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Activated CD8+ T Lymphocytes Induce Differentiation of Monocytes to Dendritic Cells and Restore the Stimulatory Capacity of Interleukin 10-treated Antigen-presenting Cells[1]

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ABSTRACT

We analyzed the role of CD8+ T cells on dendritic cells (DCs). Incubation of monocytes with activated but not resting CD8+ T cells induced their differentiation into DCs with a CD1a+ and CD14- phenotype. Activated CD8+ lymphocytes induced down-regulation of CD14 expression in interleukin (IL)-10-treated macrophages and resulted in the expression of DC-related CD1a and up-regulation of mRNA transcripts for RelB, IL-12p40, MCP-2, MIP-1α, and ELC. These DCs were potent stimulatory cells in mixed lymphocyte reaction and induced primary CTL responses. This interaction was independent of a direct cell-to-cell contact. Monoclonal antibodies against IFN-γ completely abolished the CD1a induction on IL-10-treated antigen-presenting cells. These results demonstrate that CD8+ cells can provide stimulatory signals to antigen-presenting cells to induce their differentiation in DC and to increase their immunostimulatory capacity.

INTRODUCTION

CD4+ Th[3] cells can productively interact with APC. This interaction has been described to be cognate (1, 2). However, there have been several reports demonstrating that Th-independent CTL responses can be induced *in vivo* during a viral infection (3–5). A study using a murine lymphocytic choriomeningitis virus model demonstrated that antiviral CD8+ T cells could induce maturation of DCs in the absence of CD4+ cells and CD40 ligation (6). These CD8+ T cells were able to induce in transmaturation of DCs that were not presenting the antigen, thus suggesting that this interaction does not have to be cognate. However, factors responsible for the observed DC maturation have not yet been identified.

Cytokines like IL-10 can inhibit the development and function of DC by altering the chemokine expression, decreasing the cytokine production, and affecting antigen presentation (7, 8). In DCs generated from peripheral blood monocytes, IL-10 was shown to inhibit IL-12, IL-6, and IL-15 production and induces a state of antigen-specific anergy in CD4+ and CD8+ T cells. Secretion of IL-10 by tumor cells has been shown to be a major mechanism of tumor escape from immune surveillance by affecting antigen presentation and induction of T-cell tolerance (9).

In this report, we demonstrate that activated human CD8+ T cells can induce differentiation of monocytes into DCs and restore the stimulatory capacity of IL-10-treated APCs. Using transwell and blocking experiments, we were able to show that these effects are not mediated by cognate T-cell/APC interactions but soluble factors, including IFN-γ.

MATERIALS AND METHODS

Cell Isolation and Cultures. CD8+ cells were purified from PBMNCs using MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the cells was 95.5% (SD, 0.9%). Monocytes were isolated by adherence as described previously(10) or using MACS technology as indicated. For stimulation, T lymphocytes were incubated overnight with 25 ng/ml phorbol 12-myristate 13-acetate and 1 μg/ml ionomycin. After extensive washing, T cells were used in experiments.

The following cytokines were obtained from R&D Systems (Wiesbaden, Germany): IL-4 (1000 IU/ml); TNF-α (10 ng/ml); IL-10 (10 ng/ml); IFN-α (100 IU/ml); and IFN-γ. Human recombinant GM-CSF (Leucomax, 100 ng/ml) was from Novartis (Basel, Switzerland).

For blocking studies, we used mAbs and soluble proteins specific for CD154 (CD40L, 10 μg/ml; Alexis, Grünberg, Germany or Ancell, Bayport, MN), CD137 (4-1BB, 5 μg/ml; Ancell or Alexis), TNF-α (10 μg/ml; R&D Systems), TNF-α-receptor I and II (10 μg/ml; R&D Systems), antibodies against IFN-α, and its receptors as well as IFN-γ and its receptor (10 μg/ml; R&D Systems), purified CD137 ligand-muCD8 (12.5 μg/ml; Ancell, or Alexis), Osteprotegerin:Fc (12.5 μg/ml; Alexis), and soluble human recombinant RANK ligand (2.5 μg/ml; Alexis).

For transwell experiments, we used 24-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) with cell culture inserts (pore size, 0.4 μm). APCs were cultured in the bottom, whereas activated or untreated T cells were placed in the inserts above.

Immunostaining. Cell staining was performed using FITC- or phycoerythrin-conjugated mouse mAbs against CD86, CD40 (purchased from PharMingen, Hamburg, Germany), CD3, CD19, CD20, CD80, HLA-DR, CD54, CD14 (Becton Dickinson, Heidelberg, Germany), CD83 (Coulter-Immunotech Diagnostics, Hamburg, Germany), CD1a (Dako Diagnostika, Hamburg, Germany), and mouse IgG isotype controls. Samples were analyzed on a FACScan Calibur (Becton Dickinson).

RT-PCR. Total RNA was isolated from cell lysates using Qiagen RNeasy anion-exchange spin columns (Qiagen GmbH, Hilden, Germany) according to the instructions of the manufacturer. Total RNA was subjected to first-strand cDNA using an optimized protocol described by SuperScript Preamplification System (Life Technologies, Inc., Eggenstein, Germany), using oligodeoxythymidylate as primer. cDNA obtained from the reverse transcriptase reaction was subjected to the amplification. To control the integrity of the isolated RNA, cDNA was amplified by an intron-spanning primer pair for the *β2 microglobuline* gene. Reactions were amplified in a DNA thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer). The primer sequences were used as published (11).

MLR Assay. Responding cells (10^5) from allogeneic PBMNC were cultured in 96-flat-bottomed microplates (Nunc, Wiesbaden, Germany) with various numbers of irradiated stimulator cells. Thymidine incorporation was measured on day 5 of coculture by a 16-h pulse with thymidine (0.5 μCi/well; Amersham Life Science, Buckingham, United Kingdom).

Induction of Antigen-specific CTL Responses Using an HLA-A2-restricted Synthetic Peptide. For CTL induction, 10^5 APC (macrophages treated with GM-CSF, IL-4, and IL-10 for 7 days) were pulsed with 50 μg/ml of the synthetic Her-2/neu-derived E75 peptide KIFGSLAFL. Subsequently, they were cultured in 6-well plates containing RP10 medium (11) with 5×10^5 autologous-irradiated (30 Gy) purified CD8+ T lymphocytes that were either activated with phorbol 12-myristate 13-acetate/ionomycin (25 ng/ml and 1

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[3] The abbreviations used are: Th, T-helper; APC, antigen-presenting cell; DC, dendritic cell; IL, interleukin; PBMNC, peripheral blood mononuclear cell; GM-CSF, granulocyte macrophage colony-stimulating factor; mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; MLR, mixed lymphocyte reaction.

μg/ml, resp.) or left untreated and were washed extensively thereafter. After 24 h of incubation, 2×10^6 autologous PBMNCs were added to the cultures. Cells were restimulated after 7 days of culture, and 2 ng/ml human recombinant IL-2 were added every other day. The cytolytic activity of induced CTLs was analyzed on day 5 after the second restimulation with inactivated peptide-pulsed PBMNCs in a standard ^{51}Cr-release assay.

RESULTS AND DISCUSSION

Incubation of Isolated Peripheral Blood Monocytes with Activated CD8+ T Cells Induces Down-Regulation of CD14 and the Expression of DC Markers. Generation and maturation of DCs, which play a central role in the induction of primary immune responses, are regulated by various stimuli. DCs can directly interact with T cells and do both, receive and provide stimulatory signals from and to T lymphocytes. In several murine models, it could be demonstrated that CTL responses can be elicited in the absence of CD40 ligand and Th cells (3–5), and CD8+ CTLs could induce maturation of DCs, thus suggesting that CD8+ T cells can solicit their own help by affecting the function of APC (6). In these models, CTLs may adopt T-cell helper function and thus support epitope spreading. We therefore analyzed the possible role of activated or resting CD8+ T lymphocytes on DC differentiation from purified peripheral blood monocytes.

Addition of activated CD8+ T lymphocytes induced a decrease of CD14 expression on monocytes within 24 h of culture (data not shown). Additional incubation (for 3 days) resulted in the induction of CD1a and complete down-regulation of CD14 in cultures containing activated T cells (Fig. 1*A*). The expression of CD83 varied pending on experiments. These cells expressed HLA-DR, CD80, and CD86 (data not shown).

Previous reports have shown that IL-10 can inhibit the differentiation of DCs from monocytes *in vitro* in the presence of GM-CSF and IL-4 when added on the day of culture initiation. These cells have the phenotype of macrophages and a reduced T-cell stimulatory capacity in MLR. Furthermore, these cells are unable to induce primary CTL responses against tumor-associated peptide antigens (8, 11).

We added IL-10 with GM-CSF and IL-4 to isolated monocytes on day 0. After 7 days, IL-10-treated cells were washed and incubated with purified CD4+ or CD8+ T lymphocytes.

When activated but not untreated CD4+ or CD8+ T cells were added to the cultures, macrophages started to reduce the expression of CD14 within 24 h that was completely down-regulated during a 3-day culture (Fig. 1, *B* and *C*). In parallel, these cells increased the expression of CD1a. As in experiments mentioned above, the cells expressed HLA-DR and costimulatory molecules CD80 and CD86 (data not shown).

Thus, incubation of peripheral blood monocytes and IL-10-treated macrophage-like cells with activated CD8+ T cells resulted in the induction of differentiation into cells expressing DC markers, similar to the effects mediated by CD4+ T lymphocytes.

Incubation of IL-10-treated Macrophages with Activated T Cells Induces Up-Regulation of IL-12p40, RelB, and Chemokine Gene Expression. RelB is a nuclear transcription factor that has been shown to be essential for the development and antigen-presenting function of DCs (12). We used a semiquantitative RT-PCR to analyze the gene expression of factors important for DC differentiation and function. The analysis was performed after a 3-day culture of IL-10-treated macrophages with irradiated CD8+ cells. As shown in Fig. 2, incubation of IL-10-treated APCs with activated CD8 cells resulted in the induction of RelB mRNA.

Furthermore, the addition of activated CD8+ cells to the cultures resulted in increased levels of mRNA expression coding for the MIP-1α, MCP-2 and ELC chemokines, suggesting that CD8+ T lymphocytes can affect the function of DCs at the level of antigen presentation, cytokine production, and chemokine expression (13). Although we could not detect any bioactive IL-12 protein in the cell cultures (ELISA, data not shown), IL-12p40 transcripts were detected by RT-PCR in the cells after incubation with activated T cells.

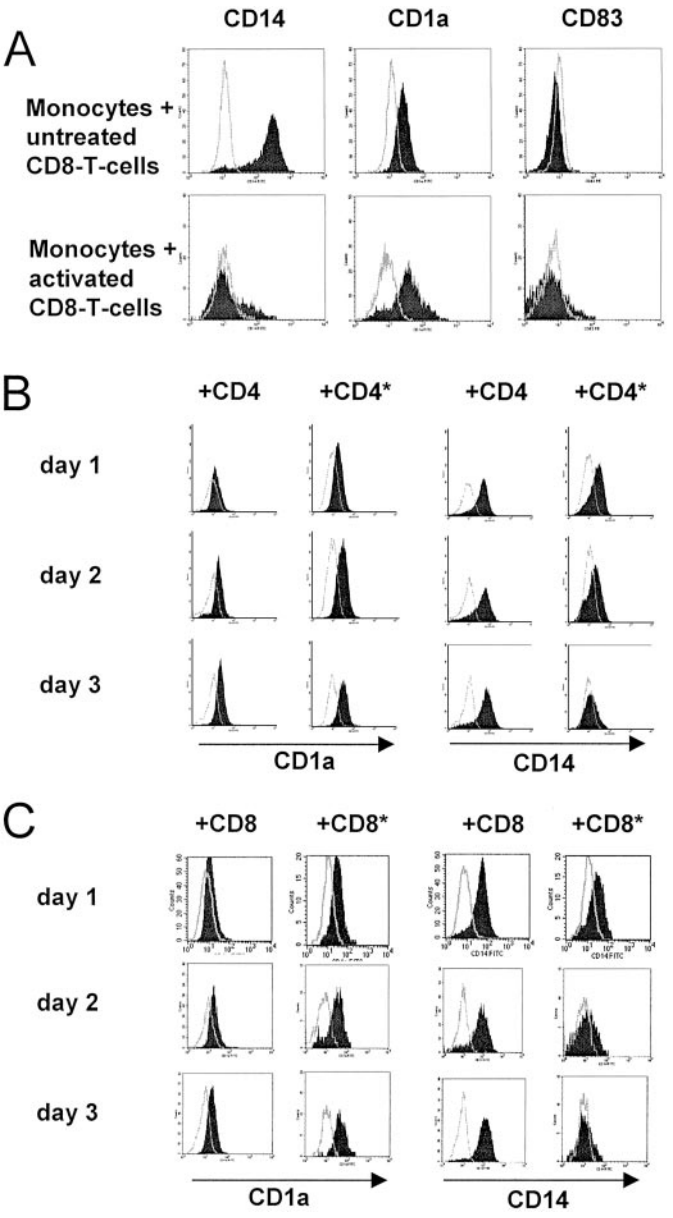

Fig. 1. Phenotypic analysis of peripheral blood monocytes incubated with CD8+ T lymphocytes. Monocytes were isolated from PBMNC and cultured with resting or activated purified CD8+ T cells as indicated. Overlay diagrams show expression of indicated molecules after 3 days of culture. *Thin histograms*: labeling with idiotype-matched irrelevant mAb (*A*). To analyze the phenotype of IL-10-treated macrophages after incubation with CD4+ (*B*) and CD8+ (*C*) T lymphocytes, monocytes were isolated from PBMNC by adherence and cultured in the presence of IL-4, GM-CSF, and IL-10. After 7 days of culture, the induced macrophages were further incubated with activated (*) or untreated purified CD4+ or CD8+ T cells for 3 days. Overlay diagrams show expression of indicated molecules after 1, 2, and 3 days of culture. *Thin histograms*: labeling with idiotype matched irrelevant mAb.

Incubation of CD8+ T Lymphocytes with IL-10-treated APCs Reverses the IL-10-induced Inhibition of the Stimulatory Capacity. In the next set of experiments, we analyzed the ability of T-cell stimulated APCs to induce proliferation of allogeneic cells in a MLR. As demonstrated in Fig. 3*A*, cells grown in the presence of IL-10,

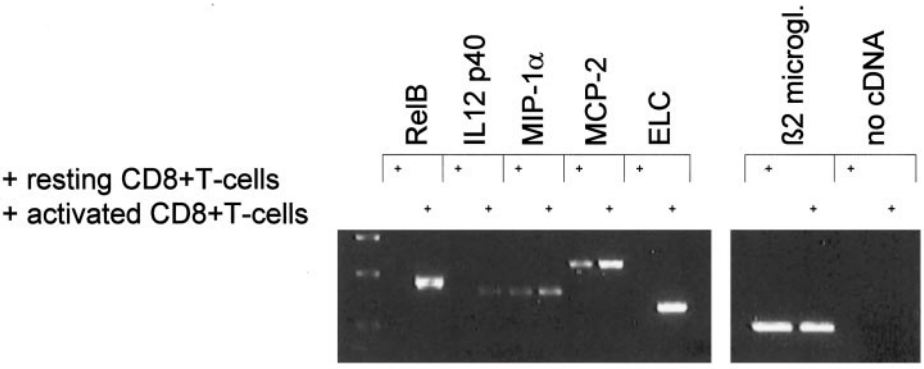

Fig. 2. Analysis of RelB, IL-12(p40), and chemokine expression by IL-10-treated macrophages after incubation with CD8+ T cells using RT-PCR. Monocytes were isolated from PBMNC and cultured in the presence of GM-CSF/IL-4/IL-10. After 7 days of culture, these cells were incubated with activate or untreated-irradiated purified CD8+ T lymphocytes. After 3 days of culture, total RNA was isolated from the cells, and analysis of RelB, IL-12 p40, MIP-1α, MCP-2, ELC, and β2 microglobulin was determined using RT-PCR. PCR products were run on a 3% agarose gel and visualized by ethidium bromide staining. Samples containing no cDNA were used as negative control.

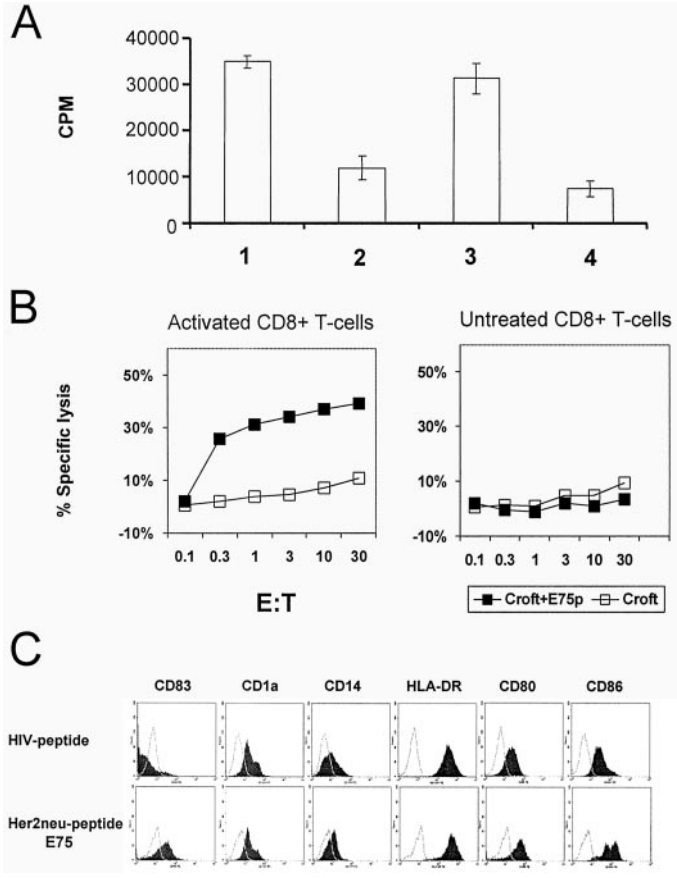

Fig. 3. Activated CD8+ T cells restore the stimulatory capacity of DCs derived from IL-10-treated macrophages. Monocytes were isolated from PBMNC by adherence and cultured in the presence of IL-4, GM-CSF, and IL-10 for 7 days. Afterward, the stimulatory ability of the cells was determined in a MLR. *A*, the induced macrophages were further incubated with irradiated-activated (3) or -untreated (2) purified CD8+ T cells for 3 days. Afterward, the stimulatory ability of the cells was determined in a MLR. The experiments were performed in triplicates, and the results represent mean ± SD. To analyze the effect of activated CD8+ cells on the observed activation of allogeneic PBMNC, these cells were included in the experiments and used as stimulator cells (4). Immature DCs (1) were used as a positive control. *B*, induction of CTL responses by peptide-pulsed APCs. Monocytes from an HLA-A2+ donor were cultured with GM-CSF/IL-4/IL-10 for 7 days and used as APCs after pulsing with the E75 peptide and incubation with resting or activated CD8+ T lymphocytes as indicated. After 24 h, an HLA-A2-binding peptide (E75) derived from Her-2/neu tumor antigen and autologous PBMNC was added to the cultures. After two restimulations, the cytotoxic activity of induced CTL was determined in a standard ^{51}Cr-release assay using Croft cells as targets pulsed for 2 h with 25 μg of the cognate E75 (■) or irrelevant HIV peptide (□). *C*, cognate antigen-dependent and -independent stimulation. HLA-A2+ monocytes were isolated from PBMNC by adherence and cultured in the presence of IL-4, GM-CSF, and IL-10. After 7 days of culture, the induced macrophages were pulsed with the cognate Her2/neu peptide (E75) or with irrelevant HIV peptide and incubated with CD8+ Her2/neu (E75)-specific CTL.

GM-CSF, and IL-4 have a reduced ability to stimulate allogeneic PBMNC. The stimulatory capacity of the IL-10-pretreated APCs increased upon interaction with activated CD8+ T lymphocytes and was similar to cells generated in the presence of GM-CSF and IL-4 without IL-10 (immature DCs). We also included activated irradiated CD8+ T cells as stimulator cells to determine the contribution of these cells to the proliferation results.

To analyze the ability of these APC populations to induce primary CTL responses against a tumor associated antigen, cells were pulsed with the HLA-A2-binding E75 peptide derived from the Her-2/neu antigen (14). As demonstrated in Fig. 3*B*, monocytes cultured with IL-10, GM-CSF, and IL-4 for 7 days and incubated with irradiated untreated CD8+ T lymphocytes were not able to elicit cytotoxic T cells responses after several restimulations. However, when these cells were cultured with activated CD8+ T lymphocytes, peptide-specific cytotoxic activity was detected in a standard ^{51}Cr-release assay against target cells pulsed with the antigenic peptide.

This functional analysis demonstrated that the interaction of CD8+ lymphocytes with APCs can overcome the IL-10-induced suppression of antigen-presenting function of DCs.

CD4+ Th cells can productively interact with APCs in a cognate interaction. The importance of direct cognate regulation of APC function was described for minor histocompatibility antigens such as HY antigens and cross-priming of CTL (1, 2, 15).

To test for cognate interaction in the next experiments, we used HLA-A2+ macrophages, grown with IL-10, GM-CSF, and IL-4 for 7 days. They were pulsed with Her2/neu peptide E75 or irrelevant HIV peptide and incubated with antigen-specific CD8+ CTL for 3 days (Fig. 3*C*).

Upon coculture with peptide-specific CTLs, macrophages differentiated into cells with DC phenotype, independent of peptide antigen used in the experiments. However, CD83 expression, a molecule up-regulated upon maturation of DCs, was only found on APCs presenting the CTL-specific antigen, thus indicating a more potent induction of APC function when cognate antigen-specific interaction is engaged.

Blocking Studies. To define the possible role of molecules involved in the cross-talk between DCs and CD8+ T cells, we used mAbs specific for CD154 and CD137 as recently described (16) and soluble recombinant proteins that were shown to block the interaction between CD40/CD40L, RANK/RANKL, and 4-1BB/4-1BBL (17, 18). However, the use of these compounds had no effect on the phenotype of the cells as compared with the controls (data not shown).

We further performed transwell experiments to analyze if soluble factors may contribute to our observations. The phenotype of the APC in the transwell experiments was similar to control cells, thus suggesting that cytokines might mediate the effects of activated T cells. We therefore repeated these experiments using blocking antibodies

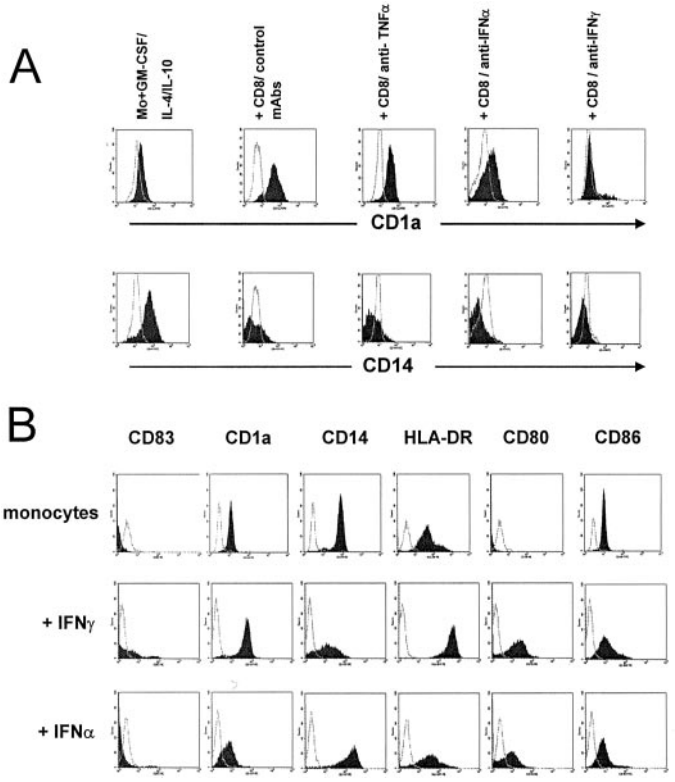

Fig. 4. Transwell experiments and blocking experiments. Monocytes were isolated from PBMNC by adherence and cultured in the presence of IL-4, GM-CSF, and IL-10. After 7 days of culture, the induced macrophages were further incubated with or without activated purified CD8+ T cells for 3 days in 24-well tissue culture plates with cell culture inserts (*A*). For blocking experiments, mAbs specific for the indicated cytokines and corresponding receptors were added to the cultures in the bottom of the wells before adding the T cells into the inserts. To determine the effect of sole IFN-γ and IFN-α on DC differentiation, monocytes were isolated from PBMNC using MACS technology and cultured in the presence of IFN-γ or IFN-α. After 3 days, the phenotype of the cells was analyzed by flow cytometry (*B*).

against TNF-α, IFN-α, and IFN-γ and their corresponding receptors because these cytokines were demonstrated to exert stimulatory effects on generation and maturation of DCs (19). As shown in Fig. 4*A*, blocking of TNF-α and its receptors had no effect on the phenotype of the cells in the transwell experiments. The addition of neutralizing mAbs specific for IFN-α and its receptor chain 1 and 2 resulted in the decreased induction of CD1a on the cell surface, thus being consistent with previous data (20). Blocking the action of IFN-γ almost completely abolished the CD1a expression on these cells. However, it had no effect on the down-regulation of CD14, suggesting that other factors may also contribute to this phenomenon. Adding sole IFN-α or IFN-γ to purified monocytes (Fig. 4*B*) confirmed the data obtained from blocking studies. IFN-γ induced a DC-like phenotype, whereas IFN-α had no significant effects.

Our results demonstrate that CD8+ T cells can productively interact with APCs, induce their activation and differentiation, and that this interaction does not have to be cognate. As a result of this cross-talk, CD8+ T cells can solicit their own help for the induction of antigen-specific CTL responses. Using our *in vitro* system of CD8+ T-cell/APC interaction, we were able to show that soluble factors like IFN-γ rather than receptor/ligand interactions are critical for the Th function of CD8+ T lymphocytes.

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