られたものの抗原のみ接種した群でも非特異的なIFNγ産生細胞の増加が見られたため、これを特異的な免疫反応と結論付けられなかった。そこで初回ワクチン接種からboostまでの期間を7日間から5日間に短縮して再度解析した結果DCワクチン群で抗原特異的にIFNγ産生細胞の増加が見られた個体があった。しかし同じ群でも結果にばらつきがあり、1群あたりの匹数が少なかった為と考えられた。また初回ワクチン接種とboostの期間を7日間から5日間に短縮したが、boost後と殺するまでの期間も8日より短縮した方がより高い免疫反応を捉えられる可能性があると考えられた。また接種するDCワクチンの量を増加させることでも効果の上昇が期待できると考えられた。今後はこの抗原特異的免疫反応が見られた個体の液性免疫や血漿中のHIV特異的抗体の検出を行う。またhu-PBL SCIDマウスの実験系をさらに短縮した再解析を計画している。

E.結論

hu-PBL SCIDマウスを用いた抗DCワクチンの評価では、一部のマウスで抗原特異的な免疫誘導を確認できた。しかしDCワクチンを接種したマウスの中には抗原特異的な免疫反応が見られない個体もあり、初回DCワクチン接種とboostの期間やboost後の期間を5日間からさらに短縮して解析するのが有効と考えられた。

F.健康危険情報

HIV-1の感染実験、ウイルスの保管は全て国立感染症研究所で定める感染微生物取り扱い安全管理委員会の規定に基づき、P3実験施設で行われている。

G.研究発表

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H.知的財産権の出願・登録状況

1)特許取得
該当なし
2)実用新案登録
該当なし
3)その他　　該当なし

抗原特異的免疫誘導の評価～IFNγELISPOT assay～ NOG9

/head　候補ワクチン群　　対照群

Group	1	2	3	4
Prime	AT-2 HIV / DC ($1x10^5$)	AT-2 HIV	DC ($1x10^5$)	———
Boost	AT-2 HIV / DC ($1x10^5$)	AT-2 HIV	DC $1x10^5$)	———

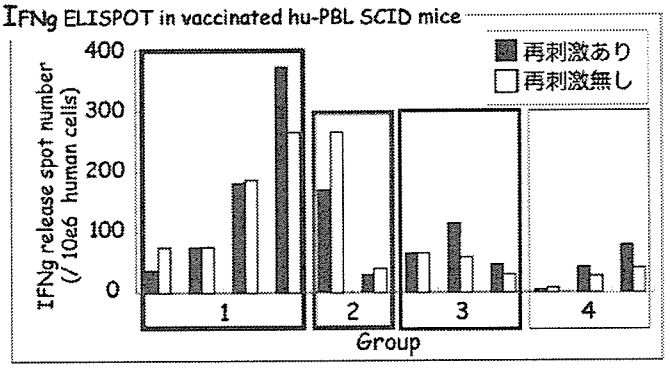
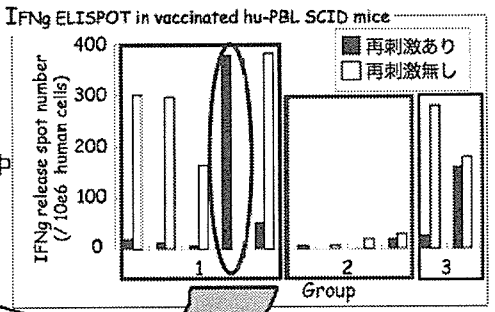


図 1

抗原特異的免疫誘導の評価 ～Neutralization assay～

/head　候補ワクチン群　　対照群

Group	1	2	3
Prime	AT-2 HIV / DC ($3x10^5$)	AT-2 HIV	DC ($3x10^5$)
Boost	AT-2 HIV / DC ($2x10^5$)	AT-2 HIV	DC ($2x10^5$)



○抗原特異的免疫誘導が得られた個体を中心に液性免疫応答を解析中

○血漿中HIV-1特異的抗体の検出

図 2

III. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論　文 タイトル名	書籍全体の 編集者名	書 籍 名	出版社名	出版地	出版年	ページ
なし							

雑誌

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Koyanagi Y, Tanaka Y, Ito M, and Yamamoto N.	Humanized mice for human retrovirus infection.	Cur Top Microbiol Immunol.			in press.

IV. 研究成果の刊行物・別刷

Cross-Linking Cell Surface Chemokine Receptors Leads to Isolation, Activation, and Differentiation of Monocytes into Potent Dendritic Cells

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Monocytes express on the cell surface several kinds of chemokine receptors that facilitate chemotaxis followed by differentiation in target tissues. In the present study, we found that a large number of monocytes from peripheral blood mononuclear cells (PBMCs) tightly adhered to plastic cell culture plates precoated with a monoclonal antibody (mAb, clone T312) specific for human CCR5 but not an isotype control after overnight incubation. Soluble T312 did not induce such adhesion, indicating that cross-linking of CCR5 is required for the enhanced adhesion of monocytes. The adhesion was blocked by a PI3-K inhibitor and an anti-CD18 blocking mAb. Following the cross-linking of CCR5, monocytes synthesized high levels of M-CSF, RANTES, MIP-1α, and MIP-1β associated with a readily detectable downmodulation of CD14, CD4, CCR5, and CXCR4 expression. The T312-enriched monocytes differentiated into dendritic cells (DCs) in the presence of interleukin-4 alone. After maturation with β-interferon, the T312-induced DCs stimulated proliferation of allogeneic naïve $CD4^+$ T cells accompanied by the synthesis of high levels of γ-interferon *in vitro*. Furthermore, the T312-induced DCs were capable of stimulating antigen-specific human T- and B-cell immune responses in our hu-PBL-SCID mouse system. Finally, screening of other anti-chemokine receptor mAbs showed that select clones of mAbs against CXCR4 and CCR3 were also capable of facilitating enrichment of monocytes similar to T312. These results show that cross-linking of chemokine receptors on monocytes by appropriate mAbs leads to activation and differentiation of monocytes and that the method described herein provides an alternate simple strategy for adherence-based isolation of monocytes and generation of functional DCs. Exp Biol Med 231:431–443, 2006

Key words: dendritic cell; monocyte; chemokine receptor; human immunodeficiency virus (HIV)

Introduction

Peripheral blood monocytes are derived from bone marrow progenitor cells and are recruited to tissues, where they undergo differentiation into macrophages or myeloid dendritic cells (DCs) (1–4). Recruitment of monocytes from the recirculating pool into normal or inflamed tissues involves a series of cascading events, which include the generation of chemokine gradients and the expression of cell adhesion molecules and their cognate ligands (5). Ligation of the chemokine receptors activates β1- and β2-integrins (6), which in turn mediate adhesion of monocytes within tissues, where they differentiate into DCs. DCs are potent antigen-presenting cells (APCs) and have a central role in the activation and function of both innate and adaptive immune responses against infectious microorganisms (7). Dysfunction and potential loss of DCs have been associated with decreased antigen-specific T-cell responses and synthesis of lower levels of virus-suppressive Type-1 interferon (8–11).

Results from a number of studies have documented the enhanced potential of DCs to process and present antigen, and thus, DCs have been regarded as natural cellular "adjuvants." This functional attribute has led to clinical trials of DC-based immunotherapy not only in a number of animal tumor models and human malignancies (12) but also against a number of infectious disease agents. This view is

This work was supported by grants from a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Research on HIV/AIDS and Health Sciences focusing on Drug Innovation from the Ministry of Health, Labor and Welfare of Japan; and the Japan Human Science Foundation.

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Received November 13, 2005.
Accepted January 5, 2006.

1535-3702/06/2314-0431$15.00

highlighted by the recent finding that immunization of human immunodeficiency virus Type 1 (HIV-1)–infected patients with autologous DCs sensitized with chemically inactivated autologous HIV-1 led to a marked sustained decrease in viral load (13, 14). Essentially similar data, in terms of decreasing viral loads, were obtained using the simian immunodeficiency virus (SIV)–infected nonhuman primate model of human AIDS following immunization with autologous SIV-pulsed DCs (15); the results of this study were reasoned to be due to enhancement of both T-cell and neutralizing antibody responses. This finding was further supported using the hu-PBL-SCID model, in which human peripheral blood mononuclear cell (PBMC)–engrafted mice, following immunization with inactivated HIV-1–pulsed human DCs, were shown to generate high levels of HIV-1–specific T-cell and B-cell immune responses sufficient to protect these animals against challenge with virulent HIV-1 isolates (16, 17).

Human DCs for such studies are generally derived from culturing enriched populations of monocytes *in vitro* in media supplemented with varying combinations of cytokines, depending on the nature of the studies to be performed. Evidence has been accumulating in support of the notion that there exist subsets of DCs that differ in their expression of cell surface markers, *in vivo* trafficking patterns, and cytokines synthesized, which influences the quality of the T-cell response that is induced by such DCs (18). Thus, *in vitro* culture of monocytes with recombinant human granulocyte/macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) leads to their differentiation into myeloid DCs, and *in vitro* culture of those cultured in media containing GM-CSF and IL-3 leads to their differentiation into lymphoid DCs (19). There also appears to be evidence that a common precursor progenitor cell exists that can give rise to either myeloid or plasmacytoid DC subsequent to Flt-3 ligation (20). In the *in vitro* culture of the monocytes, GM-CSF functions as a survival and differentiation factor, whereas IL-4 induces differentiation of DCs by blocking their differentiation into the macrophage lineage (21, 22).

To study the biology of these DCs and to use them for *in vivo* studies, a large number of monocytes need to be isolated from the peripheral blood. This is accomplished using a variety of techniques, such as elutriation centrifugation (23), use of antibody-conjugated immunobeads (21, 24), and the more simple method of adherence of these cells to plastic (5, 24). Although the purity of the preparations using such procedures varies, it is clear that the former two techniques are expensive, requiring unique instrumentation and/or clinically trained staff, and labor intensive. Although the adherence-to-plastic method is simple, the yield of monocytes in such preparations varies among donors, and the results are difficult to reproduce.

We have previously reported that cross-linking of CXCR4 by a monoclonal antibody (mAb) that recognizes the extracellular loop 3 (ECL-3) region of CXCR4 induces homologous adhesion of T cells and enhances HIV-1 infection (25). Since fresh monocytes express a variety of chemokine receptors on their cell surface (26), we hypothesized that cross-linking of these chemokine receptors by an immobilized mAb might induce monocyte adhesion onto culture plates, providing a simple alternative procedure for the enrichment of monocytes from PBMCs.

Indeed, herein we present data that show that cross-linking the chemokine receptors CCR5, CXCR4, and CCR3 on monocytes by appropriate mAbs enhances adhesion of monocytes to plastic plates and that these adherent monocytes can be induced to differentiate along the macrophage or myeloid DC lineages with the use of distinct recombinant cytokines. This procedure will provide a relatively efficient and a more practical alternative for the isolation and study of monocytes and DC lineages.

Materials and Methods

Animals. SCID mice lacking functional T, B, and natural killer (NK) cells and BALB/c–rag2$^{-/-}$ common gamma$^{-/-}$ mice (27) were used in the present study. These mice were kept in the specific-pathogen-free animal facility of the Laboratory Animal Center at the University of the Ryukyus. The protocols for the care and use of the hu-PBL-SCID mice were approved by the Committee on Animal Research of the University of the Ryukyus before initiation of the present study.

Reagents. RPMI-1640 medium was purchased from Sigma Chemical Co. (St. Louis, MO) and supplemented with 5% heat-inactivated fetal calf serum (FCS) (Sigma Chemical) (referred to as RPMI medium). Serum-free medium, AIM-V, was purchased from Life Technology (Grand Island, NY). Recombinant (r) human GM-CSF and IL-4 were produced in 293T cells transfected with pCMhGM-CSF and pCMhIL-4, respectively (RIKEN Gene Bank, Ibaraki, Japan), using the calcium phosphate method. The total protein and cytokine concentrations of the pooled culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) and a functional assay, as described previously (16). rIL-2 was provided by the U.S. National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. rIFN-β was purchased from Torey (Tokyo, Japan). rIFN-γ and rM-CSF were purchased from Peprotec (London, UK). The PI3-K inhibitor LY294.002 LPS from *Escherichia coli*, OVA, keyhole limpet hemocyanin (KLH), and bovine serum albumin (BSA, fraction V) were all purchased from Sigma Chemical. Fluorescein isothiocyanate (FITC)-labeled *E. coli*, FITC-fibrinogen, FITC-gelatin, and FITC-collagen were purchased from Molecular Probes (Eugene, OR). Monocyte negative isolation kits were purchased from Dynal (Oslo, Norway). Cell proliferation kits were purchased from Roche Diagnostics (Mannheim, Germany). ELISA kits for human IL-1β, IL-4, IL-6, IL-10, IL-12 p70, tumor necrosis factor (TNF)-α, M-CSF, MIP-1α, MIP-1β, and RANTES were

purchased from Biosource (Camarillo, CA). ELISA kits for γ-interferon (IFN–γ) were purchased from R&D Systems, Inc. (Minneapolis, MN). Naïve $CD4^+$ T-cell isolation kit was purchased from MACS (Gladbach, Germany).

The mAbs produced in our laboratory included two rat anti-human CCR5 N-terminus clones (clone T312 immunoglobulin [Ig]G1, clone T227 IgG2b); three rat anti-human CXCR4 (clone A145 IgG1, clone A120 IgG2b, clone A80 IgG1) (25); rat anti-human T-cell leukemia virus (HTLV)-I (clone LAT-27, IgG2b) (28); rat anti-hepatitis C virus (HCV) (clone Mo-8, IgG2b) (29); rat anti-human OX40 (clone W4–54, IgG2b, Tanaka *et al.*, unpublished data), and mouse anti-human OX40L (clone 5A8, IgG1) (30). The other mAbs used included mouse IgG anti-human CD4, SIM-2, and SIM-4 (obtained from the U.S. NIH AIDS Research and Reference Reagent Program), the clones OKT-4, OKT-8, and 60-bca anti-CD14 (obtained from ATCC, Rockville, MD). These mAbs were purified from SCID mouse ascites fluids by Superdex G-200 gel filtration (Amersham Bioscience, Uppsala, Sweden). Commercially available mAbs used were mouse IgG anti-human CD4, CD11c, CD80, CD83, CD86, HLA-DR (Coulter Inc., Hialeah, FL), and mouse IgG anti-human CD11a, CD11b, CD14, CD18, CD29, CD51, and CD61 (BD Pharmingen, San Diego, CA). Additional anti-human chemokine receptor mAbs, such as anti-CCR1 (mouse IgG2b, Cat #MAB145), CCR2 (mouse IgG2b, Cat #MAB150), CCR3 (rat IgG2a, Cat #MAB155), CCR5 (mouse IgG2b, Cat #MAB180), and CCR8 (rat IgG2b, Cat #MAB1429), were purchased from R&D Systems, Inc. (Minneapolis, MN); and the mAbs against CXCR4 (clone 12G5, mouse IgG2a) and CCR5 (clone 2D7, mouse IgG2a) were purchased from BD Pharmingen.

Cultivation of Monocytes. PBMCs were isolated from heparinized (5 U/ml) blood of normal healthy donors by a standard density gradient centrifugation at 400 g using lymphocyte separation medium (Sigma Chemical) for 15 mins at room temperature. The cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% BSA (BSA-PBS). PBMCs were resuspended in RPMI medium or serum-free AIM-V medium at 5×10^6 cells/ml. Then, 1 ml of the cell suspension was dispensed into individual wells of 12-well plates (BD Pharmingen), which were precoated with various mAbs (5 μg/ml) for 1 hr at 37°C. PBMCs were allowed to adhere 2 hrs or overnight at 37°C in a 5% CO_2 humidified incubator. Nonadherent cells were removed by gentle washing three times in BSA-PBS. The remaining adherent cells were then cultured in RPMI medium. For some experiments, monocytes were purified using a monocyte negative isolation kit and were used at $1–2 \times 10^5$ cells/ml. For the generation of macrophages, adherent or immunomagnetic bead-enriched $CD14^+$ monocytes were cultured in the presence of 20 ng/ml M-CSF for 6 days in a 5% CO_2 humidified incubator. For the generation of DCs, the media was supplemented with either GM-CSF (500 ng/ml) and IL-4 (200 ng/ml) or IL-4 (25 ng/ml) alone. Immature DCs were obtained after 5–6 days of culture. For maturation, the immature DCs were cultured in the presence of human β-interferon (IFN-β) (1000 U/ml) for an additional day. For sensitization with antigens, the immature DCs at Day 6 were cultured in the presence of either 100 μg/ml OVA or KLH for 1 day and then matured with IFN-β for an additional day. Viable cell number was assessed on an aliquot of such cells using staining with 0.1% eosin-Y.

Flow Cytometry. Phagocytosis and cell surface markers were determined using fluorescence activated cell sorter (FACS) Calibur and Cell Quest software (BD Pharmingen). Cell samples were Fc-blocked by incubation in media containing 2 mg/ml of human IgG in PBS containing 0.1% NaN_3 and 2% FCS (FACS buffer) on ice for 15 mins and were then stained with appropriate fluorescent dye–conjugated reagents on ice for 30 mins, according to the manufacturer's instructions. After washing with FACS buffer, cells were fixed in 1% paraformaldehyde-containing FACS buffer and analyzed. For quantitation of phagocytosis, sample cells (1×10^6) in 0.2 ml of RPMI medium were cultured in the presence of FITC-labeled *E. coli* at a cell to bacterium ratio of 1:10 to 1:100 for 1 hr at 37°C in a 5% CO_2 humidified incubator. After incubation, these cells were washed once in FACS buffer, fixed with 1% PFA, and then analyzed.

There were significant individual variations in the percentage of CCR5-positive cells in PBMCs. The percentages of T312-positive cells were as follows: 6%–14% of $CD14^+$ monocytes, 11%–43% of $CD3^+$ T cells, 6%–15% of $CD3^+$ $CD4^+$ T cells, and 5%–14% of $CD20^+$ B cells. Overnight incubation of PBMCs in medium alone led to a marked increase in the frequency of monocytes that expressed CCR5 (up to 70%), as detected using the T312 mAb. However, there was no detectable change of CCR5 expression in the other cell subsets (data not shown).

DC Functional Assays. Several assays were employed to determine whether the cell population that was being cultured belonged to the DC lineage. This included quantitation of the levels of IL-12 p70 and IL-10 produced in the culture supernatants of the potential DCs, as determined by ELISA; ability of the cells to induce allo-proliferation; and a unique *in vivo* assay. For the assessment of allo-proliferative potential, the mature or immature DCs to be tested were cultured at 1×10^5 cells/ml in RPMI medium in the presence of LPS (1 μg/ml) and IFN-γ (100 ng/ml) for 24 hrs. The ability to induce allogeneic stimulation was determined by co-culturing naïve $CD4^+$ T cells (1×10^5) with these potential DCs (0.5×10^4) in triplicate in a 96-well plate in a final volume of 0.2 ml in RPMI medium containing 20 U/ml IL-2 for 7 days. The level of proliferation of the $CD4^+$ T cells was assessed by the bromodeoxyuridine (BrdU)–incorporation ELISA method (31).

The *in vivo* function of DCs was determined using the hu-PBL-SCID mouse system, as previously described (16). Briefly, a SCID mouse received antigen-pulsed mature DCs

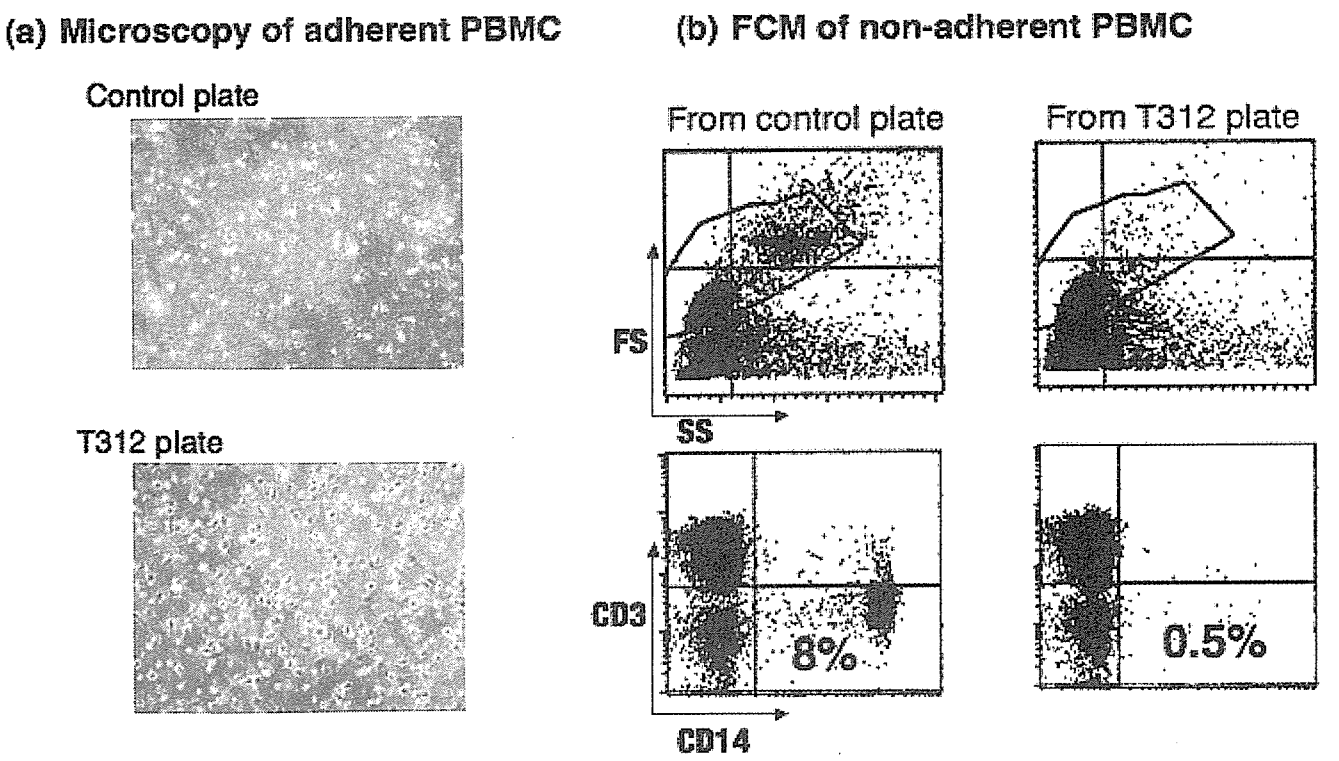

Figure 1. Stable adhesion of monocytes onto the bottom of plastic wells precoated with an anti-human CCR5 mAb. Aliquots (1 ml) of PBMCs at 2×10^6/ml of 5% FCS-RPMI medium were cultured overnight in individual wells of a 12-well plate that had been previously coated either with clone T312 rat IgG1 anti-human CCR5 or an isotype control mAb. (a) The morphology of the adherent cells as seen by phase-contrast microscopy at ×100. (b) Nonadherent PBMCs from each well were collected and analyzed by flow cytometry. The percentages of $CD14^+$ monocytes in the lymphocyte and monocyte gate in nonadherent PBMCs are noted in the double-staining profile with anti-CD3 and anti-CD14 mAbs. FS, forward scatter; SS, side scatter.

(5×10^5 cells) with autologous fresh PBMCs (3×10^6 cells) in a final volume of 0.1 ml in RPMI medium injected directly into the spleen, followed 7 days later with the ip injection of the same number and source of DCs pulsed with the same antigen. Five days later, the mice were sacrificed, and blood was collected by cardiocentesis and human lymphocytes were recovered from both the peritoneal cavity and the spleen. The immune sera were assayed for human antibody titers against OVA or KLH by ELISA using peroxidase-labeled goat anti-human IgG, as described previously (16). For the measurement of antigen-specific human T-cell immune responses, the human lymphocytes (2×10^6 cells) collected from the immunized mice were cultured for 2 days at 37°C in a 5% CO_2 humidified incubator with 2×10^5 autologous APCs (adherent PBMCs) in the presence or absence of either 5 μg/ml OVA or KLH in a volume of 0.5 ml in individual wells of a 24-well microtiter plate (BD Pharmingen). Media used consisted of RPMI medium supplemented with 20 U/ml of IL-2. The concentration of human IFN-γ produced in the culture supernatants was determined by standard ELISA.

Results

Cross-Linked CCR5 Induces Strong Adhesion of Monocytes. Twelve-well microtiter plates were first coated with the rat anti-human CCR5 N-terminus antibody (IgG1, clone T312) or an isotype control mAb. Then, PBMCs (5×10^6/ml) isolated from several normal donors were individually incubated overnight at 37°C in a 5% CO_2 humidified incubator in a volume of 1 ml of RPMI medium in each well of the plate. Following incubation, the nonadherent cells were removed. As shown in Figure1a, there appeared to be a marked increase in the number of cells that remained adherent to the wells precoated with the T312 mAb as compared with the control wells. As seen in Figure 1b, of interest was the finding that the nonadherent cells from the T312 mAb-coated wells were selectively depleted of $CD14^+$ cells (0.5%), compared with the nonadherent cells from the control wells (8%). The input unfractionated PBMCs from the various donors used for this study contained levels of $CD14^+$ cells that were essentially similar to the levels noted in the control wells, indicating that incubation of the PBMCs in the T312 precoated wells led to the select adhesion of the $CD14^+$ cells. Lymphoid cells other than $CD14^+$ monocytes were also present in the population of cells that adhered to the T312 mAb-coated wells, which mainly consisted of T and B cells, constituting up to 20% of the total adherent cells (data not shown). It is important to note that the addition of the T312 mAb to aliquots of the PBMCs from the same donors before

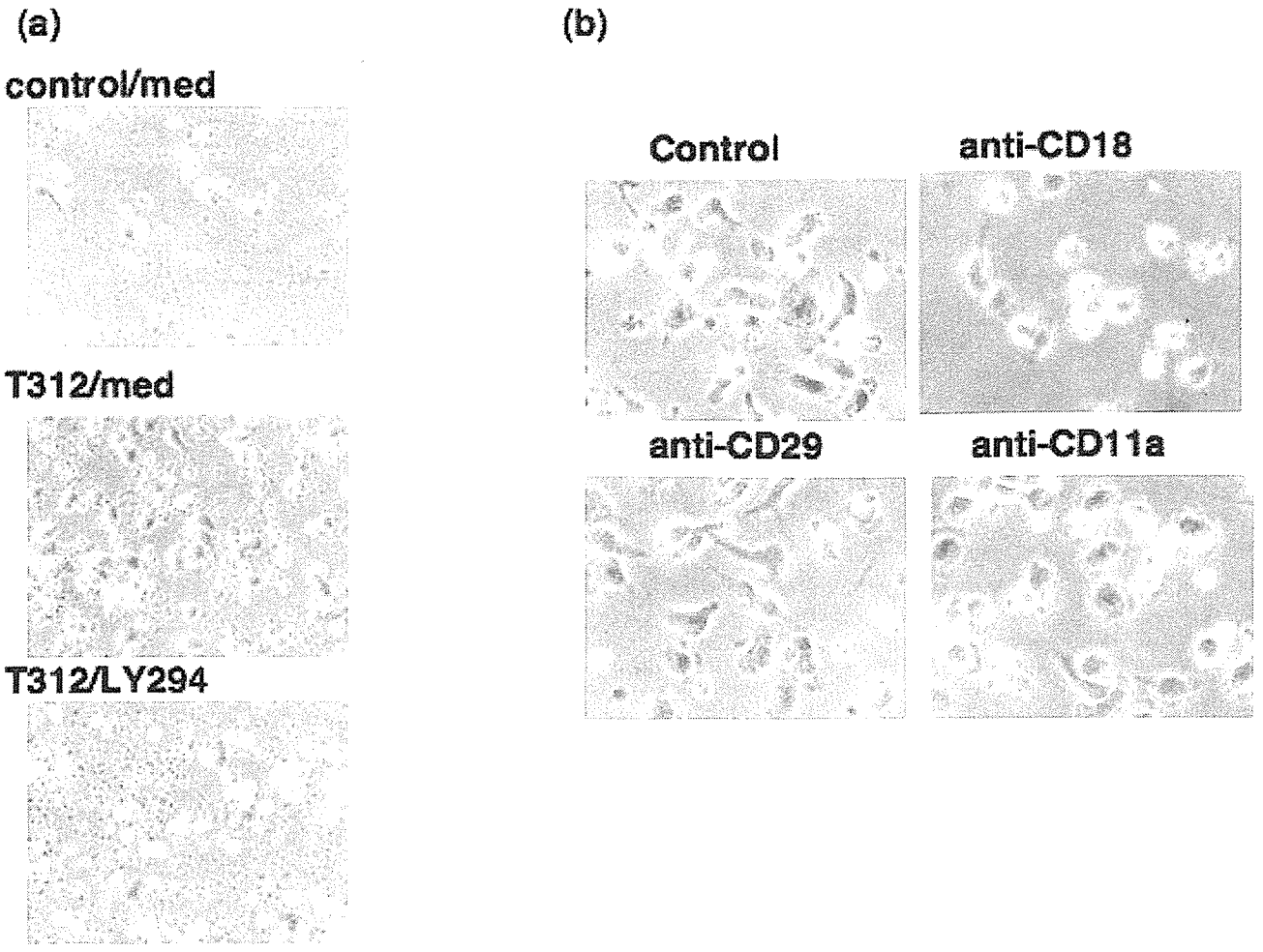

Figure 2. Microscopic analysis of the mechanisms for monocyte adhesion following CCR5 cross-linking. (a) PBMCs were incubated for 2 hrs in control mAb-coated wells (control/med) or overnight in T312 mAb-coated wells in medium alone (T312/med) or in the presence or of 5 μ*M* PI3-K inhibitor, LY294.002 (T312/LY294). (b) PBMCs were cultivated overnight in T312 mAb-coated wells in medium alone (control) or in the presence of 10 μg/ml of each mAb: anti-CD18, CD29, or CD11a. In these experiments, nonadherent cells were removed by gentle washing, and the wells were analyzed microscopically at ×200.

incubation in the microtiter wells or the addition of the same T312 mAb to the PBMCs following dispensing of the cells into the microtiter wells did not lead to increased adhesion of the $CD14^+$ monocytes, providing evidence indicating that the antibody had to be cross-linked for the adhesion to occur. A study of the kinetics of cell adhesion to the T312 mAb-coated wells was also performed. It should also be noted that there was a difference in the incubation time for maximal yield of monocytes following incubation of PBMCs in wells precoated with the T312 mAb, compared with the control antibody. Thus, where a 2-hr incubation was sufficient to obtain the maximum yield of monocytes by incubation of PBMCs in the control antibody-coated wells, it required an overnight or 24-hr incubation in the T312 mAb-coated wells (data not shown). Thus, for all further studies, adherent cells were isolated by incubation of the PBMCs either for 2 hrs in control wells or overnight in T312 mAb-coated wells, unless otherwise noted.

Since binding of CCR5 by its natural ligands is known to induce intracellular signaling via the PI3-K pathway, experiments were carried out to determine if such signaling was a prerequisite for the stable binding of the monocytes to the T312 mAb-coated wells. The above experiment was repeated, with the only difference being that an aliquot of each of the donor PBMCs was incubated in the T312 mAb-coated wells in the presence of 5 μ*M* of the PI3-K inhibitor LY294.002. Representative data shown in Figure 2a demonstrate that the addition of the PI3-K inhibitor completely blocked the cell adhesion. Flow cytometric analysis of the nonadherent cells in the wells containing the PI3-K inhibitor confirmed the presence of a high frequency of $CD14^+$ cells, denoting the lack of adhesion of the CD14-expressing cells (data not shown). It was also of interest that the addition of various concentrations of soluble or immobilized RANTES, MIP-1α, and MIP-1β did not enhance monocyte adhesion (data not shown), denoting that simple ligation of the chemokine receptors was not sufficient to facilitate adhesion.

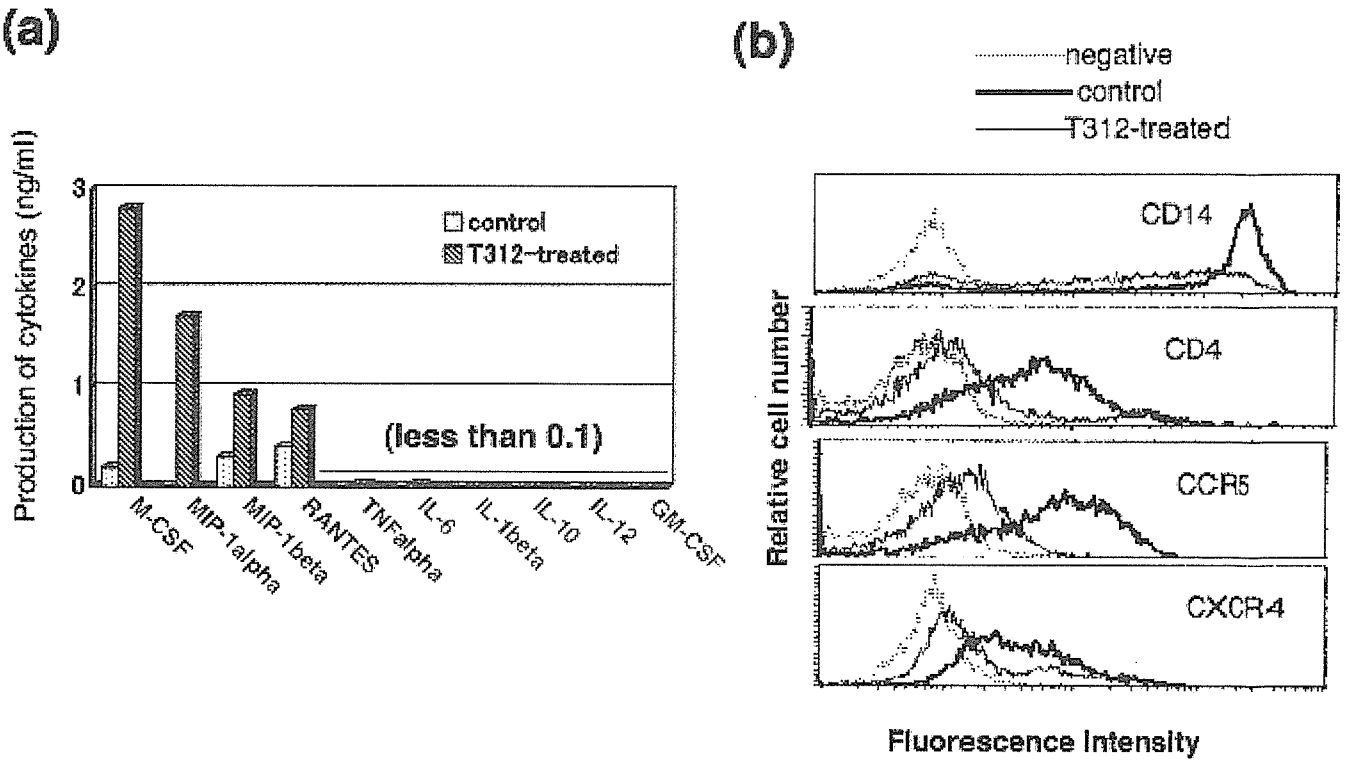

Figure 3. Cytokine profile and phenotypic analysis of purified monocytes following CCR5 cross-linking. (a) CD14 negatively selected monocytes at 2×10^5/ml in RPMI medium in T312 mAb-coated (T312-treated) or control wells (control) were cultured overnight, and cytokines produced in the culture supernatants were assayed by ELISA. Values of <0.1 ng/ml were below the sensitivity of the assay employed. (b) Aliquots of monocytes that were cultured in control wells (thick dark lines) or those cultured in T312 mAb-coated wells (thin dark lines) were analyzed for the expression of CD14, CD4, CCR5, and CXCR4 by routine flow cytometry. The broken lines show background staining of cells with isotype control mAbs.

It has been previously documented that adhesion of monocytes onto plastic plates is mediated by fibrinogen binding by the β2-integrins Mac-1 (CD11b/CD18) and/or p150/95 (CD11c/CD18) (32, 33). As shown in Figure 2b, the T312 mAb-induced adhesion of monocytes was blocked by anti-CD18 blocking mAb (with specificity for the common chain of β2-integrins), but not anti-CD29 mAb (β1-integrin) or anti-CD11a (another component of the β2-integrin, LFA-1), indicating an involvement of the β2-integrins in the enhanced adhesion of monocytes by T312 mAb-mediated CCR5 cross-linking.

Cytokine Profile and Phenotype Analysis of Monocytes Adhered to the T312 mAb-Precoated Wells. In an effort to define the cytokine profile characteristic of the monocyte population, it was reasoned that depletion of nonmonocyte lymphoid cells before analysis would be important. The magnetic bead negative selection technique for $CD14^+$ cells was thus used to deplete such nonmonocyte lymphoid cells, and the subsequent enriched monocytes were then incubated overnight in wells precoated with either T312 or control mAb, and the supernatant fluids were collected. Representative data from three experiments are shown in Figure 3a. As seen, the T312 mAb-stimulated monocytes secreted significant levels of M-CSF, MIP-1α, MIP-1β, and RANTES. The $CD14^+$ monocytes are likely to be the major cell lineage that responded to the T312 mAb stimulation by the production of cytokines, because PBMCs depleted of $CD14^+$ monocytes failed to synthesize detectable levels of the same cytokines. An aliquot of the same supernatants was also screened for levels of GM-CSF, TNF-α, IL-1β, IL-12, or IL-10, but each was found to be below 0.1 ng/ml. A variety of rat and mouse mAbs that had been generated in our laboratory with specificity for HTLV-1, HIV-1, HCV, OX40L, OX40, or IL-2R were also used to precoat the wells and were screened for their ability to induce cytokine synthesis by purified monocytes in parallel with the use of T312 mAb. As expected, only monocytes incubated in the wells precoated with T312 mAb produced M-CSF, MIP-1α, MIP-1β, and RANTES, denoting an element of specificity for the T312 mAb (data not shown).

Phenotypic analysis of monocytes adhered to the T312 mAb-precoated wells consistently showed a marked down-regulation of CD14, CD4, CCR5, and CXCR4 (a representative profile is shown in Figure 3b). Downmodulation of the CD14 on the cell surface was not attributed to the shedding of CD14, since there was no difference in the level of soluble CD14 between the supernatant fluids from the wells precoated with the T312 mAb and those from the control wells, as determined by ELISA (data not shown).

CCR5 Cross-Linking Stimulates Monocyte Dif-

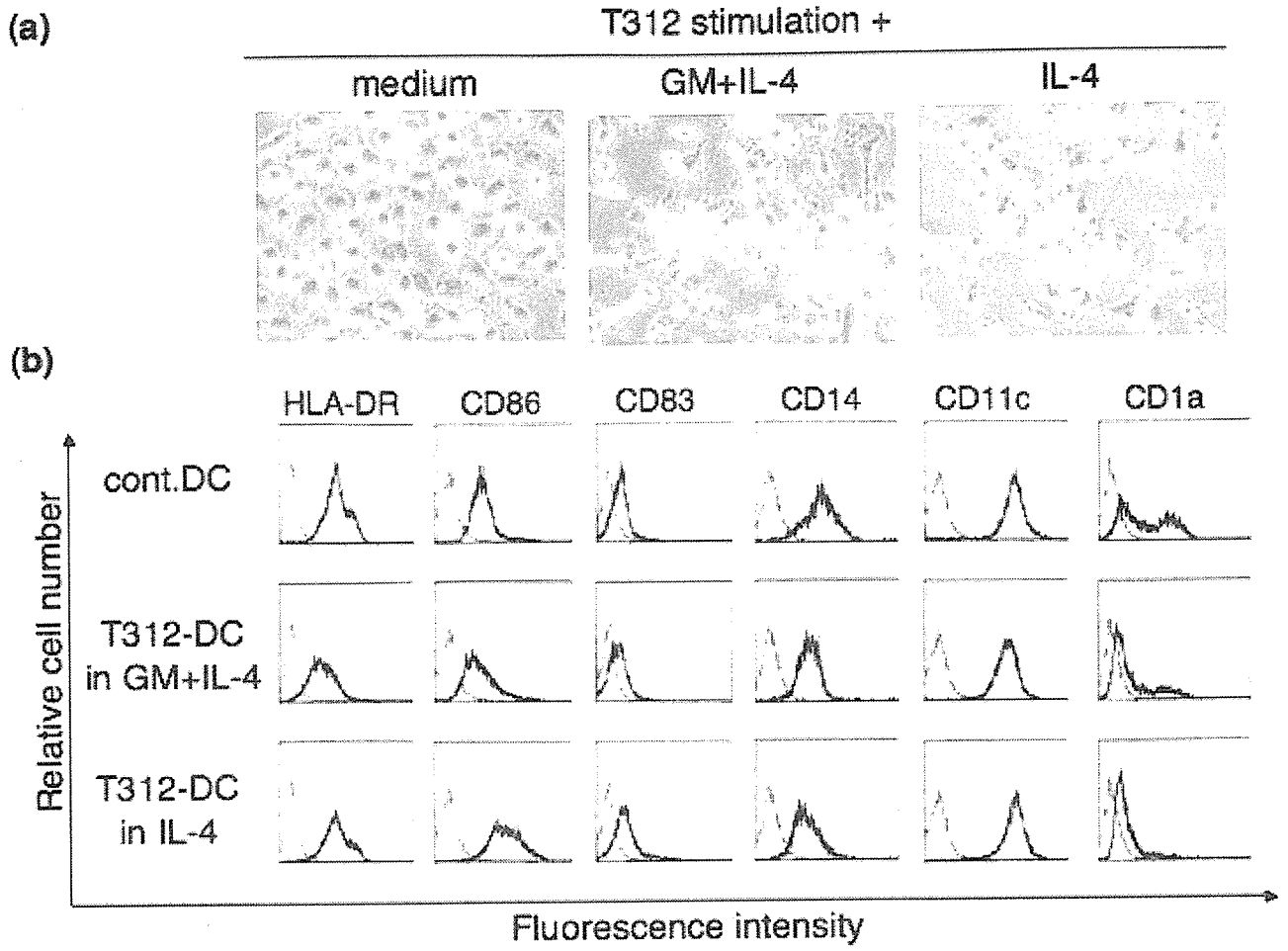

Figure 4. Morphological and flow cytometric analyses of adherent PBMCs following maturation in different culture conditions *in vitro*. (a) PBMCs at 5×10^6/ml in RPMI medium were cultured overnight in wells precoated with T312 mAb. After removal of nonadherent cells, adherent cells were cultured in RPMI medium (medium) or in the presence of 500 ng/ml GM-CSF and 200 ng/ml IL-4 (GM-CSF+IL-4) or 25 ng/ml IL-4 alone (IL-4) for 5 days. Morphology was observed microscopically at ×200. (b) The T312-induced immature DCs were generated from PBMCs adhered to the T312 mAb-coated wells during overnight incubation followed by cultivation in the presence of either GM-CSF and IL-4 (T312-DC in GM+IL-4) or IL-4 alone (T312-DC in IL-4). For comparison, control conventional DCs were generated from 2 hr–adherent PBMCs in control wells followed by cultivation in GM-CSF and IL-4 for 6 days (cont. DC). After maturation for an additional day with IFN-β, aliquots of these cells were subjected to flow cytometric analysis, and the profiles for the expression of HLA-DR, CD86, CD83, CD14, CD11c, and CD1a are shown.

ferentiation into DCs. To test whether the T312 mAb-induced CCR5 cross-linking has the potential to differentiate monocytes into functional DCs, aliquots of PBMCs were first incubated either in the T312 mAb-coated wells overnight or the control mAb-coated wells for 2 hrs (for the generation of conventional DCs), the nonadherent cells were removed, and then the adherent cells were further cultured in the presence or absence of either GM-CSF and IL-4 or IL-4 alone for 5 or 6 days, respectively.

As shown in Figure 4a, the adherent cells incubated in T312-coated wells appeared to have a DC type of morphology, which was confirmed by flow cytometric analysis (see Fig. 4b). Interestingly, the T312 mAb-stimulated monocytes, when cultured in the presence of IL-4, regardless of the presence or absence of GM-CSF, exhibited higher mean density levels of CD86 and lower levels of CD1a than did control conventional DCs that were cultured in media containing both GM-CSF and IL-4. The DCs cultured in the presence of GM-CSF and IL-4 in the T312 mAb-coated wells had a phenotype that was basically similar to that of the ones cultured in IL-4 alone, and these are both representative profiles of immature DCs. Kinetic studies of the differentiation of these T312 mAb-stimulated immature DCs showed that the optimal level of maturation was achieved by cultivation in the presence of IFN-β for an additional day.

We next sought to examine the relative phagocytic activity of monocyte-derived DCs or macrophages that were cultured in various conditions. As seen in Figure 5, macrophages obtained following differentiation of a highly enriched population of $CD14^+$ monocytes in M-CSF–containing media in general gave a higher relative phagocytic index than did DCs generated in media containing GM-CSF and IL-4. In addition, those that were

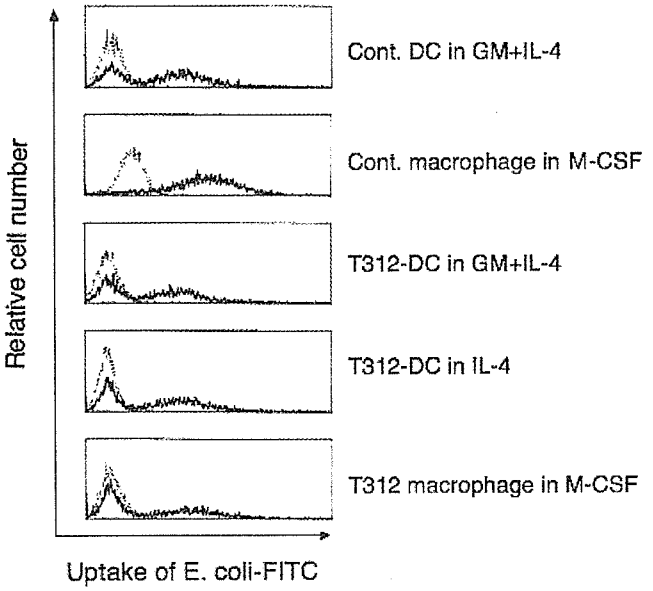

Figure 5. Phagocytic activities of cultured macrophages or immature DCs. Various DCs were generated as described in the legend for Figure 4, and macrophages were generated from 2 hr–adherent PBMCs followed by cultivation in the presence of M-CSF for 6 days. These sample cells in RPMI medium were incubated with a 10-fold excess of FITC-labeled *E. coli* particles for 1 hr at 37°C. After washing, the levels of uptake of FITC-labeled *E. coli* by cells were determined by flow cytometry with gating for macrophages and DCs using standard forward and side scatter.

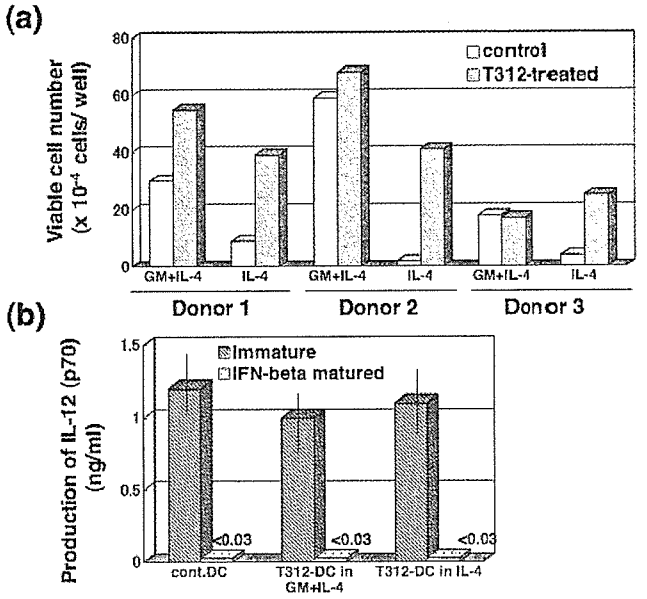

Figure 6. Yields of DCs and IL-12 production. (a) Immature DCs were generated by either incubation of aliquots of PBMCs from three different donors in the control wells (control) or in the T312 mAb-coated wells (T312-treated) and then for 6 or 5 days, respectively, with GM-CSF and IL-4 or IL-4 alone, as described in the legend for Figure 4. Numbers of viable cells that were not stained by eosin-Y were manually counted using a hemocytometer. The means from triplicate determinations are shown. (b) DCs from a donor before (immature) or after IFN-β treatment (mature) were stimulated by 1 μg/ml LPS and 100 ng/ml IFN-γ for 24 hrs. IL-12 p70 levels in the culture supernatants were determined by ELISA.

stimulated with T312 mAb and cultured in media containing either GM-CSF and IL-4 or IL-4 alone were less phagocytic than the control macrophages generated by M-CSF. Interestingly, the T312 mAb-stimulated monocytes, following incubation with media containing M-CSF alone, gave slightly lower phagocytic values than the normal macrophages cultured in the same media. These data seem to indicate that cross-linking of CCR5 influences monocyte differentiation into DC rather than macrophage, even in the presence of M-CSF.

We further determined the relative levels of viability of the cells that adhered either to the T312-coated wells overnight or to control wells for 2 hrs followed by cultivation in medium alone or media containing either GM-CSF and IL-4 or IL-4 alone for 5 or 6 days, respectively. Figure 6a shows, first of all, that whereas there was variation in the number of viable cells recovered among donors of the PBMCs, the yields of viable cells generated in the T312 mAb-coated or control wells followed by cultivation in GM-CSF and IL-4 were basically similar. In contrast, aliquots of the same cells incubated in T312 mAb-coated or control wells gave different yields following incubation with IL-4 alone. The cells stimulated by T312 mAb gave significantly higher viable yields than those incubated in control wells. The overnight adherent monocytes could differentiate into functional DCs in the presence of GM-CSF and IL-4, but the cell yield was lower than those obtained from the 2-hr adherent cells (data not shown).

Aliquots of these *in vitro*–generated immature DCs were subsequently challenged with LPS and IFN-γ for 48 hrs, and supernatant fluids were assayed for levels of IL-12 p70. Controls consisted of aliquots of the conventional DCs obtained from the same PBMCs in wells coated with control IgG1 instead of T312 mAb. As seen in Figure 6b, there were significant levels of IL-12 synthesized by both these populations of immature DCs, and the importance of the levels appeared quite similar. Furthermore, the IL-12–producing activity of these immature DCs was lost after full maturation by incubation with IFN-β.

Function of DCs. In an effort to determine whether the T312 mAb-induced DCs were as competent as conventional DCs, these immature DC populations were matured by cultivation in media containing IFN-β for 1 day and the aliquots dispensed into microtiter wells and standard mixed lymphocyte reaction (MLR) studies performed. The T312 mAb-induced DCs (termed T312/IL-4 or T312/GM-IL-4) and the conventional DCs (termed control/GM-IL-4) were co-cultured with allogeneic naïve $CD4^+$ T cells. Controls consisted of $CD4^+$ T cells cultured alone or DCs cultured alone. The cultures were performed at a DC to T-cell ratio of 1 to 20, and each combination was cultured in triplicate for 7 days and proliferation assessed using BrdU incorporation by the ELISA method. Supernatant fluids from the MLR

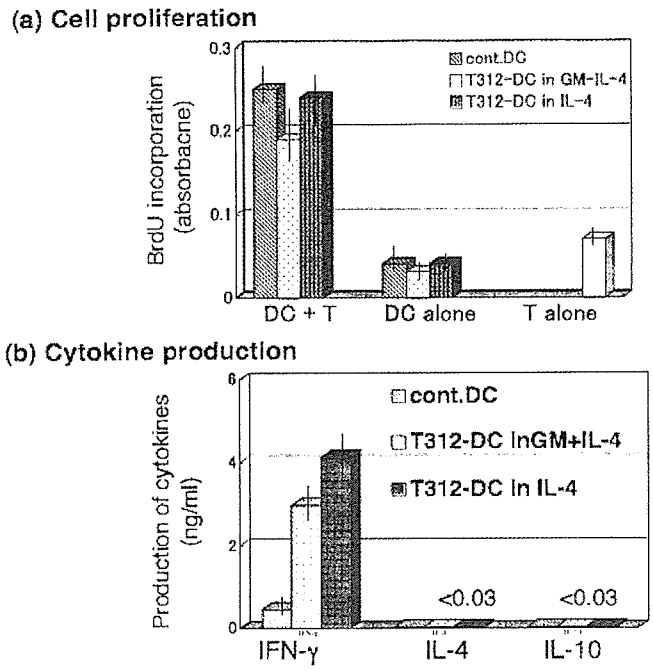

Figure 7. T312 mAb-induced DCs stimulate allogeneic naïve $CD4^+$ T cells to proliferate and produce IFN-γ. Allogeneic naïve $CD4^+$ T cells were co-cultured with IFN-β–matured DCs that had been generated from the adherent PBMCs, as described in the legend for Figure 4 in RPMI medium supplemented with 20 U/ml IL-2 for 7 days. (a) Cell proliferation was determined by BrdU incorporation. Mean values from six wells and standard errors are shown. Controls consisted of culturing either DCs or T cells alone. (b) IFN-γ production in the culture supernatants was determined by ELISA.

cultures were also assayed for levels of IFN-γ, IL-4, and IL-10. As shown in Figure 7a, there was no significant difference in the proliferation-inducing activity among the three preparations of DCs. However, an obvious difference was seen in the levels of IFN-γ synthesized from the naïve $CD4^+$ T cells (Fig. 7b). The T312 mAb-induced DCs generated in the presence of either GM-CSF and IL-4 or IL-4 alone stimulated higher production of IFN-γ than the control DCs. None of these DCs induced production of IL-4 or IL-10 from the $CD4^+$ T cells by co-culture. The T312 mAb-induced DCs did not produce detectable levels of human IFN-γ, even following stimulation with LPS or CD40L (data not shown), indicating that the allogeneic naïve $CD4^+$ T cells are the likely source of IFN-γ following co-culture and stimulation with DCs. These results indicate that the T312 mAb-induced DCs acquired a relatively higher Th1-polarizing capacity than control conventional DCs.

Furthermore, we tested and compared the ability of these DCs to induce human antigen-specific T- and B-cell immune responses *in vivo*. For this purpose, we used our hu-PBL-SCID mouse model (16). SCID mice were engrafted with normal PBMCs together with antigen (OVA)–pulsed autologous T312 mAb-induced or conventional mature DCs. A booster injection with antigen-pulsed homologous source of mature DCs was performed after 7 days. Immune

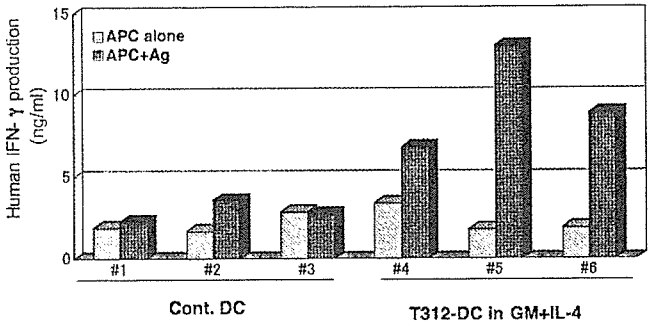

Figure 8. T312 mAb-induced DCs trigger human antigen–specific T-cell responses in hu-PBL-SCID mice. DCs were generated as described in the legend for Figure 4, followed by exposure to antigen (100 μg/ml OVA or KLH) for 24 hrs and by maturation with IFN-β for an additional 24 hrs. These DCs (5×10^5 cells) together with autologous fresh PBMCs (3×10^6 cells) were mixed in 0.1 ml RPMI medium and transplanted into the spleen of SCID mice. Booster immunization was made by injection of the same numbers of antigen-loaded DCs (ip) on Day 7. After 5 days, cells obtained from the spleen and peritoneal cavity of the mice were cultured with autologous APCs (adherent PBMCs during 2-hr incubation in normal wells) in the presence (APC + Ag) or absence (APC alone) of 5 μg/ml OVA for 2 days, and the supernatants were assayed for human IFN-γ by ELISA. Values depicted reflect the mean levels of IFN-γ of triplicate cultures (SD was <10%).

sera and spleen cells were harvested after 5 days following the booster injection. Figure 8 shows that immunization with the T312 mAb-induced and OVA-pulsed DCs was more efficient in inducing antigen-specific T-cell immune responses in the hu-PBL-SCID mice, as determined and defined by human IFN-γ production by the immune cells *in vitro*, than the conventional DCs pulsed with OVA. IL-4 was not produced from those immune cells (data not shown). Human OVA-specific IgG were also induced by both types of the conventional and T312 mAb-induced DCs up to a titer of 1:400, as determined by ELISA (data not shown). Essentially similar results were obtained when mice were immunized with another antigen, KLH (data not shown). These data indicate that the T312 mAb-induced DCs, in general, appear to be relatively more efficient than conventionally derived DCs in inducing antigen-specific IFN-γ responses *in vivo*.

Effect of Cross-Linking by Other mAbs Against Chemokine Receptors. Based on the results from the above studies, additional experiments were carried out to examine the specificity of the ligation of the CCR5 coreceptor by the T312 mAb. Thus, a battery of mAbs against human chemokine receptors expressed by monocytes, including CCR1, CCR2, CCR3, CCR5, CCR8, and CXCR4, were used to precoat microtiter wells, and aliquots of purified monocytes were incubated in such antibody-coated wells. The level of M-CSF synthesized by the cells following overnight incubation was used as an index of monocyte stimulation. As seen in Figure 9 among the anti-human CCR5 mAbs tested, Mab180 (mouse IgG anti-CCR5 N-terminus), but not T227 (rat IgG2a anti-CCR5 N-terminus) or 2D7 (mouse IgG anti-CCR5 ECL-2), was as