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Research Article

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CD40-activated Human B Cells: An Alternative Source of Highly Efficient Antigen Presenting Cells to Generate Autologous Antigen-specific T Cells for Adoptive Immunotherapy

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Abstract

Multiple clinical trials have shown the efficacy of adoptively transferred allogeneic antigen-specific T cells for the treatment of viral infections and relapsed hematologic malignancies. In contrast, the therapeutic potential of autologous antigen-specific T cells has yet to be established since it has been technically difficult to generate sufficient numbers of these T cells, ex vivo. A major obstacle to the success of this objective derives from our inability to simply and rapidly isolate and/or expand large numbers of highly efficient antigen presenting cells (APCs) for repetitive stimulations of antigen-specific T cells in vitro. We show that autologous CD40-activated B cells represent a readily available source of highly efficient APC that appear to have several important advantages over other APCs for ex vivo T cell expansion including: (*a*) methodological simplicity necessary to generate continuously large numbers of APCs from just 50 cm^3 of peripheral blood without loss of APC function; (*b*) capacity to induce high peak T cell proliferation and interferon-γ production without IL-10 production; (*c*) ease in cryopreservation; and (*d*) markedly reduced cost. We, therefore, contend that CD40-activated B cells are an alternative source of highly efficient APCs with which to generate antigen-specific T cells ex vivo for autologous adoptive immunotherapy. (*J. Clin. Invest.* 1997. 100:2757–2765.) Key words: B lymphocytes • dendritic cells • antigens, CD40 • immunotherapy, adoptive • T cell proliferation

Introduction

Numerous unique peptide antigens have been identified that induce T cell specific immunity to pathogens (1–3) and tumor cells (4). Induction of productive T cell immunity requires efficient presentation of peptide antigens by professional antigen-presenting cells (APCs).[1] Although dendritic cells (DCs) (5–12), activated macrophages (13), or activated B cells (14–22) are all capable of presenting peptides, DCs are considered to be the most efficient APC since fewer DCs are required to induce an optimal T cell immune response (8). In addition to their capacity to present antigen, DCs are also highly efficient at antigen capture, processing, and migration (reviewed in reference 23). Therefore, DCs have been selected as the "APC of choice" to generate antigen-specific T cells for immunotherapy (5, 6, 24, 25).

Since DCs constitute only 0.1–0.5% of human peripheral blood (PB) mononuclear cells, considerable difficulty and expense has been experienced in obtaining sufficient numbers of highly enriched mature DCs (26). Cytokines like GM-CSF and IL-4 have permitted selection and ex vivo expansion of functional DCs (7, 27–29). However, by 2–3 wk, DCs cease to proliferate and become less efficient at presenting antigen. Therefore, to obtain fresh DCs with which to repetitively stimulate autologous T cells, multiple phlebotomies would be required. Alternatively DCs could be generated from $CD34^+$ enriched progenitors (30, 31). Indeed, significantly larger numbers of DCs have been generated from this source (32, 33). However, isolation of DC precursors will likely require either chemotherapy or cytokine pretreatment, leukophereses, and $CD34^+$ stem cell collection (33). Moreover, since mature DCs have yet to be successfully cryopreserved, repetitive generation of DCs either from cryopreserved precursors or from fresh DC sources will be required. Although potentially sufficient numbers of DCs might be generated using any of the above technologies (26), the complexity and cost of preparation of DC precursors and generation of functional DCs limits their utility as APCs for the ex vivo generation of antigen specific T cells. To overcome these obstacles, we sought an alternative, cost-effective source of autologous APCs that could be simply generated from small quantities of human PB, which would result in large numbers of APCs to present peptide antigen efficiently to T cells. Here, we show that CD40-activated peripheral blood B cells (CD40-Bs) fulfill these criteria. Moreover, through comparison with DCs, we have identified several unique characteristics of CD40-Bs that suggest that they might be the optimal APC with which to generate antigen-specific T cells ex vivo for adoptive immunotherapy.

Methods

Donors and cell lines. All specimens were obtained following approval by the institutional Scientific Review Committee. Informed consent for blood donations was obtained from all volunteers. Short-term cultured melanoma cell lines were generated from biopsies of two HLA-A*0201$^+$ and two HLA-A*0201$^-$ patients with melanoma. Human PBMC from healthy donors were obtained by phlebotomy or leukopheresis followed by Ficoll-density centrifugation.

The CD40 ligand system for the culture of normal human PB B

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; CD40-B, CD40-activated B cell; CD40L, CD40 ligand; DC, dendritic cell; MLR, mixed lymphocyte reaction.

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cells. B cells from PBMC were stimulated via CD40 using NIH3T3 cells transfected by electroporation with the human CD40 ligand (t-CD40L cells) (19). The expression of the human CD40 ligand on the transfectants has been stable over a period of > 3 yr. Phenotypic analysis have been performed regularly on these cells and in all analyses > 95% of t-CD40L cells were positive for human CD40 ligand with a mean intensity of fluorescence (MIF) between 80- and 300-fold over background (MIF = 10). t-CD40L cells were negative for human MHC class I and II antigens and therefore no targets for human $CD4^+$ or $CD8^+$ T cells. t-CD40L cells were also tested for all murine viruses known and found to be negative. For B cell cultures, t-CD40L cells were lethally irradiated (96Gy) and subsequently plated on 6-well plates (Costar, Cambridge, MA) at a concentration of 0.4×10^5 cells/well in medium containing 40% DME (Gibco/BRL, Gaithersburg, MD), 40% F12 (Gibco/BRL) 10% FCS, 2 mM glutamine (Gibco/BRL), and 15 μg/ml gentamicin (Gibco/BRL). After an overnight culture at 37°C in 5% CO_2 t-CD40L cells were adherent and could be used for coculture. Before adding PBMC, t-CD40L cells were washed twice by rinsing the plates with PBS. CD40-Bs were generated from PBMC by simply coculturing whole PBMC at 2×10^6 cells/ml with t-CD40L in the presence of IL-4 (2 ng/ml; Immunex, Seattle, WA) (19, 34–37) and cyclosporin A (CsA) at 5.5×10^{-7} M in Iscove's MDM (Gibco/BRL) supplemented with 10% human AB serum, 50 μg/ml transferrin (Boehringer Mannheim, Indianapolis, IN), 5 μg/ml insulin (Sigma Chemical Co., St. Louis, MO), and 15 μg/ml gentamicin (Gibco/BRL) at 37°C in 5% CO_2. The concentration of CsA used in the culture system was found to suppress T cell proliferation without affecting B cell growth. Cultured cells were transferred to new plates with fresh irradiated t-CD40L cells every 3–5 d. Once the cultured PBMC were > 75% $CD19^+$ they were cultured at concentrations of $0.75–1.0 \times 10^6$ cells/ml. If cells were used for analysis or cryopreserved, only a small proportion of cells was recultured and the potential total increase was then calculated. Total number of viable cells was assessed by trypan blue exclusion and the number of $CD19^+$ B cells by immunophenotypic analysis on days 0, 5, 8, and weekly thereafter (37). Before use in functional assays, CD40-Bs were always Ficoll-density centrifuged followed by washing with PBS twice to remove nonviable cells including remaining t-CD40L cells.

Dendritic cell preparation and culture. PBMC were depleted of T, B, and natural killer (NK) cells by magnetic bead depletion (19, 37). DCs were generated from the remaining cell fraction (1.3×10^6 cells/ml, > 80% $CD14^+$) with GM-CSF (50 ng/ml, Genzyme, Cambridge, MA) and IL-4 (10 ng/ml, Immunex, Seattle, WA) in Iscove's MDM (Gibco/BRL) supplemented with 5% human AB serum, 50 μg/ml transferrin (Boehringer Mannheim), 5 μg/ml insulin (Sigma Chemical Co.), 2 mM glutamine (Gibco/BRL), and 15 μg/ml gentamicin (Gibco/BRL) at 37°C in 5% CO_2. Cytokines were added at the beginning of culture and every third day thereafter. To determine maximum expansion DCs were cultured up to 30 d with GM-CSF and IL-4. For functional analysis DCs were maturated on day 6 for 48 h with either TNF-α (30 ng/ml; Genyzme) or t-CD40L before use as APCs in allogenic mixed lymphocyte reaction (allo-MLRs).

T cells. Whole $CD3^+$ T cells and T cell subsets ($CD4^+$, $CD4^+$ $CD45RA^+$ $CD45RO^-$, $CD4^+$ $CD45RO^+$ $CD45RA^-$, $CD8^+$) were obtained from PBMC by magnetic bead depletion of non–T cells (19, 37). Preparations were always > 97% as assessed by immunophenotypic analysis.

Immunofluorescence studies. Dual-color FACS® analysis using directly conjugated mAbs (19, 37) was performed to determine the surface expression of CD1a (T6), CD3 (T3), CD4 (T4), CD8 (T8), CD14 (My4), CD19 (B4), CD20 (B1), CD23 (B6), CD33 (My9), CD45RA (2H4), CD45RO (UCHL1), CD56 (NKH1), CD83 (HB15), MHC class I (B9.12.1) and II (I3) (Coulter Inc., Miami, FL), CD54 (Leu-54; Becton Dickinson, San Jose, CA), CD58 (Amac, MA), CD80 (C4; Repligen Inc., Cambridge, MA), CD86 (IT2.2; PharMingen, San Diego, CA). To determine the source of IFN-γ and IL-10 in cocultures these cytokines were detected by intracellular staining using anti–IFN-γ and anti–IL-10 mAbs (PharMingen) and the Caltag Fix and Perm Kit (Caltag, Burlingame, CA) for intracellular staining.

Peptides. The tyrosinase peptide YMNGTMSQV (369–377) and the influenza A matrix peptide GILGFVFTL (58–66) were synthesized (38) by the Dana-Farber molecular core facility.

Allo-MLR. Allogeneic whole $CD3^+$ T cells or T cell subsets from healthy individuals were plated at 1×10^5 T cells/well with 10^1 to 10^5 irradiated (32 Gy) DCs or CD40-Bs/well in RPMI-1640 supplemented with 5% human AB serum (Sigma Chemical Co.), 2 mM glutamine (Gibco/BRL), 15 μg/ml gentamicin (Gibco/BRL) (RPMI-5) (19). Determination of [^{2}H]thymidine incorporation was performed in triplicates on days 2 to 8.

ELISA for interferon-γ and IL-10. Interferon-γ or IL-10 were detected by ELISA (Endogen, Woburn, MA) in supernatants of cultures of allogeneic T cells either stimulated with CD40-Bs or DCs. Supernatants were collected after 3–8 d to determine peak cytokine accumulation.

Reverse transcriptase (RT-PCR) for tyrosinase. Tyrosinase in melanoma cells was detected by RT-PCR using previously published primers (39).

Immunocytology. Immunocytology for latent membrane protein (LMP-1) and Epstein-Barr virus nuclear antigen-2 (EBNA-2) on cytospin preparations of CD40-Bs was performed as previously described (40).

In vitro CTL response induction. CD40-Bs of HLA-A*0201^+ donors were loaded with peptide (50 μg/ml) in the presence of human β2-microglobulin (3 μg/ml; Sigma Chemical Co.), irradiated (32 Gy) and added to purified $CD8^+$ T cells (> 98%) of the same donor at a ratio of T:CD40-Bs = 4:1 in RPMI-5 containing IL-7 (10 ng/ml; Genzyme) (38). At day 7, T cell cultures were harvested, Ficoll density centrifuged to remove nonviable cells, washed twice, and restimulated with fresh peptide-pulsed CD40-Bs and IL-7. This was repeated on days 14, 21, and 28. For restimulation of T cells, CD40-Bs were pulsed for 2 h at 37°C with peptide (10 μg/ml) and β2-microglobulin (3 μg/ml). IL-2 was first introduced into the cultures at days 18–20 (10 IU/ml). To further expand T cells, IL-2 was added at 100 IU/ml from day 24 on every third day until cytotoxicity was assessed at day 35.

Cellular cytotoxicity assay. Expanded autologous $CD8^+$ T cell lines were analyzed in a standard 4-h ^{51}Cr-release assay or by JAM-test (37, 41) for their ability to kill various target cells. As target cells, melanoma cells or CD40-Bs, alone or peptide-pulsed were harvested from culture using standard procedures for adherent respectively nonadherent cells, washed twice by centrifugation in PBS and resuspended in RPMI-5. Targets were labeled with either ^{51}Cr or ^{3}H-Tdr as previously described and 2×10^4 labeled cells were plated with various concentrations of effector cells. Percent cytotoxicity is calculated as the [(cpm-spontaneous release)/(total cpm-spontaneous release)] ×100% for chromium release assays and [(spontaneous release-cpm)/spontaneous release] × 100% for the JAM test (37, 41).

Results

Large numbers of CD40-Bs can be generated that induce highly significant peak T cell proliferation and IFN-γ production without IL-10 secretion. DCs and CD40-Bs generated from PB were compared with regard to cell surface phenotype, expansion, and capacity to present alloantigen. After 6 d of culture with GM-CSF and IL-4, DCs were isolated and then either continued in culture with GM-CSF and IL-4 for 9 additional days or cultured with either TNF-α or CD40L from days 6 to 8 to induce maturation (7, 27) (Fig. 1 *A*). The majority of cultured cells (> 83%) developed a DC-like morphology (7) and phenotype ($CD83^+$ $CD14^-$) after 6 d of culture (42). Cells cultured in GM-CSF and IL-4 became very large and adherent by day 12 with decreased expression of CD83, thereafter. Differentiation with either TNF-α or CD40L between days 6 and 8

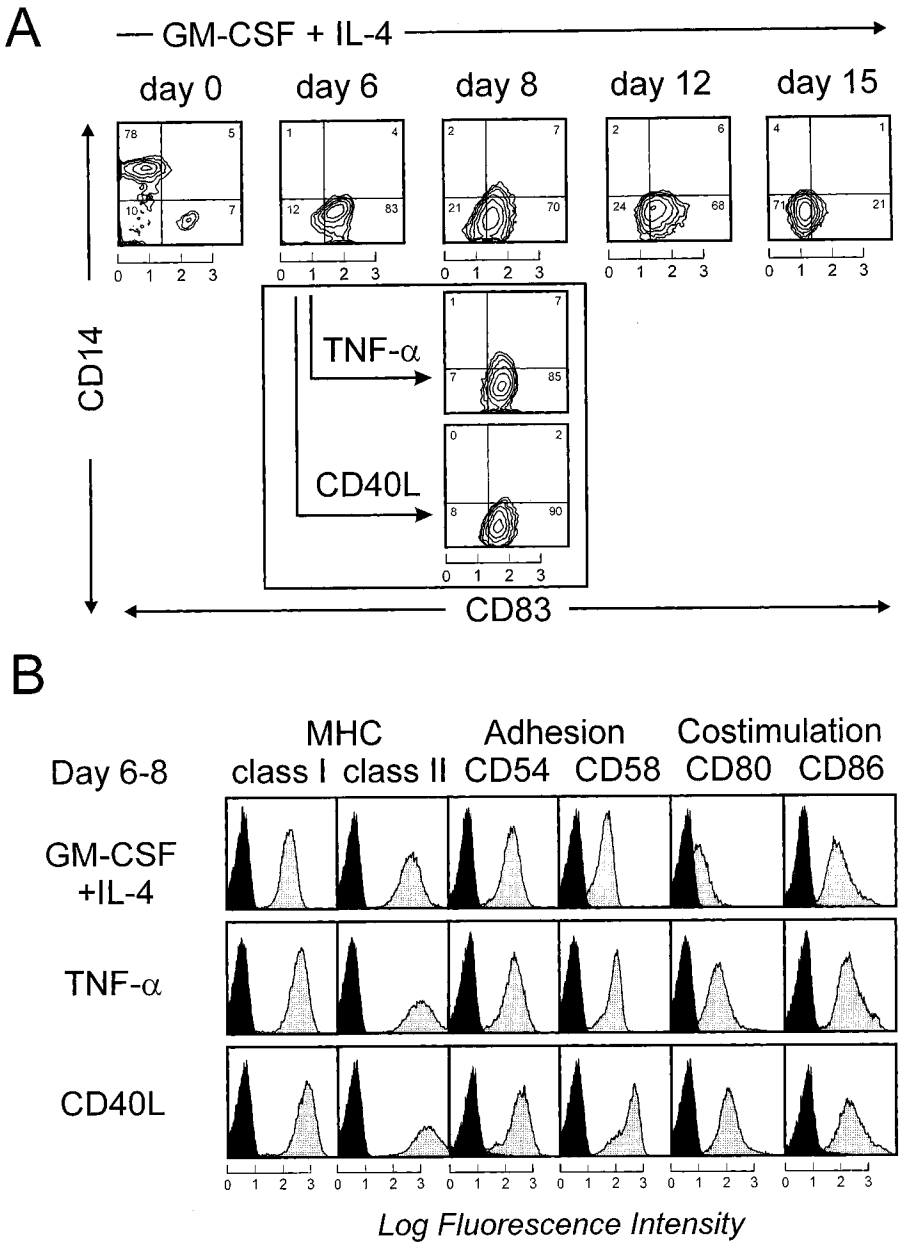


Figure 1. CD83$^+$ CD14$^-$ DCs generated from a monocyte-enriched PBMC fraction express high levels of adhesion, MHC, and costimulatory molecules. (*A*) Expression of CD14 and CD83 was assessed by two color immunofluorescence analysis before and during culture. (*B*) Expression of MHC class I and II, CD54 and CD58, CD80 and CD86 on DCs cultured for 8 d; DCs cultured with GM-CSF and IL-4 (*top*), DCs cultured with GM-CSF and IL-4 for 6 d followed by TNF-α (*middle*); or CD40L (*bottom*) for 2 d. Black shaded area indicates fluorescence of isotype matched conjugated antibodies.

did not significantly increase the expression of CD83. In contrast, the expression of MHC, adhesion, and costimulatory molecules (Fig. 1 *B*) further increased compared to already high expression on DCs cultured with GM-CSF and IL-4. However, DCs cultured for > 12 d expressed significantly lower levels of both adhesion and costimulatory molecules (data not shown).

CD40-Bs, generated by coculture of PBMC with CD40L in the presence of IL-4 and low concentrations of cyclosporin A, were > 80% CD19$^+$CD3$^-$ by day 8 and were uniformly CD19$^+$CD3$^-$ by day 12 (Fig. 2 *A*). These cells were highly activated as shown by their expression of CD23. Comparable levels of MHC, adhesion, and costimulatory molecules as observed for DCs between days 6 and 12 of culture was demonstrated for CD40-Bs at day 8 (Fig. 2 *B*) and expression of these molecules remained stable thereafter (data not shown).

DCs and CD40-Bs generated from 50 cm^3 of PB were compared for expansion in short-term cultures (Fig. 3). After 8–12 d in culture, $\sim 1 \times 10^7$ DCs could be generated and no further expansion ensued. Approximately 1×10^8 CD40-Bs could be generated by day 8 and 1×10^9 by day 14. Comparable differences in the levels of expansion were observed for DCs and CD40-Bs generated from leukophereses (Fig. 3, *bottom*).

Primary allogeneic MLRs with highly purified T cell subsets were used to compare the APC capacity of DCs and CD40-Bs. MLRs were performed for 2–8 d and peak proliferation induced by DCs was at day 7 for CD4$^+$ T cells and at day 5 for CD8$^+$ T cells (shown in Fig. 4). At early time points (2–4-d cultures, data not shown) CD40-Bs were as efficient as DCs at low APC:T ratios. However at peak proliferation (days 5–7) the most efficient alloantigen presenting cells at low APC:T cell ratios were: (*a*) DCs cultured with GM-CSF + IL-4 fol-

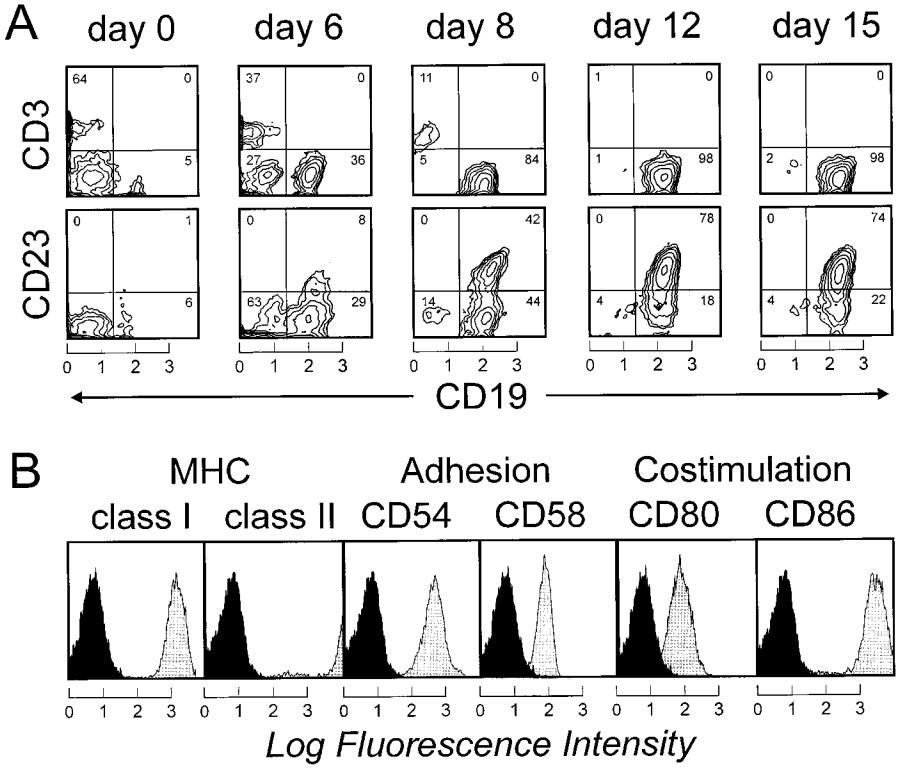
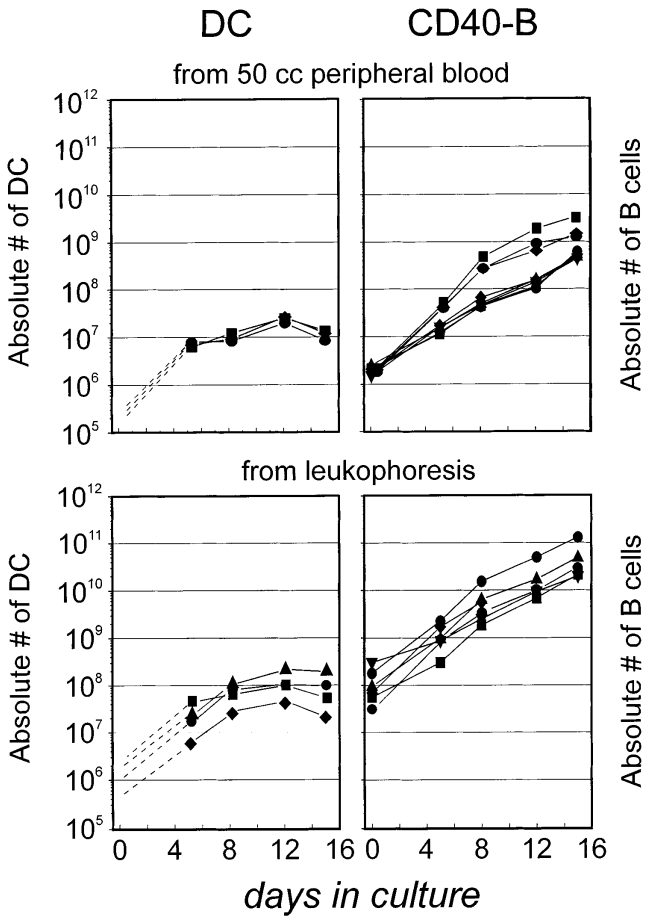


Figure 2. Stimulation of PBMC with t-CD40L and IL-4 in the presence of cyclosporin A results in outgrowth of highly pure activated B cells expressing high levels of adhesion, MHC, and costimulatory molecules. (*A*) Two color immunophenotypic analysis including CD19, CD23, and CD3 on PBMC before and during culture until day 15. (*B*) Expression of MHC class I and II, CD54, CD58, CD80, and CD86 on $CD19^+$ cells cultured for 8 d. Black shaded area indicates fluorescence of isotype matched conjugated antibodies.



lowed by CD40L activation; (*b*) DCs cultured with GM-CSF + IL-4 followed by TNF-α activation; (*c*) DCs cultured with GM-CSF + IL-4 alone; and (*d*) CD40-Bs. In all cultures (day 2–8) when $> 10^4$ DCs/well were used, T cell proliferation dramatically decreased whereas when $> 10^4$ CD40-Bs/well were used, T cell proliferation was enhanced (Fig. 4 *A*). Moreover, peak proliferation was consistently greater for CD40-Bs compared with DCs, ranging from 20 (Fig. 4) to 150% higher peak proliferation in a total of 10 experiments. Likewise, production of IFN-γ closely correlated with T cell proliferation and peak IFN-γ production was induced by CD40-Bs (Fig. 4 *B*). Of note, CD4-positive T cell subsets, both $CD45RA^+$ $CD45RO^-$ and $CD45RO^+$ $CD45RA^-$, revealed identical patterns of T cell proliferation and IFN-γ production (data not shown). Unlike DCs, CD40-Bs did not induce IL-10 production by $CD4^+$ and $CD8^+$ T cells at any stimulator/responder ratio tested. Cytokine production by T cells in cocultures with DCs or CD40-Bs was confirmed by intracellular staining for IFN-γ and IL-10 (data not shown). Taken together, although DCs are superior APC on a cell-to-cell basis, larger numbers of CD40-Bs can be

Figure 3. Expansion of DCs and CD40-Bs from PB. DCs were generated from a monocyte-enriched PBMC fraction cultured with GM-CSF and IL-4. DCs in the culture were determined by morphology and phenotype. CD40-Bs were generated from total PBMC by culture in the CD40L system. Expansion of $CD19^+$ B cells was calculated from total cell number of cells and the percentage of $CD19^+$ B cells. Expansion of DCs from either leukopheresis or PB was analyzed in four donors. CD40-B cell expansion from eight donors' PB and from six donors leukopheresis was assessed.

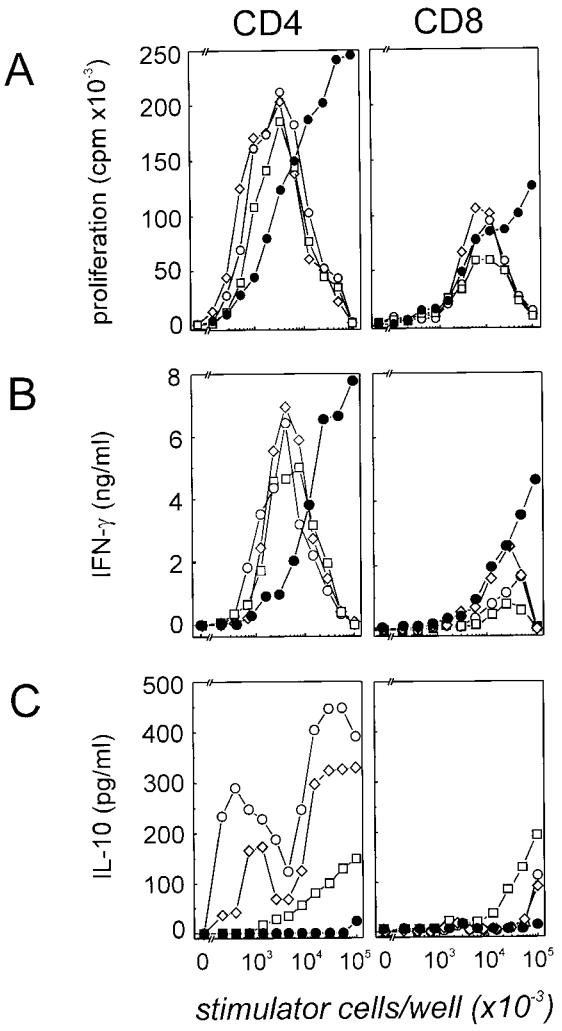


Figure 4. Response of allogeneic $CD3^+$ $CD4^+$ T cells or $CD3^+$ $CD8^+$ T cells (10^5/well) to purified CD40-Bs or DCs. Purified T cells were cocultured with CD40-Bs (> 98% purity, *solid circles*) or PB-derived DCs cultured with GM-CSF and IL-4 (*open squares*), DCs cultured with GM-CSF and IL-4 followed by TNF-α (*open circles*), or DCs generated with GM-CSF and IL-4 followed by CD40L (*open diamonds*). [^{3}H]Thymidine incorporation was assessed for the last 16 h of 2–8 d primary MLRs. Shown here are the days of peak T cell proliferation induced by DCs (day 7 for $CD4^+$ T cells, day 5 for $CD8^+$ T cells). After 2–4 d of culture low numbers (100–10,000 cells/well) of CD40-Bs and DCs induced equivalent proliferation and IFN-γ production by $CD4^+$ T cells (data not shown). Appropriate controls (T cells, stimulator cells) were always < 2,000 cpm. Production of (*B*) IFN-γ and (*C*) IL-10 in cocultures of allogeneic T cells and either CD40-Bs or DCs was measured by ELISA in supernatants collected shortly before addition of thymidine. One representative experiment of a total of 10 experiments is shown. Phenotypic analysis of CD4 CD8 at the end of each culture demonstrated stable expression of T cell subset markers (data not shown).

generated which induce higher peak T cell proliferation and IFN-γ production without IL-10 secretion.

CD40-Bs, but not DCs, can be continuously expanded in long term culture without loss of APC function. DCs generated with GM-CSF plus IL-4 coculture could be expanded for ~ 12–15 d and cell numbers decreased dramatically thereafter with only few viable cells remaining at 4 wk (Fig. 5 *A*). Confirming the work of others (7), we observed that addition of TNF-α or CD40L to these cultures did not result in further expansion or prolonged viability of DCs (data not shown). In contrast, CD40-Bs could be continuously expanded throughout the 65 d evaluated. EBV-related proteins LMP-1 and EBNA-2 could not be detected by highly sensitive immunocytology in CD40-Bs up to day 51 of culture. When cultures were analyzed on day 65, between 5–30% of cells showed LMP-1 staining and 30–80% EBNA-2 (data not shown). However, it is critical to note that continuous CD40L and IL-4 stimulation was required and that factor-independent B cell lines were never detected. Phenotypic analysis performed weekly revealed stable expression of B cell lineage markers as well as MHC, adhesion, and costimulatory molecules (data not shown). From five unselected donors, we were able to generate between 8.5×10^{10} and 4.0×10^{11} CD40-Bs from 50 cm^3 of PB after 65 d of culture (four- to five-log fold increase). To assess APC capacity of these long-term cultured CD40-Bs, cells were harvested at days 4, 8, 15, 33, 51, and 65 of culture, cryopreserved, and used as stimulators for allogeneic $CD4^+$ T cells from three individuals. As shown in Fig. 5 *B*, unstimulated B cells were very poor APCs whereas long-term cultured CD40-Bs were highly efficient alloantigen presenting cells inducing significant T cell proliferation and IFN-γ production. In contrast to cryopreserved DCs (< 15% cell recovery, data not shown), > 75% of CD40-Bs could be consistently recovered post cryopreservation. Similar data have been obtained with CD40-Bs cultured in serum free media supplemented with autologous serum (data not shown).

Capacity of CD40-Bs to generate peptide-specific cytolytic T cells in vitro. The generation of antigen-specific cytolytic $CD8^+$ T cells for adoptive immunotherapy requires multiple stimulation with APCs in vitro. Our schema is depicted in Fig. 6. CD40-Bs were generated from PB and the remaining PBMC were cryopreserved. Once CD40-Bs had been generated, $CD8^+$ T cells were isolated and subsequently stimulated in the presence of IL-7 with peptide-pulsed CD40-Bs. From this single blood draw, continuously cultured CD40-Bs are available and can be harvested for weekly restimulations of the autologous T cells. CD40-Bs were pulsed with the immunogenic HLA-A*0201 binding 369–377 peptide YMNGTMSQV of tyrosinase, a melanoma associated tumor antigen (38). As shown in Fig. 7, *top*, T cell lines could be generated from five normal HLA-A*0201^+ donors that lysed either autologous or haplo-mismatched HLA-A*0201^+ CD40-Bs pulsed with the tyrosinase peptide from four of these donors. Most importantly, the T cell lines generated against the tyrosinase peptide-pulsed CD40-Bs demonstrated significant cytotoxicity against two HLA-A*0201^+ tyrosinase$^+$ melanoma cell lines. In contrast, no cytotoxicity was generated against control targets including unpulsed autologous or haplo-mismatched HLA-A*0201^+ (Fig. 7, *bottom*) or allogeneic tyrosinase$^+$ melanoma cells and allogeneic CD40-Bs (data not shown). To demonstrate specificity for peptide, HLA-A*0201^+ CD40-Bs were pulsed with an irrelevant HLA-A*0201^+ binding influenza A peptide. As shown, these cells were not lysed by any of the T cell lines (Fig. 7, *bottom*). These data indicate that priming with peptide-pulsed CD40-Bs induced peptide-specific cytotoxicity and that cytotoxicity was not directed against B cells, alloantigen or other processed peptides. Cold target experiments using unlabeled peptide-pulsed HLA-A*0201^+ CD40-Bs confirmed specificity since peptide specific cytotoxicity was abrogated (data

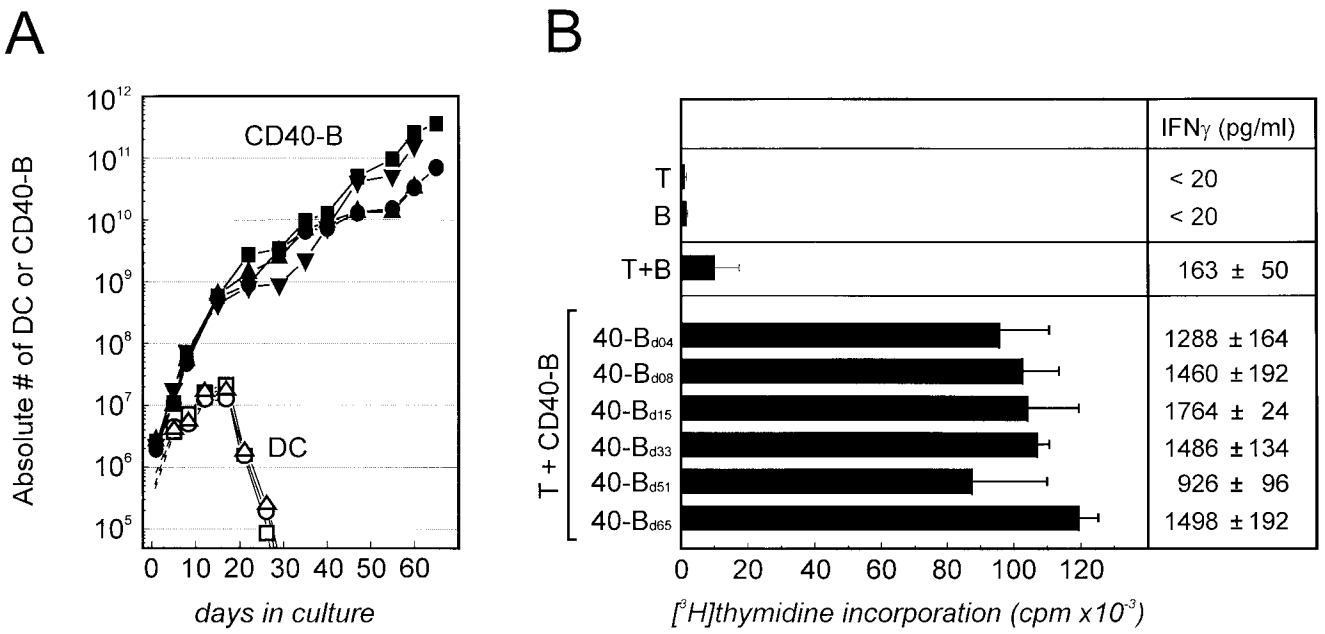


Figure 5. CD40-Bs but not DCs can be expanded in long-term culture without loss of APC function. (*A*) DCs and CD40-Bs were generated as described in Methods. Cells cultured with GM-CSF and IL-4 lost DC characteristics between day 16 and 25 of culture and the few remaining cells became very large and adherent so that cultures were stopped at day 28. Expansion of CD40-Bs was calculated as described for Fig. 2. (*B*) Induction of allogeneic T cell proliferation by unstimulated B cells or CD40-Bs precultured for 4, 8, 15, 33, 51, or 61 d. Purified $CD3^+$ $CD4^+$ T cells were cocultured with B cells or CD40-Bs in a final volume of 0.2 ml. MLRs were cultured for 5 (data not shown) or 7 d and T cell proliferation and IFN-γ production measured as described under Fig. 3. The ability of purified T cells (from other normal donors with unrelated MHC) to proliferate in response to CD40-Bs was tested in a total of three experiments.

not shown). Total number of $CD8^+$ T cells on day 35 ranged between 9×10^7 and 3×10^8.

CD40-Bs represent a cost-effective, alternative source of highly efficient antigen presenting cells. We compared the cost of preparing either DCs or CD40-Bs for repetitive ex vivo activation of antigen-specific T cells for adoptive immunotherapy (Table I). Generation of between 1×10^8 to 1×10^9 DCs ranges from two to seven times the cost of generating 1×10^{11} CD40-Bs. Moreover, generation of 1×10^8 DCs requires unacceptable phlebotomy (100 cm^3 per week for 10 wk) whereas 10^{11} CD40-Bs can be generated from a single 50-cm^3 blood draw.

Discussion

Multiple clinical trials have demonstrated the efficacy of adoptively transferred matched allogeneic antigen-specific T cells for both treatment and prophylaxis (reviewed in references 1, 2). Prophylactic transfer of cytomegalovirus specific allogeneic T cells has been successful in preventing reactivation of cytomegalovirus in immunocompromised patients after allogeneic transplant (43, 44). Treatment of EBV-induced lymphoproliferation in immunosuppressed patients with allogeneic EBV-specific T cells has resulted in clinical complete remissions (45–49) and, importantly, prophylactic trials are already

Table I. Cost Analysis of DC from Different Sources and CD40-B to Obtain APC for Ex Vivo Expansion of Autologous T Cells for Adoptive Immunotherapy

	Source*	Cell numbers‡	Preparation§	Culture‖	Estimated cost¶
					$
DC from PB	10× 100 cm^3 PB	2×10^8	10× monocyte enrichment	GM-CSF + IL-4±CD40L±TNF-α	6000
DC from LP	5× leukophereses	5×10^8	5× monocyte enrichment	GM-CSF + IL-4±CD40L±TNF-α	12000
DC from $CD34^+$	10× G-CSF 2-4 leukophereses	1×10^9	2× $CD34^+$ column cryopreservation	SCF + flt-2/flk-3L + GM-CSF + TNF-α	20000
CD40-B	1× 50 cm^3 PB	1×10^{11}		IL-4 + CD40L	3000

*Estimated amount of PB or leukopheresis (*LP*) necessary to obtain sufficient numbers of APCs to stimulate T cells ex vivo. ‡Total cell number generated from each source. §Preparation of all cells include Ficoll density centrifugation before further enrichment procedures according to previously published procedures for DCs (7, 27–33) and CD40-Bs. ‖Culture conditions according to previously published procedures to obtain highly enriched DCs (7, 27–33) or CD40-Bs (19, 37). ¶Cost analysis includes clinical visits and procedures, material, medium, cytokines, and labor.

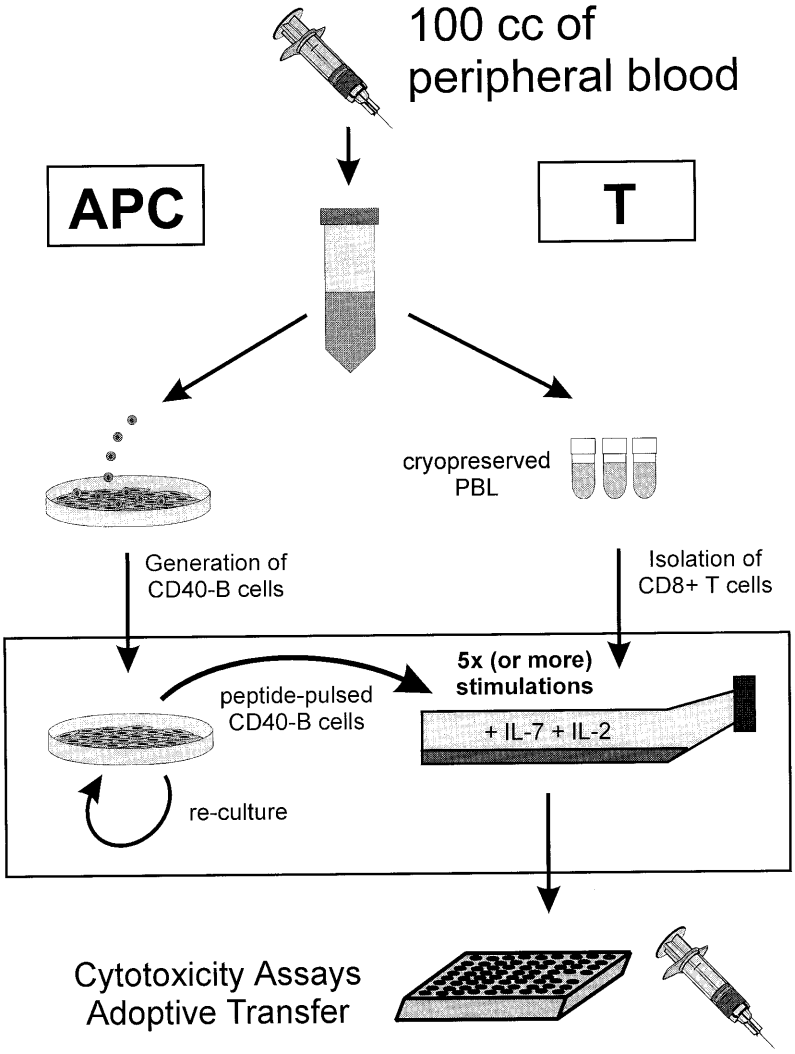


Figure 6. Induction of peptide-specific T cells by repetitive stimulation with peptide-pulsed CD40-Bs in vitro. From one single blood draw (100 cm^3) CD40-Bs as well as T cells are obtained. Half of the PBMC are used to generate CD40-Bs, the remaining cells cryopreserved for isolation of T cells. Once CD40-Bs are generated after 8–10 d, $CD8^+$ T cells can be isolated from the cryopreserved PBMC and stimulated repeatedly with peptide-pulsed CD40-Bs in the presence of IL-7. IL-2 is first added at day 18 of coculture and from day 24 on every third day thereafter.

encouraging. Finally, adoptive transfer of HLA-matched PBMC results in many complete remissions in patients with overtly relapsed chronic myelogeneous leukemia (50–53), myeloma (54, 55), and chronic lymphocytic leukemia. Therefore, the capacity to generate sufficient numbers of autologous antigen-specific T cells for adoptive immunotherapy may also provide an important therapeutic approach to treat patients with viral and fungal infections as well as with cancer.

The objective of the present study was to identify a simple, highly efficient, cost-effective source of autologous APCs with which to generate autologous antigen-specific T cells ex vivo for adoptive immunotherapy. From a single 50-cm^3 phlebotomy, very large numbers of autologous CD40-Bs can be generated. CD40-Bs are readily available for repetitive autologous T cell stimulations since they do not lose APC capacity either during long-term culture or cryopreservation and can be continuously expanded. This technology allowed us to multiply restimulate antigen-specific T cells to tyrosinase using this continuously available source of functional APC. In contrast, although DCs are highly efficient at APC function, our inability to continuously expand and/or cryopreserve mature DCs severely limited their functional utility in this setting. CD40-Bs appear to have additional unique characteristics including the induction of extremely high peak T cell proliferation, the induction of INF-γ production without IL-10 production, their simplicity of preparation, and cost. CD40-Bs might replace Epstein Barr virus–transformed lymphoblastoid cells lines (EBV-LCL) as APCs for repetitive T cell stimulation (1, 2). To our best calculation, from 50 cm^3 of PB larger numbers (10^{10}–10^{11} in 50 d) of CD40-Bs can be generated more consistently and rapidly than EBV-LCL. Moreover, if CD40-Bs cultured for < 50 d are used as APCs, they do not induce EBV-directed T cell responses during ex vivo culture. It remains to be determined, if the detection of EBV-related proteins during late cultures (day 65) predict for the outgrowth of EBV cell lines thereafter and if this might limit the culture system. Current experiments are aimed to answer these important questions.

Recent reports have demonstrated that the transfection of genes into DCs is superior to peptide pulsing in generating peptide-specific T cells (56, 57). Preliminary results in our laboratory suggest that CD40-Bs can also be efficiently transfected without changing their functional, phenotypic and/or growth characteristics. We have been able to transduce CD40-Bs with a marker gene or the human GM-CSF gene. Current studies are aimed to determine whether transfection of genes encoding for tumor antigens will lead to efficient peptide pre-

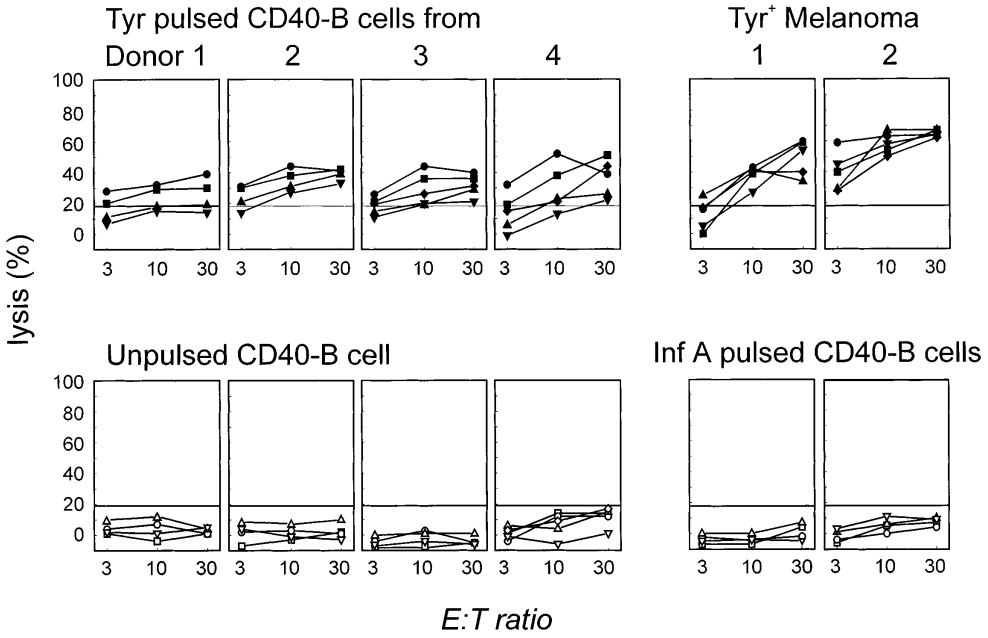


Figure 7. Induction of cytotoxic T cells after stimulation with peptide-pulsed CD40-Bs. HLA-A*0201$^+$ CD8$^+$ T cells from five normal donors were multiply stimulated with autologous CD40-Bs and subsequently analyzed for their cytotoxicity. Targets were autologous or HLA-A*0201$^+$ haplo-mismatched CD40-Bs from four donors pulsed with the specific tyrosinase peptide (*top left panel*), HLA-A*0201$^+$ melanoma cell lines positive for the tyrosinase gene (*top right panel*), unpulsed autologous or HLA-A*0201$^+$ haplo-mismatched CD40-Bs from four donors (*bottom left panel*), or autologous of HLA-A*0201$^+$ haplo-mismatched CD40-Bs from two donors pulsed with an irrelevant peptide (*Influenza A peptide*) binding to HLA-A*0201 (*bottom right panel*). Cytotoxicity was measured by JAM-test (shown here) or ^{51}Cr release with similar results.

sentation by CD40-Bs. Taken together, we conclude that CD40-Bs are an alternative highly efficient source of APCs with which to generate autologous T cells for adoptive immunotherapy.

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