, combined with presentation of MHC class-I restricted antigenic epitopes and co-stimulatory molecules, result in priming of $CD8^+$ CTL responses (6). Compared with other APCs, DCs are up to 1000–fold more efficient in activating resting T cells (7). As such, DCs are likely to be important in the induction of cellular immune responses to vaccine antigens.

Blood DCs, traditionally characterized by the absence of leukocyte lineage (Lin)-specific Ag and by surface expression of HLA-DR and CD4 (8), have been subcategorized into myeloid DCs (mDCs), denoted as $Lin^-HLA\text{-}DR^+CD11c^+$, and plasmacytoid DCs (pDCs), denoted as $Lin^-HLA\text{-}DR^+CD11c^-IL\text{-}3R^{Hi}$ (9, 10). In addition to phenotypic differences, different functional attributes, including the ability to stimulate specific T helper cell (Th) subsets, were thought to be associated with the individual subsets based on their lineage (10, 11). However, it has been suggested that the DC lineage per se may not actually be the basis of the Th1/Th2 polarization previously observed. Rather, the activation state of the DC, the type of activation signal it encounters, and the cytokine environment all have been postulated to contribute to Th polarization by DCs (12–15).

Blood DCs and their progeny are likely to play a critical role in priming T cell responses to HIV-1 and opportunistic pathogens *in vivo* and as such may be useful targets for vaccine preparations. However, a number of defects in both blood DC subsets have been observed in chronically

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HIV-1-infected subjects, which might limit the ability of infected subjects to respond to a vaccine challenge. A decrease in the proportion or absolute number of blood DCs expressing CD11c (mDCs) has been observed in a number of cohorts of HIV-1-infected donors (16–20), in particular in subjects with active viral replication. Blood pDCs, reported to be the natural interferon alpha/beta (IFN-α) producing cells (IPCs) of the immune system (21, 22), are also decreased in number in patients with HIV-1 infection (18–20, 23). The deletion of this DC subset in the blood of HIV-1-infected subjects has been associated with disease progression and development of opportunistic infections (24).

In addition to numeric defects, a number of studies have reported functional defects in both subsets of blood DCs obtained from HIV-1-infected subjects, primarily from those with plasma viremia or late stage disease. These reported functional abnormalities include decreased cytokine production (20, 23), defective maturation potential, and defects in the ability to stimulate autologous or allogeneic T cell proliferation [reviewed in (25)]. Although the impact of DC dysfunction on *in vivo* T cell induction remains unclear, it stands to reason that vaccine approaches that target or activate DCs might be necessary to overcome these potential APC defects.

Recombinant yeast vectors, as a vaccine delivery system, are an attractive option in this setting for several reasons. Multiple antigens can be engineered for expression within a single yeast formulation (26) and they share many advantages with DNA vaccines, including ease of construction, low expense of mass production, and biological stability. We have previously shown that whole *Saccharomyces cerevisiae* yeast recombinant for a model tumor Ag were effective at inducing $CD8^+$ T cell-mediated tumor protection in vaccinated mice (27). Furthermore, whole yeast were shown to be avidly internalized by murine DCs *in vitro*, leading to DC maturation and IL-12 production (27). In order to translate these important findings from the murine to human systems, we investigated the interactions between whole recombinant yeast and human DCs [monocyte-derived DCs (MDDCs) and blood DC subsets (mDCs and pDCs)] in terms of maturation, activation, cytokine production, and the ability to stimulate Ag-specific T cells.

METHODS

Study Population

Peripheral blood mononuclear cells (PBMCs) were isolated from individual donor leukapheresis rinse packs (obtained from Bonfils Blood Center, Denver, CO) or from heparinized blood obtained from HIV-1-infected and uninfected subjects at the University of Colorado Health Sciences Center (UCHSC). The Colorado Multiple Institutional Review Board (COMIRB) at UCHSC (Denver, CO) approved this study, and informed consent was obtained from all study participants.

Generation and Culture of Recombinant Yeast

S. cerevisiae yeast transformed with the empty vector (containing no gene insert, referred to as YVEC) and *S. cerevisiae* transformed with the Gag vector expressing amino acids 1-431 of HXB2 HIV-1 Gag p55 under control of the CUP1 promoter (referred to as HIVAX-Gag) were kindly provided by GlobeImmune, Ltd. Gag expression in HIVAX-Gag was determined by comparison to Gag protein standards, quantified by STORM phosphoimage analysis of immunoblots using a mouse monoclonal antibody (mAb) to Gag and a goat anti-mouse alkaline phosphatase conjugate secondary mAb, with blots being developed by ECL. Yeast were grown at 30°C in yeast nitrogen base (YNB) media supplemented with amino acids and 2.5% glucose in a shaking water bath to an $OD_{600} = 0.25$–0.5, and then induced with Cu^{2+} and grown overnight. Yeast were frozen in aliquots of YNB containing 15% glycerol at -70°C until used. Yeast were heat-killed by incubation for 15 min in 56°C water bath and washed with phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA) prior to use in all experiments.

Culture and Maturation of Monocyte-Derived DCs (MDDCs)

MDDCs were generated from PBMCs as described (28). Briefly, PBMCs were isolated from blood using a standard Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation and plated at a concentration of 1×10^7/mL in serum-free media (AIMV, Gibco) and incubated for 60–90 min at 37°C. Several gentle washes were performed to remove the nonadherent cell fraction, and the adherent cells were cultured in serum-free media containing 1000 U/mL rhGM-CSF (Immunex Inc., Seattle, WA) and 1000 U/mL rhIL-4 (R&D, Minneapolis, MN) for 5–7 days. In some experiments, $CD14^+$ cells were selected using beads from Miltenyi Biotech according to manufacturer's instructions and cultured at 1×10^6 cells/mL in serum-free media containing rhGM-CSF and rhIL-4 for 5–7 days. On day 5, cells were counted and the following added to one or more flasks: Media alone, LPS (100 μg/mL, Sigma), CD40L trimer (5 μg/mL, Immunex, Inc.), or YVEC (heat-killed and added at yeast–to–DC ratio of 10:1), and incubated for an additional 48 h at 37°C. Cells were then harvested

with several gentle washes with ice–cold HBBS to remove the nonadherent cell fraction from the flasks, counted, and stained for surface maturation markers. Supernatants were removed and frozen at -20°C. The dose of LPS used was determined via dose titration experiments with the goal of yielding maximum phenotypic maturation of MDDCs.

Maturation of Blood DC Subsets in Whole PBMC Preparations

PBMCs were isolated from five leukapheresis rinse packs using standard Ficoll-Paque density gradient centrifugation. PBMCs were co-incubated with media alone (RPMI with 10% human AB serum; Gemini, Woodland, CA), LPS (100 μg/mL), or YVEC at a ratio of 0.5 yeast/PBMC for 18–24 h at 37°C in T25 or T75 flasks at a concentration of 1×10^6 PBMC/mL. The cells from each maturation condition were then harvested with several gentle washes with ice–cold HBBS to remove the nonadherent cell fraction from the flasks. Supernatants were removed and frozen at -20°C. The surface phenotype of pDCs and mDCs in the PBMC preparation was determined by flow cytometry as described later.

Enrichment and Maturation of mDC and pDC Subtypes from PBMCs

PBMCs were isolated as described, then mDC and pDC were enriched from PBMC according to Miltenyi instructions using BDCA1 and BDCA4 microbeads. Briefly, PBMC were first depleted of $CD19^+$ cells using MACS microbeads and magnetic column selection. mDC were then isolated from the CD19 negative fraction using BDCA1+ (CD1a+) microbeads and column selection. The mDC negative fraction was retained for isolation of pDCs using BDCA4+ microbeads and magnetic column selection. Purity was assessed using flow cytometry. In three experiments, purity of mDCs was 95, 92, and 90%, respectively, and purity of pDCs was 95, 95, and 86%, respectively. Enriched DCs were cultured at 1×10^6 cells/mL in RPMI with 10% human serum with either YVEC yeast (5:1) or LPS (10 μg/mL) for mDC, either YVEC yeast or CpG oligodeoxynucleotide 2006 (5′-GGGGGACGATCGTCGGGGGG-3′) and CpG oligodeoxynucleotide 2216 (5′-TCGTCGTTTTGTCGTTTTGTCGTT-3′) (3 μg/mL of each) (Invitrogen) for pDCs, or media alone. The CpG oligodeoxynucleotide sequences were selected based on their ability to induce maturation of pDC (ODN 2006) and production of high amounts of IFN-α (ODN 2216) (29). After 18–24 h, mDCs and pDCs were stained with dendritic cell-specific markers and maturation markers. Supernatants were removed and stored at -20°C.

Flow Cytometry

The phenotype of MDDC was evaluated with two-color flow cytometric analysis using a panel of monoclonal antibodies directed against surface markers known to be expressed or up-regulated on mature DCs (1) including: CD80 (Ancell), CD86 (Ancell), CD40 (Ancell), MHC Class-I and -II, MR, CD11c (Becton-Dickinson (BD)), CD1a, and CD83 (PharMingen (Ph)) that were either FITC- or PE-labeled. Four-color analysis was performed on fresh PBMC and enriched mDCs or pDCs using FITC-labeled anti-Lineage (Lin−) panel (CD3/CD14/CD16/CD19/CD20/CD56) mAb (BD), FITC-labeled anti-CD34 mAb (BD), tricolor-labeled anti-HLA-DR mAb (Caltag, Burlingame, CA), allophycocyanin conjugate (APC)-labeled anti-CD11c (BD), or biotinylated anti-CD123 (IL-3R) (Ph) that was then labeled with streptavidin-conjugated APC (Ph) as per the manufacturer's instructions, and either PE-labeled anti-CD40, -CD4 (Ph), or CD83 mAbs. Cells were also labeled with appropriate isotype control antibodies in each experiment. After staining for 30 min with mAbs, cells were washed, resuspended in FACS Fix (PBS 1% paraformaldehyde, 0.6% sodium azide), and stored at 4°C in the dark before analysis. Two—color flow samples were analyzed on a FACScan flow cytometer (Becton Dickinson), and four—color flow samples were analyzed on a FACSCalibur flow cytomer (BD).

Expansion of HIV-1-Specific $CD8^+$ T Cells by Enriched Blood DCs

Briefly, mDC and pDC populations were enriched (as described earlier) from PBMCs of three HIV-1-infected donors receiving antiretroviral therapy. Each DC subset was pulsed overnight with media alone, YVEC (5:1 yeast:DC), HIVAX-Gag (5:1 yeast:DC), or HIV-1 Gag p55 peptides (pooled HXB2 strain HIV-1 Gag peptides, 15 amino acids overlapping by 11 aa, 1 μg/mL final of each peptide (122 peptides) (AIDS Reference and Reagent Program). DCs were cultured at 1×10^5/mL in RPMI with 10% human AB serum with 100 ng/mL GMCSF (mDC) or 10 ng/mL IL-3 (R&D Systems) (pDC) to improve survival. After overnight incubation with antigen, DCs were washed and admixed with $1.5–2.5 \times 10^6$ CD8-selected autologous T cells (1:100 DC—to—T cells) in RPMI with 10% human AB serum. The cells were co-cultured for 5–7 days, at which time responder $CD8^+$ T cells were tested for HIV-1 Gag specificity in an IFN-γ ELISPOT assay. For the ELISPOT assay, T cells were cultured at

1×10^4 cells per well in serum—free AIM V media with thawed, autologous DC- and CD8-depleted PBMCs as APCs at 5×10^3 per well. Assay conditions included triplicate wells containing cells plus either media alone, PHA (1 μg/mL), or pooled P55 peptides (0.5 μg/mL per peptide).

Mixed Leukocyte Reaction (MLR)

Blood mDC and pDC subsets enriched from heparinized peripheral blood as described earlier were cultured overnight at 1×10^6 cells/mL in RPMI with 10% human AB serum with either 10 μg/mL LPS, 3 μg/mL CpGs, YVEC yeast (5:1 yeast:DC), or media alone. Allogeneic $CD3^+$ T-cells were isolated from PBMC using anti-CD3 microbeads (Miltenyi), resuspended at 5×10^5/mL in RPMI with 10% human AB serum, and 0.1 mL per well was plated in a 96—well plate. DCs were irradiated at 3000 rads, washed, resuspended in RPMI with 10% human AB serum, and added to the T cells at various ratios of T cells to DCs. Cells were co-incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 days; then plates were pulsed with tritiated thymidine for 18 h, harvested, and radioactivity was counted on a Beta-counter (Packard, Meriden, CT). Results are expressed in counts per minute (CPM).

Measurement of Cytokine Production

Cytokine production from each maturation experiment was evaluated using frozen supernatants and testing for production of IL-12 p70, IL-10, TNF-α (Pharmingen), and IFN-α (Bender) using ELISA kits as per manufacturer's instructions. For experiments with enriched mDCs and pDCs, IL-1β, IL-6, IL-8, IL-10, IL-12 p70, and TNF-α production was evaluated using a human inflammatory cytometric microbead array kit (Becton-Dickinson).

Internalization of Yeast by Mononuclear Cells

YVEC yeast cultures were grown at 27°C in 20 mL YNB media supplemented with amino acids and 2.5% glucose in a shaking water bath to an OD_{600} of 1. After approximately 24 h, 20 μL of mito-tracker Green (MTG) dye (dissolved in DMSO) was added to one of the cultures and incubated for an additional 20 min. Yeast were harvested, washed with PBS, counted, and heat killed at 56°C for 15 min prior to being used in experiments.

PBMCs were isolated from leukapheresis rinse packs as described and plated at 2×10^6 cells/mL in media (RPMI with 10% human Ab serum). Either unlabeled or MTG-labeled yeast were added to the cells at a ratio of 0.5 yeast/PBMC and cultured at 37°C for 6 h. Cells were then harvested, and bound extracellular yeast were quenched with 0.2% trypan blue solution for 20 min at room temperature. Four—color flow cytometric analysis was performed to determine uptake of MTG-labeled yeast (FITC channel) by blood DCs [PE-labeled anti-Lineage panel mAb (BD), tricolor-labeled anti-HLA-DR mAb (Caltag), APC-labeled anti-CD11c (BD) or -CD123 (IL-3R, Miltenyi)], T cells (APC-labeled anti-CD3 (BD), monocytes (PE-labeled anti-CD14, Ancel), and B cells (PE-labeled anti-CD20, Ancel). After staining for 20 min at 4°C, the cells were washed, resuspended in FACS Fix, and analyzed on a FACSCalibur flow cytomer (BD).

RESULTS

Exposure of MDDCs to Recombinant Yeast Resulted in Phenotypic Maturation, Cytokine Production, and Enhanced Allogeneic T Cell Proliferation

MDDCs derived from PBMC of five HIV-1 seronegative individuals were cultured with media alone, recombinant yeast (YVEC), or LPS for 48 h; then DC surface phenotype was evaluated by flow cytometry. MDDCs cultured with yeast had at least a two-fold increase in the median expression of MHC class-I, MHC class-II, ICAM, CD40, and CD86 compared to media alone, whereas increases in CD80 and CD83 were less consistent (Fig. 1A). Expression of the mannose receptor on MDDCs uniformly decreased in all experiments following yeast exposure. These changes were all consistent with patterns of MDDC maturation induced by LPS (data not shown). In addition, when cytokine production from these experiments was assessed, YVEC-pulsed MDDCs produced increased amounts of IL-12 and IL-10 compared to MDDCs cultured with media or LPS (Fig. 1B). It is noteworthy that stimulation of MDDCs with low-doses of LPS (10 μg/mL) results in high amounts of IL-12 production (30). Therefore, the lower level production observed in our experiments could be a result of the high dose of LPS used (100 μg/mL) to induce maximal phenotypic maturation. Yeast-induced TNF-α production was above background but slightly less than that induced by LPS (Fig. 1B). Results are numerically summarized in Table I.

In order to assess the immunogenicity of YVEC-pulsed MDDCs, standard MLRs were performed using HIV-1 seronegative donor MDDCs in varying ratios to allogeneic T cells. T cell proliferation was markedly increased when T cells were stimulated by MDDCs cultured with YVEC versus those cultured with media alone, LPS, or CD40L (Fig. 2, representative of three experiments). The

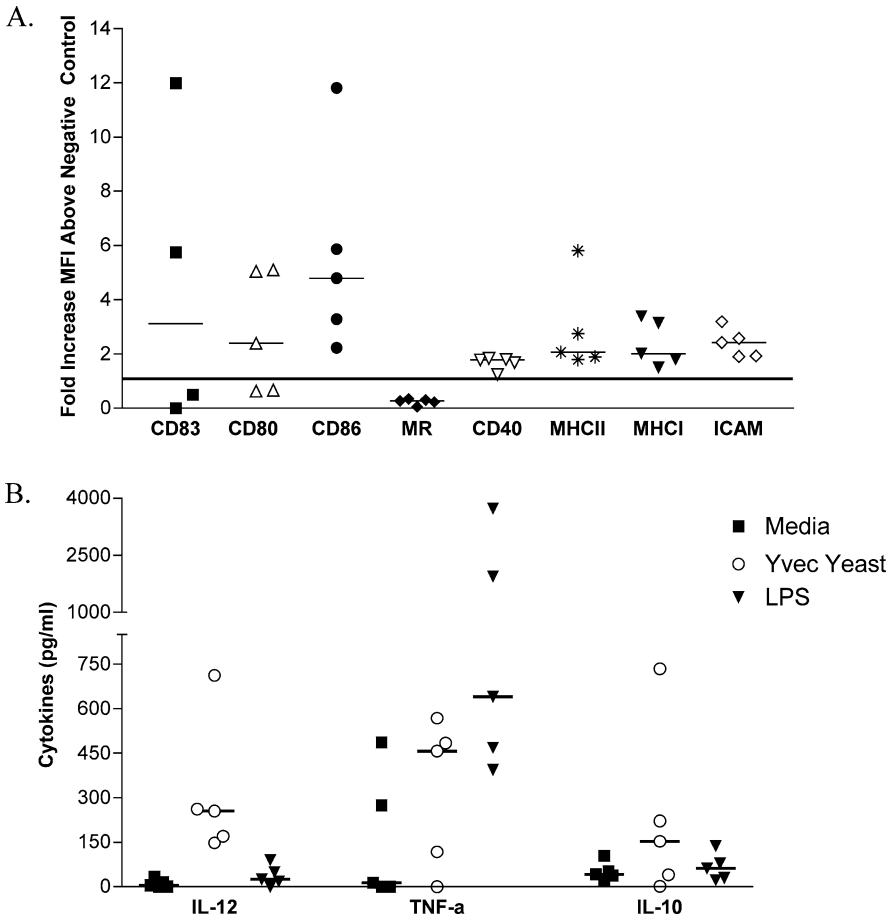

Fig. 1. Surface phenotype and cytokine production of MDDCs following exposure to recombinant *S. cerevisiae* yeast. MDDCs were cultured from PBMCs from 5 HIV-1 seronegative donors and incubated with media, yeast (10 yeast:1 DC), or LPS (100 μg/mL) for 48 h. (A) DC surface expression of the following maturation markers were determined by flow cytometry: CD83, CD80, CD86, mannose receptor (MR), CD40, MHC class-I and -II, and CD54 (ICAM). Results shown indicate MFI of each marker for the yeast condition divided by the MFI without stimulation (fold increase MFI). CD83 expression was only evaluated in four experiments. (B) Culture supernatants collected from each experiment and frozen at -20°C were evaluated for IL-12, TNF-α, and IL-10 production using standard ELISA kits. Bars represent the median of five experiments.

increased T cell proliferation in response to YVEC-pulsed MDDCs was noted at all three DC:T cell ratios (1:20, 1:50, and 1:100).

Recombinant Yeast Matured Both Blood DC Subsets in Whole PBMC Preparations

In addition to investigating MDDC-recombinant yeast interactions, we evaluated the interaction between yeast and blood pDCs and mDCs in unfractionated PBMCs. PBMCs were obtained from five HIV-1-uninfected donor leukapheresis rinse packs as described in the Section Methods and incubated with media alone, YVEC yeast (0.5 yeast:1 PBMC), or LPS for 18–24 h. Surface expression of CD40 (Fig. 3) and CD83 on mDCs (Lineage-, HLA-DR+, CD11c+) and pDCs (Lineage-, HLA-DR+, CD123 high) within unfractionated PBMCs were evaluated for by four—color flow cytometry. Pulsing PBMC with yeast resulted in a median increase in CD40 expression on mDCs of at least two-fold compared to media alone, similar to mDC changes in CD40 expression induced by LPS (Fig. 4). Yeast also similarly increased CD40 expression on pDCs in whole PBMC. Only variable increases in CD83 expression were observed on both DC subsets after either yeast or LPS stimulation (data not shown). Cytokine analysis of the culture supernatants

Table I. Cytokine Production

	IL-1β	IL-6	IL-10 (median pg/mL; range)	TNF-α (median pg/mL; range)	IL-12 (p70) (median pg/mL; range)	IFN-α (median pg/mL; range)
MMDC[a]						
Media	–	–	43 (19–105)	14 (0–486)	5 (0–34)	–
YVEC yeast (10:1)	–	–	153 (2–735)	458 (0–641)	255 (148–713)	–
LPS (100 μg/mL)	–	–	63 (23–138)	641 (394–3748)	26 (0–91)	–
Whole PBMC[b]						
Media	–	–	0 (0–195)	117 (0–180)	1 (0–62)	–
YVEC yeast (50:1)	–	–	15 (0–195)	1300 (1132–16,010)	73 (7–154)	–
LPS (100 μg/mL)	–	–	240 (1–710)	885 (665–4269)	19 (1–245)	–
Enriched mDC[c]						
Media	0 (0–24)	14 (13–295)	4 (0–47)	26 (0–385)	0 (0)	0 (0)
YVEC yeast (5:1)	174 (45–422)	946 (297–3667)	1014 (127-2919)	6442 (1447–7327)	0 (0–39)	0 (0–251)
LPS (10 μg/mL)	2260 (160–2495)	23,705 (23,305–28,706)	2606 (420-20,520)	1979 (266-2571)	9 (0–45)	0 (0–43)
Enriched pDC[c]						
Media	0 (0)	2 (2–19)	3 (0-4)	0 (0)	0 (0)	0 (0)
YVEC yeast (5:1)	0 (0)	3 (2–15)	3 (0–6)	9 (0–22)	0 (0)	0 (0)
CpG (3 μg/mL)	0 (0)	1095 (506–2079)	0 (0)	4087 (1317–4927)	0 (0–17)	3993 (3852–4004)

Note. Cytokines were measured by standard ELISA kits (MMDC or whole PMBC) or by bead flow cytometry (purified DC subsets).
[a]Supernatants from five separate experiments were collected at 48 h after addition of the indicated stimuli.
[b]Supernatants from five separate experiments were collected 18–24 h after addition of indicated stimuli.
[c]Supernatants from three separate experiments were collected 18 h after addition of the indicated stimuli.

harvested from those experiments revealed significant production of TNF-α by YVEC-pulsed PBMCs (median: 1300 pg/mL) but minimal production of IL-12 or IL-10 (median: 73 and 15 pg/mL, respectively). Results are summarized in Table I.

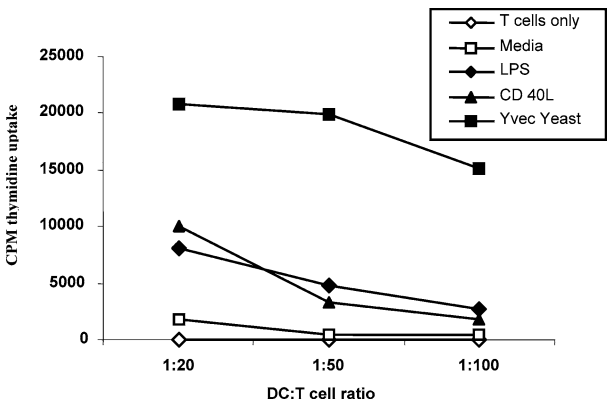

Fig. 2. Stimulation of allogeneic T cell proliferation by yeast-pulsed MDDCs. Normal donor MDDCs were pulsed overnight with media alone, YVEC yeast, LPS, or CD40L as described in the Section Methods, washed, irradiated, then plated in standard MLR assays with varying DC:T cell ratios with allogeneic T cells for 4 days. T cell proliferation was determined by tritiated thymidine uptake, with results expressed as CPM. This figure is representative of three experiments.

Exposure of Enriched Blood DC Subsets to Recombinant Yeast Resulted in Phenotypic Maturation of mDCs but not pDCs

In order to assess whether the recombinant yeast was directly causing maturation of the DC subsets or indirectly via interactions with other cells in the PBMCs (e.g., monocytes, B cells, etc.), mDCs and pDCs were enriched from peripheral blood of three normal donors using Miltenyi DC isolation kits, and then incubated for 18–24 h with YVEC yeast, LPS, or CpGs 2006/2216. Using four-color flow cytometry, the surface phenotype of both mDCs and pDCs was then examined as described earlier. Exposure to yeast resulted in phenotypic maturation of mDCs, as evidenced by increases in CD40, CD83, and CD86 expression similar to those induced by exposure to LPS (Fig. 5A). Conversely, yeast did not induce typical upregulation of these surface markers on enriched pDCs relative to that observed following incubation of enriched pDCs with CpGs (Fig. 5B).

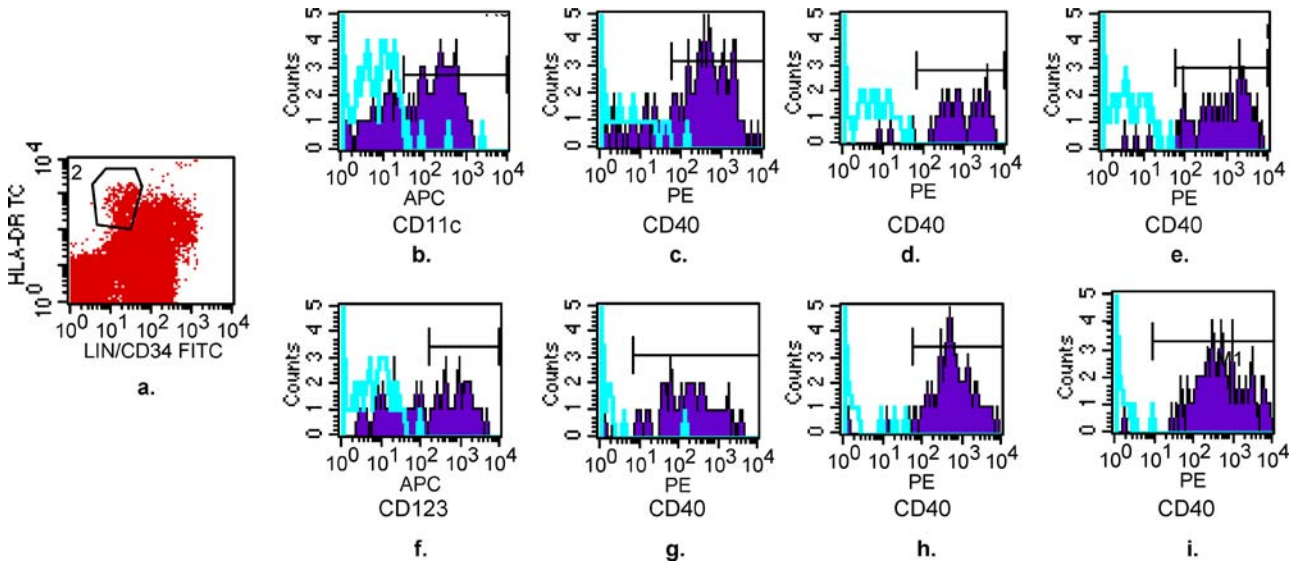

Fig. 3. Flow cytometric analysis of DC subsets matured with media, YVEC, or LPS. (a) $Lin^{-}HLA\text{-}DR^{+}$ cells were gated upon. (b) The $Lin^{-}HLA\text{-}DR^{+}CD11c^{+}$ cells (mDC) were then gated with subsequent expression of CD40 on cells matured for 24 h with media alone (c), YVEC (50:1) (d), and LPS (100 μg/mL) (e). A similar analysis was done for the $Lin^{-}HLA\text{-}DR^{+}CD123^{+}$ (pDC) (f–i). The outline represents the respective isotype APC or PE controls.

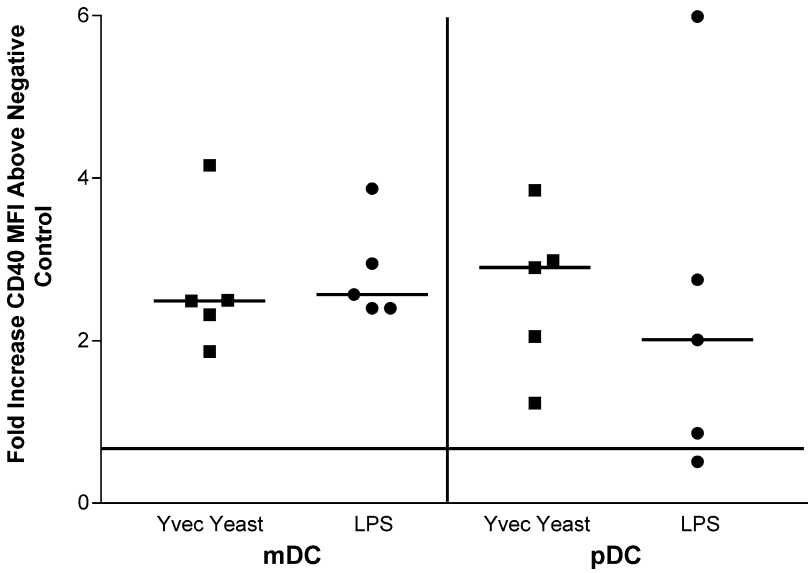

Fig. 4. Expression of CD40 on mDCs and pDCs after incubation of unfractionated PBMCs with yeast. PBMCs from five HIV-1 seronegative controls were cultured with media, YVEC yeast (0.5 yeast:PBMC), and LPS (100 μg/mL) for 18–24 h. Surface phenotype of mDCs and pDCs was then determined using four-color flow cytometry. Data shown reflect MFI of CD40 expression on each DC subset after stimulation with yeast or LPS divided by MFI of unstimulated PBMC (fold increase MFI). Bars represent median responses.

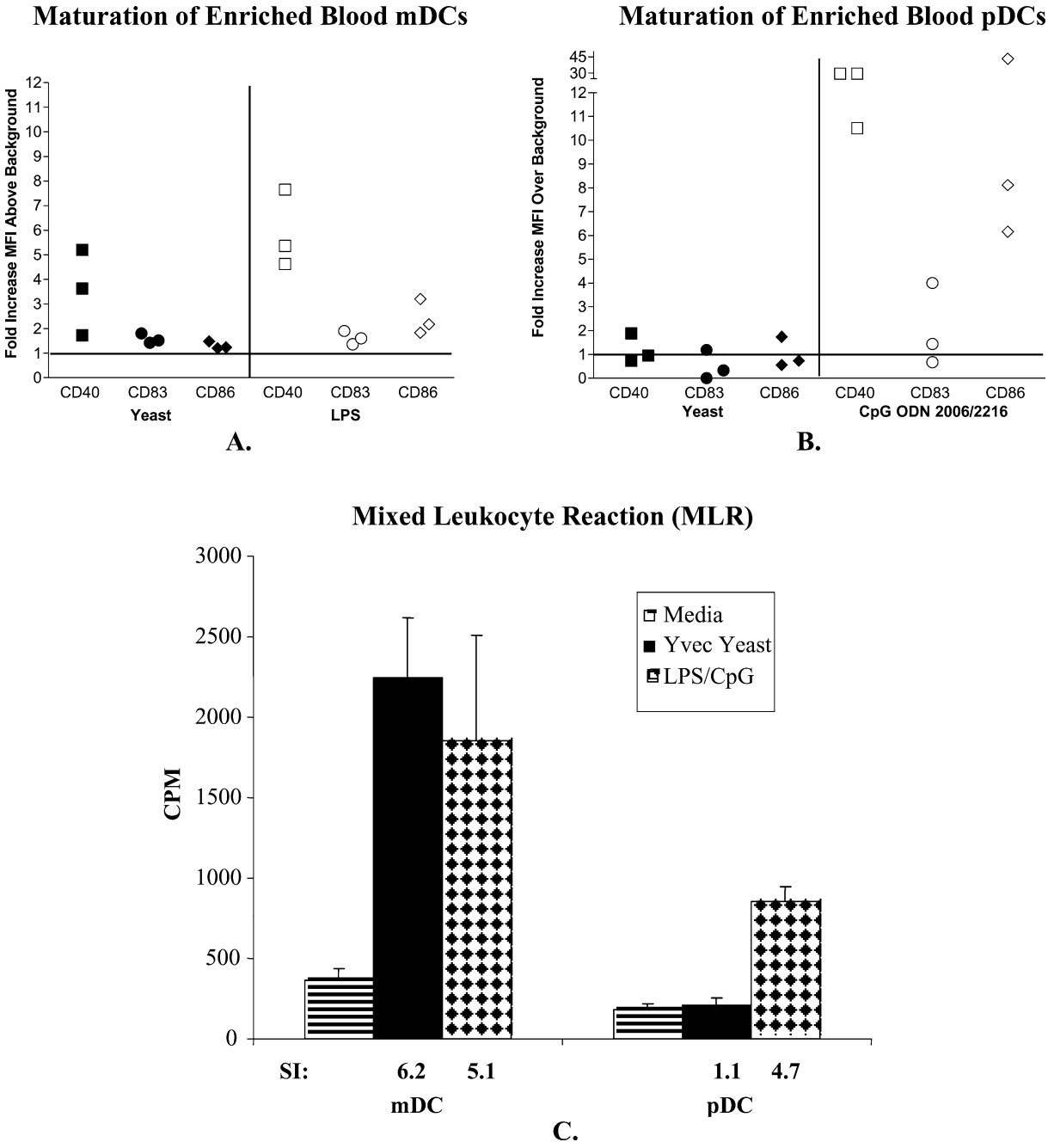

Fig. 5. Maturation phenotype and immunostimulatory capacity of enriched blood mDC and pDC subsets following yeast stimulation. Blood mDC and pDC subsets were enriched from the blood of three normal donors, incubated overnight in media alone, YVEC yeast, or LPS (for mDCs) and CpG ODN 2006/2216 (for pDCs). Expression of CD40, CD83, and CD86 were determined on (A) blood mDCs or (B) pDCs following stimulation with yeast or LPS relative to the unstimulated condition. (C) Pulsed DCs were irradiated and plated with allogeneic T cells in a 4-day MLR at a ratio of 1 DC:50 T cells. Bars represent the mean value of three replicates with standard error. The figure in (C) is representative of one of three experiments.

Cell-free culture supernatants from these maturation experiments were collected, frozen, and later analyzed for cytokine production, using either a human inflammatory cytokine microbead array kit (B–D) for measuring IL-1, IL-6, IL-10, IL-12 p70, and TNF-α or a standard ELISA kit for measuring IFN-α (Bender). As shown in Table I, enriched mDCs cultured with yeast produced large amounts of TNF-α (median: 6442 pg/mL), IL-10 (median: 1014 pg/mL), and IL-6 (median: 946 pg/mL) relative to media only condition, but they produced little IL-1 (median: 174 pg/mL) and no IL-12 p70 (median: 0 pg/mL) after yeast stimulation. Enriched pDCs cultured with yeast did not produce significant quantities of any of the aforementioned cytokines (summarized in Table I). IFN-α was not detected in the culture supernatant of either mDCs or pDCs pulsed with yeast, yet enriched pDCs stimulated with CpGs 2006/2216 produced significant amounts of IFN-α (median: 3993 pg/mL).

Standard MLRs were also performed using enriched mDC and pDC subsets to stimulate allogeneic CD3-selected T cells. Enriched mDCs stimulated with yeast induced significant allogeneic T cell proliferation, comparable to that induced by mDCs pulsed with LPS (Fig. 5C). Alternatively, minimal T cell proliferation was induced by enriched pDCs stimulated with yeast, even though CpG-stimulated pDCs induced some T cell proliferation.

Internalization of Yeast by Different Cell Populations in PBMC Preparations

In an effort to determine the mechanism underlying the different maturational effects exerted by recombinant yeast on both DC subsets within whole PBMCs versus the purified mDCs and pDCs, YVEC yeast were labeled with mito-tracker green dye and incubated with whole PBMCs from three normal donors. Following quenching of extracellular yeast bound to cell surfaces, flow cytometry was used to determine the percent of yeast internalized by the various mononuclear cells in PBMCs, based on fluorescence, including: mDCs, pDCs, monocytes, T cells, and B cells. As depicted in Fig. 6, monocytes displayed the greatest and most consistent degree of internalization of labeled yeast (30–40%), followed by mDCs, and to a lesser degree B cells and pDCs. Minimal uptake of labeled yeast by T cells was observed.

Enriched Blood DCs Pulsed with Recombinant Yeast Expressing HIV-1 Gag Stimulated Expansion of Gag–Specific, IFN-γ-Producing CD8$^+$ T Cells

Blood mDCs and pDCs enriched from PBMCs of three HIV-1-infected subjects (receiving antiretroviral therapy, peripheral CD4$^+$ T cell counts greater than 500 cells/μL)

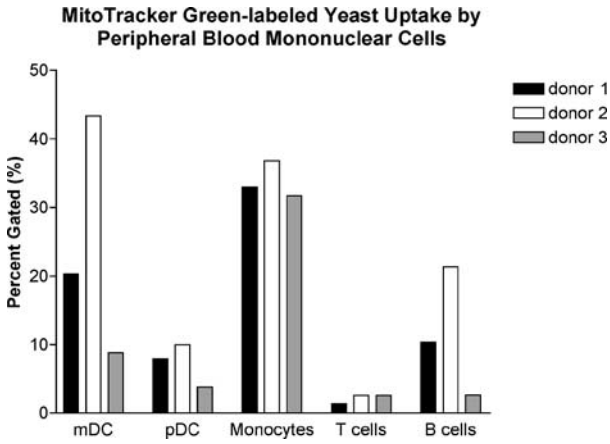

Fig. 6. Internalization of yeast by peripheral blood cells. YVEC yeast were labeled with mito-tracker green (FITC) fluorescent dye and incubated for 6 h with whole PBMCs isolated from three normal donors. Flow cytometry was performed to determine the percentage of each of the following cell types that had internalized yeast (based on FITC fluorescence): mDCs, pDCs, monoctyes, T cells, and B cells.

were incubated overnight with media, overlapping peptides spanning HIV-1 Gag p55, YVEC yeast, or yeast expressing HIV-1 Gag p55 (HIVAX-Gag) and then co-cultured for 5–7 days with autologous CD8$^+$ T cells. Following this culture period, responder cells were plated in an IFN-γ ELISPOT assay either with media alone or media plus overlapping peptides spanning HIV-1 Gag p55 in order to determine the frequency of Gag-specific cells expanded under each DC stimulation condition. Figure 7 shows three examples of the IFN-γ ELISPOT responses to HIV-1 Gag following stimulation of CD8$^+$ T cells by autologous mDCs and pDCs. In two of three experiments, mDCs pulsed with HIVAX-Gag stimulated a greater expansion of Gag-specific, IFN-γ-producing CD8$^+$ T cells than did mDCs pulsed with either media or YVEC control yeast. In all experiments, pDCs pulsed with HIVAX-Gag yeast expanded Gag-specific CD8$^+$ T cells above control conditions. In general, HIVAX-Gag-pulsed mDCs and pDCs stimulated expansion of Gag-specific CD8$^+$ T cells to a lesser extent than did Gag peptide-pulsed DCs (Fig. 7).

DISCUSSION

In a previous study, we reported that whole *S. cerevisiae* yeast stimulated murine bone marrow-derived DCs to phenotypically mature and to produce IL-12 *in vitro* (27). Furthermore, injection of yeast recombinant for a model tumor antigen induced CD8$^+$ T cell-mediated tumor protection in vaccinated mice. As DCs serve to link innate

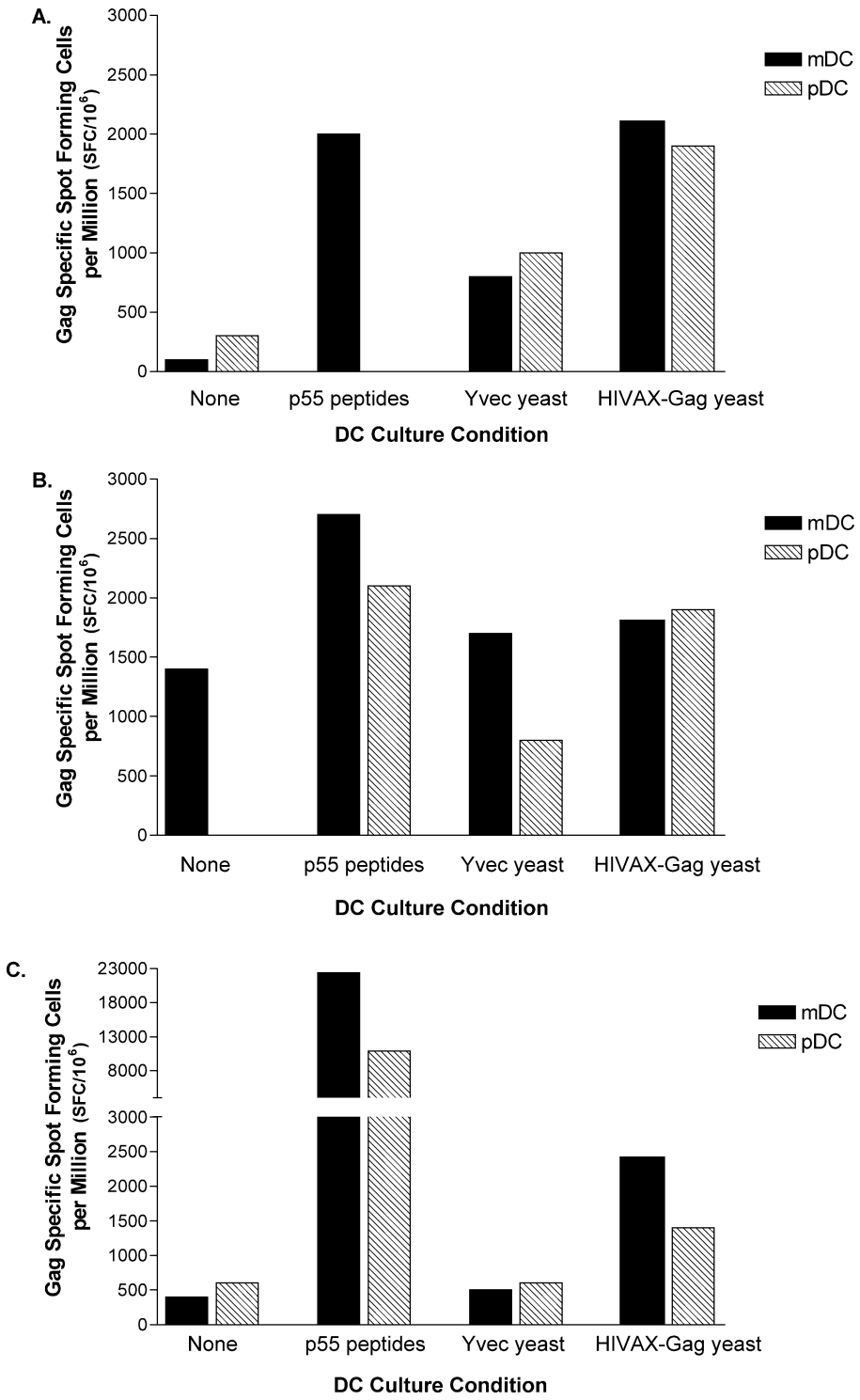

Fig. 7. HIV-1 Gag-specific $CD8^+$ T cell expansion following stimulation with yeast-pulsed blood DCs. Enriched mDCs and pDCs from three HIV-1-infected subjects were incubated overnight with media alone, overlapping HIV-1 Gag p55 peptides, YVEC control yeast, or HIV-1 Gag-expressing yeast (HIVAX-Gag), and then co-cultured for 5–7 days with autologous $CD8^+$ T cells. T cells from each culture condition were then plated in an IFN-γ ELISPOT assay with either media alone or overlapping peptides spanning HIV-1 Gag p55. The number of Gag-specific T cells was determined by subtracting the median number of SFC in the media only wells from median SFC in the Gag peptide wells.

and adaptive immunity, a vaccine that targets and activates human DCs directly should have potent immunostimulatory properties. Since a major goal in HIV-1 vaccine development is to induce strong HIV-1-specific cell mediated immunity, we sought to extend these murine yeast studies into a human *in vitro* system to determine whether recombinant yeast-based vectors might have utility as prophylactic or therapeutic HIV-1 vaccines.

We examined the stimulatory effects of *S. cerevisiae* yeast on several types of human dendritic cells, including cultured MDDCs, blood mDC, and pDC subsets within unfractionated PBMCs, and enriched blood mDCs and pDCs. Although cultured MDDCs are often used in experimental models to evaluate functional properties of myeloid DCs, we felt that investigating mDCs and pDCs both in whole PBMC preparations as well as isolated directly from blood should more accurately represent the immature DCs in tissue and lymph nodes that might interact with yeast in the *in vivo* vaccine setting. In our analysis, yeast interaction with MDDCs was characterized by moderate increases in expression of MHC class-I and -II, CD54 (ICAM), CD40, and more marked increases in CD83, CD80, and CD86 expression. Yeast exposure also resulted in decreased phagocytosis (measured by FITC-Dextran uptake, data not shown) and induced down regulation of the mannose receptor, both findings consistent with maturation (31, 32). Recombinant yeast were also found to directly induce MDDCs to secrete significant amounts of the TH1-biasing cytokine IL-12, the pro-inflammatory cytokine TNF-α, and the regulatory cytokine IL-10. Furthermore, MDDCs matured with recombinant yeast induced strong allogeneic T cell proliferation, as measured by MLR, that was comparable or greater than that observed with MDDCs cultured with other known maturation factors, LPS or CD40L trimer. These findings suggest that recombinant yeast can stimulate human MDDCs to mature, possibly resulting in the induction of potent T cell responses that are integral to effective vaccine strategies.

To investigate the effect of recombinant yeast on DCs in a more physiologic system, we next evaluated the interaction between whole recombinant yeast and immature blood DC subsets. Both blood DC subsets have been shown to play a role in HIV-1 pathogenesis and may serve as potent targets for vaccine formulations (25). Yeast stimulation of PBMCs resulted in a marked increase in production of TNF-α, moderate IL-12 production, but only minimal IL-10 production. In bulk PBMCs, yeast induced phenotypic maturation of mDCs comparable to that observed following incubation with bacterial LPS. Surprisingly, pDCs also showed evidence of phenotypic maturation following stimulation of PBMCs with both yeast and LPS. This was interesting as LPS is felt to primarily activate cells via binding to toll-like receptor 4 (TLR4), a pattern recognition receptor (PRR) expressed on mDCs but not pDCs (33, 34). Since phenotypic maturation of both subsets was noted following LPS stimulation of PBMCs, this suggested an indirect or bystander maturation of pDCs. These data show that recombinant yeast can mature both blood mDCs and pDCs in unfractionated PBMC and can induce production of cytokines with a Th-1 biasing profile. However, it was not clear based on these experiments whether DC maturation was a direct effect of binding recombinant yeast or an indirect effect via yeast interactions with other mononuclear cells in the PBMC preparation (e.g., monocytes, B cells, etc.). Furthermore, it was not clear what contribution blood DCs were actually making to the overall production of cytokines in response to yeast.

To evaluate direct versus indirect maturation effects of yeast on blood DCs, mDCs and pDCs were enriched from PBMCs using Miltenyi blood DC isolation kits, and then incubated with recombinant yeast or media alone. As noted earlier, circulating pDCs and mDCs have been shown to express reciprocal TLRs, and the recognition or activation patterns of each BDC subset are consistent with their TLR expression, with pDCs being activated by TLR9-binding CpG motifs and mDCs responding to TLR2/4 ligands, such as bacterial LPS (33). As such, purified mDCs and pDCs were stimulated with LPS or CpG motifs, respectively, as a positive control for DC function in each experiment. We found that the recombinant yeast directly matured mDCs, as evidenced by increases in CD40, CD83, and CD86 expression, but did not induce maturation of enriched pDCs. Yeast also induced mDCs to produce large amounts of TNF-α, IL-10, and IL-6, whereas yeast did not trigger cytokine production by pDCs. In addition, purified mDCs cultured with recombinant yeast were able to induce strong allogeneic T cell proliferation, whereas yeast-exposed pDCs did not. Taken together, these data show that blood mDCs are directly activated to mature by exposure to yeast, whereas pDCs mature in PBMCs via a presumed bystander effect via either cell-to-cell interactions and/or the cytokine environment.

These activation profiles of blood mDCs and pDCs in response to *S. cerevisiae* yeast are most consistent with yeast binding to TLR2, a PRR expressed on mDCs but not pDCs (33). It has been known for many years that yeast cell wall products and extracts, such as zymosan, glucans, chitins, and mannans possessed potent immunomodulatory properties (35, 36). A more recent work revealing activation of TLR2 by yeast-derived cell wall products suggests that these adjuvant properties of yeast may be

mediated through TLRs (37). However, in addition to TLRs, the adjuvant effects, uptake, and processing of yeast may be mediated in part by the mannose receptor (MR) and other C-type lectin receptors on dendritic cells (38, 39). In macrophages, the MR mediates phagocytosis of particles such as yeast, which are highly glycosylated and contain mannosylated glycoproteins in their cell wall (40). Furthermore, the MR has been shown to mediate the induction of type I IFNs by blood DCs in response to enveloped viruses (41). It has been speculated that the MR on DCs may be targeted by mannosylated antigens (like *S. cerevisiae*) or toxins to enhance immunogenicity (31).

In an attempt to understand the mechanism by which the DCs were activated by recombinant yeast, as well as to explore the capability of each DC subset to process yeast, we evaluated the ability of blood DCs and other mononuclear cells to internalize whole yeast. PBMCs were incubated with mito-tracker green (MTG)-labeled fluorescent yeast for 22 h, then using flow cytometry, the percentage of mDCs (Lineage-/HLA-DR+/CD11c+), pDCs (Lin-/HLA-DR+/CD123 high), T cells ($CD3^+$), B cells ($CD20^+$), or monocytes ($CD14^+$) that had internalized yeast based on fluorescence was determined. Monocytes were the most efficient in uptake of the yeast, followed by mDCs, and to a lesser degree, B cells and pDCs. Together with the DC maturation data, these results suggested that mDCs readily internalized yeast, leading to their maturation. Alternatively, a smaller fraction (5–10%) of pDCs were able to internalize yeast, giving them the capability of processing and presenting yeast-derived antigens, yet this uptake did not lead to significant DC maturation.

One of the striking findings in our earlier studies of yeast vaccine vectors in the murine system was the ability of recombinant yeast, a protein-based vaccine, to induce priming and expansion of MHC class-I-restricted, $CD8^+$ T cells directed at yeast-derived vaccine antigens. This is unusual in that exogenously introduced antigens, for example, those found in vaccines consisting of antigenic proteins or killed pathogens, are predominantly processed via the MHC class-II pathway for presentation to $CD4^+$ T cells (42). Thus, vaccine formulations containing protein antigens typically stimulate potent humoral immunity but are relatively ineffective at stimulating $CD8^+$ CTL. However, DCs are unique in their ability to efficiently process exogenous Ags, especially in particulate form, for presentation via the MHC class-I pathway (43), in a process termed "cross-presentation." This shortcoming has led to an investigation of vaccine strategies that specifically target DCs to present antigens via MHC class-I in addition to class-II.

In order to determine whether human blood DCs could cross-present yeast-derived antigens, enriched blood DC subsets from HIV-1-infected subjects were incubated with HIV-1 Gag-expressing yeast, co-cultured with autologous $CD8^+$ T cells for a week, and then the frequency of Gag-specific IFN-γ-producing $CD8^+$ T cells was determined by ELISPOT assay. Both mDCs and pDCs exposed to Gag-expressing yeast were able to stimulate the expansion of HIV-specific $CD8^+$ T cells, suggesting that yeast-derived HIV-1 antigens were processed via the MHC class-I pathway and cross-presented by DCs. The finding that yeast-pulsed pDCs could also stimulate the expansion of HIV-specific $CD8^+$ T cells, despite little direct maturation by yeast and limited yeast uptake, was surprising. This may speak both to the efficiency of processing of exogenous antigen by DCs as well as the minimal co-stimulation requirements for activating and expanding memory T cells (44). Whether naïve $CD8^+$ T cells could also be induced to expand by relatively immature, yeast-pulsed pDCs is yet to be determined.

In summary, these results suggest that recombinant yeast has a potent direct adjuvant effect on human myeloid DCs as well as an indirect adjuvant effect on plasmacytoid DCs. Furthermore, both subsets of blood DCs could process and cross-present yeast-derived HIV-1 antigens to stimulate the expansion of HIV-1-specific, memory $CD8^+$ effector T cells. In animal models, similar *in vitro* properties of recombinant yeast have translated into potent efficacy when utilized as a vaccine *in vivo*. These results suggest that recombinant yeast has the potential to induce strong HIV-specific cell-mediated immune responses and should be further investigated as an HIV-1 vaccine vector in both prophylactic and therapeutic settings.

ACKNOWLEDGMENTS

We thank the physicians, staff, and patients in the Infectious Diseases Group Practice at the University of Colorado Health Sciences Center for their assistance and participation in this study; and GlobeImmune, Inc. for providing recombinant yeast used in this study. This work has been facilitated by the infrastructure and resources provided by the Colorado Center for AIDS Research Grant P30 AI054907. This work was supported in part by fellowship #70534-30-RFV from the American Foundation for AIDS Research (amfAR, M. A. B.) and in part by NIH R21 AI054192 (C. C. W.).

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