

# Tumor Necrosis Factor $\alpha$ and CD40 Ligand Antagonize the Inhibitory Effects of Interleukin 10 on T-Cell Stimulatory Capacity of Dendritic Cells<sup>1</sup>

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## ABSTRACT

Interleukin (IL)-10 secretion by tumor cells was demonstrated to be one of the mechanisms by which tumor cells can escape immunological recognition and destruction. In dendritic cells (DCs), which are currently used for vaccination therapies for malignant diseases, IL-10 inhibits IL-12 production and induces a state of antigen-specific anergy in CD4- and CD8-positive T cells. We therefore analyzed the effects of different activation stimuli including lipopolysaccharide (LPS), tumor necrosis factor (TNF)- $\alpha$ , and CD40 ligation on IL-10 mediated inhibition of DC development and stimulatory capacity. In our study, the addition of IL-10 to the cultures containing granulocyte/macrophage-colony stimulating factor and IL-4 with or without LPS completely inhibited the generation of DCs from peripheral blood monocytes. These cells remained CD14 positive and expressed high levels of IL-10 receptor (IL-10R), suggesting that IL-10 mediates its effects by up-regulating the IL-10R. In contrast, the simultaneous incubation of monocytes with IL-10 and TNF- $\alpha$  or soluble CD40 ligand (sCD40L) resulted in the generation of CD83-positive DCs, induction of nuclear localized RelB, and inhibition of IL-10R up-regulation. DCs grown in the presence of IL-10 and TNF- $\alpha$  or sCD40L elicited efficient CTL responses against viral and tumor-associated peptide antigens, which, however, were reduced as compared with DC cultures generated without IL-10. IL-10 decreased the production of IL-6 and the expression of IL-12 in the presence of TNF- $\alpha$  or sCD40L, but it had no effect on IL-15, IL-18, and TNF- $\alpha$  secretion. Our results show that TNF- $\alpha$  or CD40 ligation can antagonize the IL-10-mediated inhibition on DC function, suggesting that depending on activation stimuli, the presence of IL-10 does not necessarily result in T-cell anergy.

## INTRODUCTION

DCs<sup>3</sup> are the most potent APCs with the unique ability to initiate and maintain primary immune responses when pulsed with antigens (1–8). They originate from the bone marrow, and their precursors migrate via the blood stream to almost all organs, where they can be found in an immature state characterized by a high rate of antigen uptake. Upon stimulation with bacterial products, cytokines, or CD40 ligation, DCs undergo characteristic modulations of the phenotype, antigen-presenting function, and the ability to migrate to the secondary lymphoid organs. These mature DCs express high levels of co-stimulatory and MHC molecules and are regarded as the initiators of primary immune responses. *In vitro* DCs can be generated from human CD34+ bone marrow and peripheral blood progenitor cells

after culture with different cytokine combinations. Alternatively, they also develop from peripheral blood CD14+ monocytes when grown in the presence of GM-CSF and IL-4. These cells have the characteristics of immature DCs and can be further induced to mature by inflammatory stimuli like TNF- $\alpha$ , IL-1, LPS, CD40 ligation, or by monocyte-conditioned medium (3, 9–17).

The capacity of monocytes and DCs to migrate to the sites of inflammation, where they capture the antigens, and subsequently to the local lymph nodes is regulated by the expression of different chemokines and chemokine receptors (18–24). Immature DCs and monocytes produce inflammatory chemokines including MIP-1 $\alpha$ , MCP-1, and MCP-2 and express receptors for inflammatory chemokines such as CCR1, CCR2, CCR5, CCR6, and CXCR1. In contrast, during the maturation process, DCs down-regulate the expression of inflammatory chemokines and their receptors and up-regulate constitutive chemokines such as TARC, ELC, and the CCR7 receptor.

Recently, it was demonstrated that the function of DCs can be negatively affected by IL-10 (25–32). IL-10 is an immunosuppressive and anti-inflammatory cytokine that plays a major role in T helper cell regulation by down-regulation of Th1 responses (33). The biological functions of IL-10 are mediated by its cell surface receptor (IL-10R), which belongs to the IFN receptor-like subgroup of the cytokine receptor family (34, 35). *In vitro*, it was demonstrated that IL-10 can inhibit the cytokine production and antigen-presenting function of DCs, monocytes, and macrophages (27, 36, 37). In DCs, generated from peripheral blood monocytes using GM-CSF and IL-4, IL-10 inhibits IL-12 production and induces a state of antigen-specific anergy in CD4- and CD8-positive T cells. However, these inhibitory effects of IL-10 on DC function are only observed in immature DCs, whereas mature DCs are completely resistant to the action of IL-10.

Interestingly, IL-10 was also shown to be produced and secreted by tumor cells and may therefore be involved in the defective DC function observed in cancer patients. The production of IL-10 by tumor cells was demonstrated to be one of the mechanisms by which tumor cells can escape from immunological recognition and destruction (38–40).

In the present study, we analyzed the effect of TNF- $\alpha$  and sCD40L on the IL-10-mediated inhibition of DC development and function by adding these cytokines and sCD40L on the first day of the DC culture initiation. We demonstrate that the addition of TNF- $\alpha$  or sCD40L together with IL-10 to the cultures promoted the differentiation of mature DCs from peripheral blood monocytes expressing CD83 and RelB, despite the presence of IL-10. DCs grown in the presence of GM-CSF, IL-4, IL-10, and TNF- $\alpha$  or sCD40L efficiently induced antigen-specific CTL responses and stimulated allogeneic T cells, although at a reduced capacity as compared with cultures treated without IL-10. Our data suggest that: (a) TNF- $\alpha$  or sCD40L can antagonize the inhibitory effect of IL-10 on DC development and function; (b) DCs generated in the presence of IL-10 and TNF- $\alpha$  or sCD40L have an intermediate activation status; and (c) IL-10 differentially regulates the function of DCs, depending on external stimuli.

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<sup>3</sup> The abbreviations used are: DC, dendritic cell; IL, interleukin; IL-10R, IL-10 receptor; TNF, tumor necrosis factor; LPS, lipopolysaccharide; sCD40L, soluble CD40 ligand; GM-CSF, granulocyte/macrophage-colony stimulating factor; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; IMP, influenza matrix protein; RT-PCR, reverse transcription-PCR; MLR, mixed lymphocyte reaction; APC, antigen-presenting cell; TARC, thymus and activation-regulated chemokine; NK, natural killer.

## MATERIALS AND METHODS

**Cell Isolation and Cultures.** PBMNCs were isolated by Ficoll/Paque (Life Technologies, Inc., Grand Island, NY) density gradient centrifugation of heparinized blood obtained from buffy coat of healthy volunteers from the blood bank of the University of Tübingen. CD14<sup>+</sup> cells were purified using MACS isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the cells used in the experiments was 96–99%, as analyzed by flow cytometry. Alternatively, isolated PBMNCs were plated ( $1 \times 10^7$  cells/3 ml per well) into six-well plates (Costar, Cambridge, MA) in RP10 medium (RPMI 1640 supplemented with 10%-heat inactivated FCS, 2 mM L-glutamine, 50  $\mu$ M 2-mercaptoethanol, and antibiotics). After 2 h of incubation at 37°C, nonadherent cells were removed, and the adherent cells (12–19% of the incubated cells) were cultured in RP10 medium supplemented with various combinations of cytokines for 7 days. The population of adherent cells remaining in the wells comprised of 94–97% CD14-positive cells, 2–6% CD3-positive cells, and 0–2% CD19-positive cells. The percentage of CD1a- or CD83-positive cells was <1% (14).

The following cytokines were obtained from R&D systems (Wiesbaden, Germany): IL-4 (1000 IU/ml), TNF- $\alpha$  (10 ng/ml), and IL-10 (10 ng/ml). Human recombinant GM-CSF (Leucomax; 100 ng/ml) was from Novartis (Basel, Switzerland), and LPS was obtained from Sigma (10  $\mu$ g/ml).

For stimulation of adherent cells with CD40L, we used a soluble recombinant human trimeric CD40 ligand (Biozol, Eiching, Germany; 500  $\mu$ g/ml). The cultures were fed with fresh medium and cytokines every 2–3 days, and cell differentiation was monitored by light microscopy. The antigen-presenting capacity and expression of cell surface molecules were analyzed after 7 days of culture.

**Immunostaining.** Cell staining was performed using FITC- or phycoerythrin-conjugated mouse mAbs against CD86 and CD40 (all purchased from PharMingen, Hamburg, Germany); CD3, CD19, CD20, CD80, HLA DR, CD54, and CD14 (Becton Dickinson, Heidelberg, Germany); CD83 (Coulter-Immunotech Diagnostics, Hamburg, Germany); CD1a (OKT6, Ortho Diagnostic Systems); and T6-RD1 (Coulter Immunology, Hialeah, FL), and mouse IgG isotype controls. For the detection of the IL-10R expression, we used an unconjugated mouse mAb (R&D systems, Wiesbaden, Germany) and stained the cells afterward with a FITC-labeled goat-antimouse mAb (Becton Dickinson). Samples were analyzed on a FACScan Calibur (Becton Dickinson).

**Cytokine Determination.** Cytokine concentrations in cell cultures supernatants were measured by commercially available two-site sandwich ELISAs from R&D systems (Wiesbaden, Germany; IL-15 and IL-18) or Coulter-Immunotech Diagnostics (Hamburg, Germany; IL-12, IL-10, IL-6, and TNF- $\alpha$ ), according to the manufacturer's instructions.

**MLR Assay.**  $10^5$  responding cells, either from allogeneic or autologous PBMNCs, were cultured in 96-well flat-bottomed microplates (Nunc) with various numbers of irradiated stimulator cells. To use the same percentage of DCs in the assays, the number of added DCs was assessed based on fluorescence-activated cell sorter data (cells expressing CD1a and/or CD83 on the cell surface) and confirmed by counting of the cells after staining with trypan blue under a light microscope. Thymidine incorporation was measured on day 5 by a 16-h pulse with [ $^3$ H]thymidine (0.5  $\mu$ Ci/well; Amersham Life Science, Buckingham, United Kingdom).

**Induction of Antigen-specific CTL Response Using an HLA-A2-restricted Synthetic Peptide.** The IMP 58–66, GILGFVFTL, pol HIV-1 reverse transcriptase peptide (HIV) 476–484, ILKEPVHGV, and Her-2/neu-derived E75 peptide KIGSFLAFL (42) were synthesized using standard Fmoc chemistry on a peptide synthesizer (432A; Applied Biosystems, Weiterstadt, Germany) and analyzed by reverse phase high-performance liquid chromatography and mass spectrometry. For CTL induction,  $5 \times 10^5$  DCs were pulsed with 50  $\mu$ g/ml of the synthetic IMP peptide for 2 h, washed, and incubated with  $2.5 \times 10^6$  autologous PBMNCs in RP10 medium. Cells were restimulated after 7 days of culture, and 1 ng/ml human recombinant IL-2 (R & D Systems, Wiesbaden, Germany) were added every other day (41). The cytolytic activity of induced CTLs was analyzed on day 5 after the last restimulation in a standard  $^{51}$ Cr-release assay.

**CTL Assay.** The standard  $^{51}$ Cr-release assay was performed with some modifications as described (42). Target cells (T2 cells, 174xCEM.T2 hybridoma, TAP1- and TAP2-deficient), A498 (renal cell carcinoma, HLA-A2+, Her-2/neu+), K562 (no MHC expression, sensitive to NK cell-mediated lysis),

and SK-OV-3 (ovarian cancer, HLA-A2 negative, Her-2/neu+) were pulsed with 25  $\mu$ g/ml peptide for 2 h or left unpulsed and labeled with [ $^{51}$ Cr]sodium chromate in RP10 for 1 h at 37°C. Cells ( $10^4$ ) were transferred to a well of a round-bottomed, 96-well plate. Varying numbers of CTLs were added to give a final volume of 200  $\mu$ l and incubated for 4 h at 37°C. At the end of the assay, supernatants (50  $\mu$ l/well) were harvested and counted in a microbeta counter (Wallac). The percentage of specific lysis was calculated as:  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$ . Spontaneous and maximal release were determined in the presence of either medium or 1% Triton X-100, respectively.

**Preparation of Nuclear Extracts.** Nuclear extracts were prepared from DCs as described previously (43). Briefly, cell pellets were washed in 1 ml of ice-cold Buffer A [10 mM HEPES (pH 7.9), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT], incubated for 10 min on ice in 1 ml Buffer A + 0.4% Igepal CA-630 (Sigma, Munich, Germany). Cell membranes thus obtained were centrifuged at  $750 \times g$  for 5 min. Pellets were resuspended in 200  $\mu$ l Buffer B [20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT] and nuclei were mechanically lysed for 2 h at 4°C. Cell debris were pelleted 15 min at  $7500 \times g$ , and supernatant was recovered and stored at  $-70^\circ\text{C}$  until use. Proteinase inhibitors (aprotinin and leupeptin; Sigma, Munich, Germany) were added to buffers just before use.

**PAGE and Western Blotting for Detection of RelB Protein.** Protein concentration of nuclear extracts were determined using a BCA assay (Pierce, Rockford, IL). Twenty  $\mu$ g of total protein were separated on 12% polyacrylic amide gel, blotted on polyvinylidene difluoride membrane, and probed with a polyclonal rat RelB antibody C-19 (Santa Cruz, CA); bands were visualized by ECL staining (Amersham Pharmacia, Freiburg, Germany).

**RT-PCR.** RT-PCR was performed with some modifications as described recently (44). 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. Five hundred ng of total RNA were subjected to first-strand cDNA using an optimized protocol described by Life Technologies, Inc. (SuperScript Preamplification System; Life Technologies, Inc., Eggenstein, Germany), using oligo(dT) as primer. Two  $\mu$ l of cDNA obtained from the reverse transcriptase reaction were subjected to the amplification. To control the integrity of the isolated RNA, 1  $\mu$ l of cDNA was amplified by an intron-spanning primer pair for the 18S rRNA gene. Primer sequences were deduced from published cDNA sequences: 28S rRNA, 5'-ACTTAGAGGCGTTCAGTCATAATC-3' and 5'-AGACAGGTTAGTTT-TACCCTACTG; MCP-4, 5'-AAATGACTTTTCCATTCTCCTCTG-3' and 5'-TGCAATTCATCTTTCCACAATAAAC-3'; and CCR7, 5'-TGAGCTCAG-TAAGCAACTCAACAT-3' and 5'-TAGCTTATCAGCCCTGTCTTTTC-3'. Primer sequences for IL-12 and DC-CK1 (14), MIP-1 $\alpha$ , MCP-2, ELC, TARC (18), and RelB were published recently. Reactions were amplified in a DNA thermal cycler (GeneAmp PCR System 2400; Perkin-Elmer). The temperature profiles were as follows: 5 min at 94°C pretreatment; 60°C for 30 s annealing for the DC-CK1, MCP-4, CCR7, and IL-10R; and 55°C for the 28S rRNA primers; 72°C for 30 s synthesis; and 94°C for 30 s denaturation. Finally, a single posttreatment was performed at 72°C for 5 min. Ten  $\mu$ l of the RT-PCR products were electrophoresed through a 3% agarose gel and stained with ethidium bromide for visualization under UV light. Numbers of cycles performed: 15 for 28S rRNA; 30 for IL-12; 32 for RelB; 28 for DC-CK1; 30 for MIP-1 $\alpha$ ; 32 for MCP-2; 30 for MCP-4; 32 for ELC 32; 30 for CCR7 30; and 28 for TARC.

**Statistical Analysis.** Each experiment was performed at least three times. Representative experiments are shown. Student's *t* test was performed to evaluate the significance of the results.

## RESULTS

**The Inhibitory Effect of IL-10 on Differentiation of DCs from Peripheral Blood Monocytes in the Presence of GM-CSF and IL-4 Is Antagonized by TNF- $\alpha$  or CD40 Ligation but not by LPS.** Several recent reports demonstrated that IL-10 inhibits the antigen-presenting function of immature DCs, whereas mature DCs are resistant to the action of IL-10. To analyze the effect of IL-10 on development and function of DCs from peripheral blood monocytes at

the early time point, IL-10 was added from the initiation of the 7-day culture (day 0) together with GM-CSF and IL-4. To further evaluate the influence of maturation-inducing stimuli, LPS, recombinant human TNF- $\alpha$ , or the soluble trimeric human CD40L (sCD40L) were added together with the above-mentioned cytokines.

As demonstrated in Fig. 1, the addition of IL-10 to the cultures together with GM-CSF and IL-4 abolished the differentiation of monocytes to DCs, because the cells still expressed CD14 and were negative for CD1a and CD83 at day 7 of culture. Most of the cells remained adherent with a phenotype of macrophages. When TNF- $\alpha$  or the trimeric human sCD40L were added to the cultures together with GM-CSF, IL-4, and IL-10, the cells developed into DCs expressing high levels of CD83, HLA-DR, and costimulatory molecules, comparable with the phenotype of DCs generated in the absence of IL-10. Interestingly, in cultures incubated with LPS, IL-10 completely inhibited the generation of DCs. Similar results were obtained when peripheral blood monocytes were preincubated for 1, 2, 3, or 5 days with GM-CSF and IL-4 and IL-10, LPS, or TNF- $\alpha$  or sCD40L were added for the remaining 6, 5, 4, or 2 days of culture, respectively (data not shown). The inhibitory effect of IL-10 on DC development was prevented by the simultaneous addition of a neutralizing anti-IL-10 antibody (20  $\mu$ g/ml; R&D Systems, Wiesbaden, Germany) to the culture medium (data not shown).

Sequential analysis of the cell cultures treated with IL-10 on day 0 demonstrated that the effect of TNF- $\alpha$  and sCD40L on induction of CD83 expression and up-regulation of costimulatory molecules was already detectable after 2–3 days of culture (data not shown).

**The IL-10 Induced Up-Regulation of IL-10R Expression Is Inhibited by TNF- $\alpha$  or sCD40L.** We used a monoclonal antibody specific for the human IL-10R to analyze its expression on cells generated from peripheral blood monocytes. As shown in Fig. 1, immature DCs generated in the presence of GM-CSF and IL-4 expressed low levels of IL-10R on the cell surface, whereas mature DCs cultured with sCD40L or TNF- $\alpha$  expressed no IL-10R. The addition of IL-10 to the cultures containing GM-CSF and IL-4 resulted in a marked increase of the IL-10R expression, thus suggesting that IL-10 mediates its inhibitory effects by up-regulation of its own receptor. The presence of CD40L or TNF- $\alpha$  inhibited the up-regulation of the IL-10R on the cell surface by IL-10, which resulted in the development of DCs with a mature phenotype. In contrast, addition of LPS to IL-10-treated cultures could not prevent the IL-10R up-regulation (Fig. 1).

**RelB Expression in Cells Generated from Peripheral Blood Monocytes.** RelB belongs to the family of transcription factors associated with the development and effective antigen presentation of DCs (45–48). We therefore analyzed RelB expression in different cell populations generated *in vitro* from peripheral blood monocytes by RT-PCR and Western blot. The RelB mRNA (Fig. 2) and nuclear localized protein expression in DCs (Fig. 3) were increased by incubation of the adherent cells with sCD40L, LPS, or TNF- $\alpha$ , whereas no RelB expression was detected in cultures grown in the presence of GM-CSF and IL-4 with or without IL-10. The up-regulation of RelB was not inhibited by the addition of IL-10 in the presence of sCD40L or TNF- $\alpha$ , in contrast to the cells generated with GM-CSF, IL-4, and

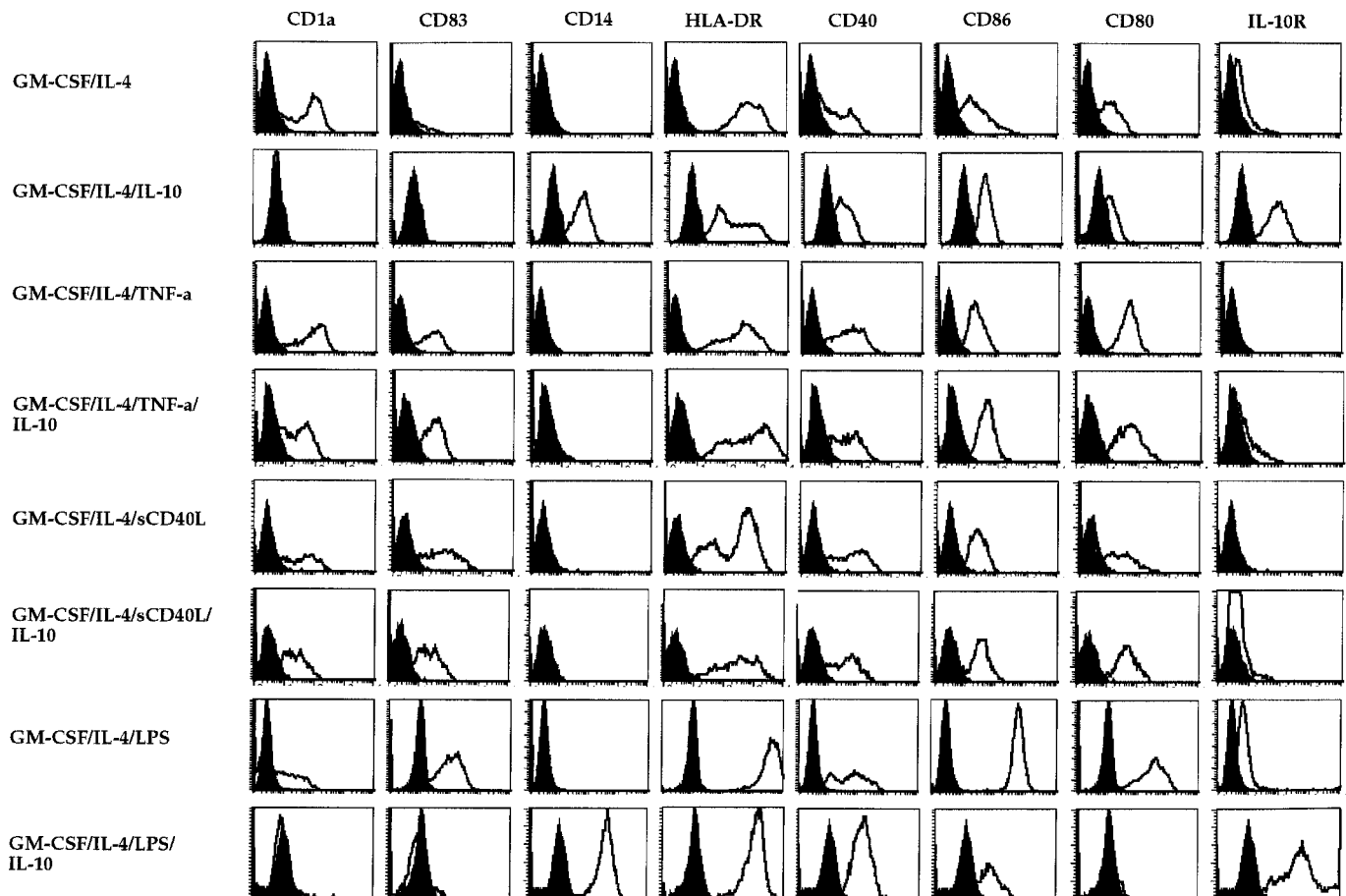


Fig. 1. Phenotypic analysis of *in vitro*-generated DCs. PBMCs were cultured in the presence of LPS, sCD40L, TNF- $\alpha$ , and different cytokines as indicated. Overlay diagrams show expression of indicated molecules after 7 days of culture. Solid histograms, labeling with idiotype matched irrelevant mAb.



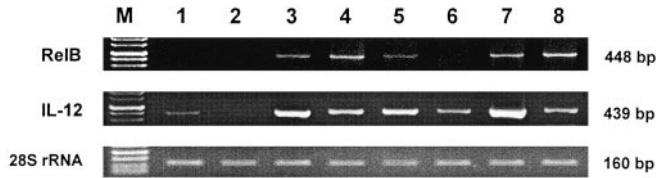


Fig. 2. Analysis of RelB and IL-12 (p40) mRNA expression by *in vitro*-generated cell populations using RT-PCR. PBMNCs were cultured with various combinations of cytokines for 7 days (Lane 1, GM-CSF/IL-4; Lane 2, GM-CSF/IL-4/IL-10; Lane 3, GM-CSF/IL-4/TNF- $\alpha$ ; Lane 4, GM-CSF/IL-4/TNF- $\alpha$ /IL-10; Lane 5, GM-CSF/IL-4/LPS; Lane 6, GM-CSF/IL-4/LPS/IL-10; Lane 7, GM-CSF/IL-4/sCD40L; Lane 8, GM-CSF/IL-4/sCD40L/IL-10). PCR products were run on a 3% agarose gel and visualized by ethidium bromide staining. Samples containing no cDNA were used as negative control.

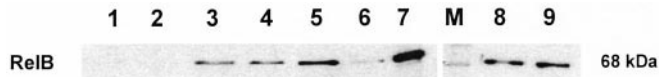


Fig. 3. Expression of nuclear localized RelB protein in cells generated from peripheral blood monocytes. Lane 1, GM-CSF/IL-4; Lane 2, GM-CSF/IL-4/IL-10; Lane 3, GM-CSF/IL-4/TNF- $\alpha$ ; Lane 4, GM-CSF/IL-4/TNF- $\alpha$ /IL-10; Lane 5, GM-CSF/IL-4/LPS; Lane 6, GM-CSF/IL-4/LPS/IL-10; Lane 7, positive control consisting of DCs generated with GM-CSF and IL-4 for 7 days and stimulated with TNF- $\alpha$  and prostaglandin; Lane 8, GM-CSF/IL-4/sCD40L; Lane 9, GM-CSF/IL-4/sCD40L/IL-10.

LPS. These data are in line with the results obtained from the set of previous experiments (Fig. 1), thus confirming the important role of RelB induction for DC development and demonstrating that the inhibitory effects mediated by IL-10 can be antagonized by TNF- $\alpha$  or sCD40L but not by LPS, and that bacterial and T-cell induced activation of DC follows distinct pathways.

**IL-10 Modulates the Stimulation of Allogeneic and Antigen-specific T Cells by DCs Generated from CD14<sup>+</sup> Monocytes in the Presence of TNF- $\alpha$  or sCD40L.** To analyze the ability of IL-10-treated DC populations to stimulate alloreactive T cells in a MLR, cells generated from monocytes using different stimuli as indicated were incubated with allogeneic PBMNCs and harvested after 5 days. As demonstrated in Table 1, the cells grown in the presence of IL-10, GM-CSF, and IL-4 with or without LPS have a reduced ability to stimulate allogeneic PBMNCs, whereas the IL-10 treatment had only a moderate inhibitory effect on the stimulatory capacity of the mature DCs generated with TNF- $\alpha$  or CD40L to trigger the proliferation of allogeneic PBMNCs.

Different cell populations generated from adherent HLA-A2-positive PBMNCs in the presence of IL-10 were pulsed with a synthetic HLA-A2-restricted peptide derived from the IMP and used as APCs to induce an antigen-specific CTL response *in vitro*. As shown in Fig. 4, CTL lines obtained after two weekly restimulations demonstrated high peptide-specific killing when DCs were grown in the presence of TNF- $\alpha$ , LPS, or soluble CD40L. The *in vitro* induced T cells only elicited a cytotoxic response against targets coated with the cognate HLA-A2 binding peptide derived from IMP, but they did not recognize targets coated with an irrelevant HIV-peptide. IL-10 completely inhibited the stimulatory capacity of cells generated in cultures containing GM-CSF and IL-4 (Fig. 4A). In contrast, DCs grown in cultures supplemented with IL-10 and TNF- $\alpha$  or soluble CD40L induced an efficient peptide-specific CTL response, which, however, was reduced as compared with cells generated without IL-10 (Fig. 4, B and C). CTLs induced with cells generated in presence of IL-10 and LPS did not induce any measurable antigen-specific cytotoxic activity, confirming again that LPS in contrast to TNF- $\alpha$  or sCD40L cannot antagonize the inhibitory effects of IL-10 (Fig. 4D).

To analyze the ability of DCs generated in the presence of IL-10 and TNF- $\alpha$  (Fig. 5, C and D) or soluble CD40L (Fig. 5, E and F) to induce a primary CTL response against a tumor-associated antigen,

cells were pulsed with the E75 peptide derived from the Her-2/neu antigen (42). As demonstrated in Fig. 5, the CTLs recognized target cells pulsed with the E75 peptide in an antigen-specific manner. Furthermore, the E75-specific CTLs efficiently lysed tumor cells expressing Her-2/neu (A498 cell line), whereas they did not recognize Her-2/neu+/HLA-A2 cells (SK-OV-3 cells) or the NK cell-sensitive K562 tumor cells, confirming that the cytotoxic activity of these CTLs is antigen specific and HLA-restricted and demonstrating that TNF- $\alpha$  or soluble CD40L can prevent the recently described IL-10-mediated inhibition of antigen-specific CTL responses. However, in line with

Table 1 Stimulatory capacity of mononuclear cells cultured with various cytokine combinations

Cytokines <sup>a</sup>	Allogeneic MLR <sup>b</sup>		
	10 <sup>3</sup> DCs	5 × 10 <sup>2</sup> DCs	10 <sup>2</sup> DCs
GM-CSF/IL-4	15931 ± 596	12706 ± 395	8053 ± 213
GM-CSF/IL-4/IL-10	4821 ± 1261	2275 ± 679	1106 ± 528
GM-CSF/IL-4/TNF- $\alpha$	26409 ± 4388	19557 ± 220	14854 ± 297
GM-CSF/IL-4/TNF- $\alpha$ /IL-10	19255 ± 591	12412 ± 550	10373 ± 2528
GM-CSF/IL-4/sCD40L	34812 ± 4223	30569 ± 2485	22378 ± 2610
GM-CSF/IL-4/sCD40L/IL-10	27780 ± 1939	19514 ± 1355	16451 ± 4145
GM-CSF/IL-4/LPS	42780 ± 1039	39674 ± 1005	29463 ± 3258
GM-CSF/IL-4/LPS/IL-10	6758 ± 1980	4512 ± 1554	1471 ± 443

<sup>a</sup> PBMNCs were cultured with the indicated combinations of cytokines for 7 days.

<sup>b</sup> 10<sup>5</sup> responding cells from allogeneic PBMNCs were cultured with irradiated stimulator cells (DCs or PBMNCs). Thymidine incorporation was measured on day 5 by a 16-h pulse with [<sup>3</sup>H]thymidine. The assay was conducted in triplicate, and results show the means and SDs in cpm of triplicates.

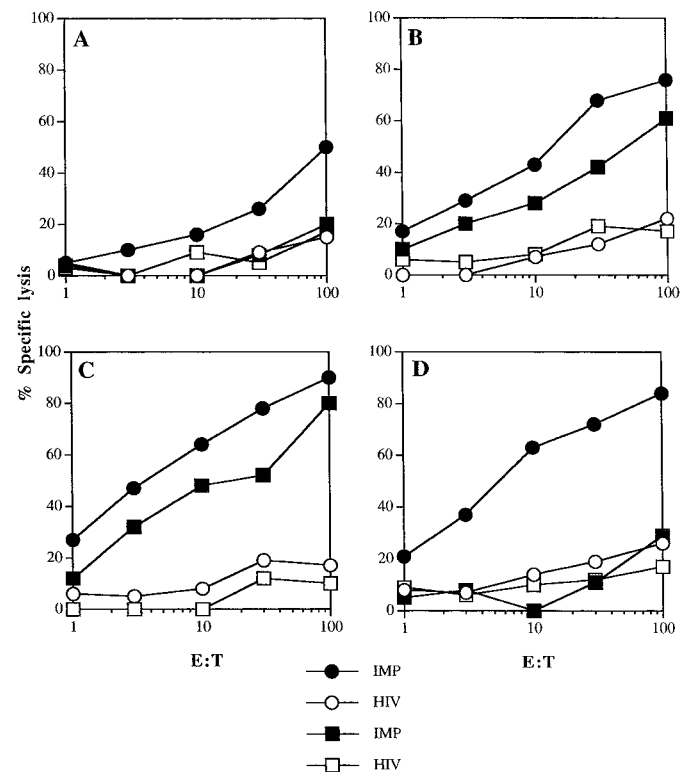


Fig. 4. Induction of CTL responses by peptide-pulsed DCs. PBMNCs from a HLA-A2-positive donor were cultured with the indicated combinations of cytokines for 7 days (A, GM-CSF/IL-4 and GM-CSF/IL-4/IL-10; B, GM-CSF/IL-4/TNF- $\alpha$  and GM-CSF/IL-4/TNF- $\alpha$ /IL-10; C, GM-CSF/IL-4/sCD40L and GM-CSF/IL-4/sCD40L/IL-10; D, GM-CSF/IL-4/LPS and GM-CSF/IL-4/LPS/IL-10). Squares, IL-10-treated cultures; circles, cultures without IL-10. The *in vitro*-generated cell populations were pulsed with the synthetic peptide derived from the IMP and used as APCs to induce a MHC class I-restricted CTL response *in vitro*. The cytotoxic activity of induced CTLs was determined after two restimulations in a standard <sup>51</sup>Cr-release assay using T-2 cells as targets pulsed for 2 h with 25  $\mu$ g of the cognate IMP (closed symbols) or irrelevant HIV peptide (open symbols).

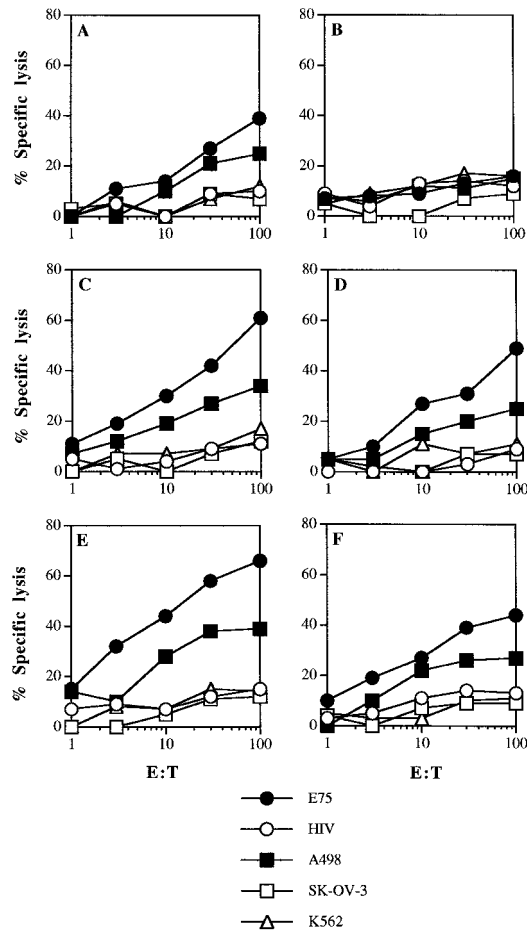


Fig. 5. Her-2/neu peptide-specific CTL responses induced with peptide-pulsed DCs. PBMCs from a HLA-A2-positive donor were cultured with the indicated combinations of cytokines for 7 days (A, GM-CSF/IL-4; B, GM-CSF/IL-4/IL-10; C, GM-CSF/IL-4/TNF- $\alpha$ ; D, GM-CSF/IL-4/TNF- $\alpha$ /IL-10; E, GM-CSF/IL-4/sCD40L; F, GM-CSF/IL-4/sCD40L/IL-10). The *in vitro*-generated cell populations were pulsed with the E75 synthetic peptide derived from Her-2/neu and used as APCs to induce a primary MHC class I-restricted CTL response *in vitro*. The cytotoxic activity of induced CTLs was determined after two restimulations in a standard  $^{51}\text{Cr}$ -release assay using different target cells: T-2 cells pulsed with the cognate E75 peptide (closed circles) or irrelevant HIV peptide (open circles), A498 tumor cells (renal cell carcinoma, HLA-A2+, Her-2/neu+; closed squares), SK-OV-3 (ovarian cancer, HLA-A2 negative, Her-2/neu+; open squares), and K562 (no MHC expression, sensitive to NK cell-mediated lysis; triangles).

the previous results, the cytotoxic activity of these antigen-specific CTLs was reduced as compared with the untreated DC cultures.

These results were not completely in line with the observations derived from the previous experiments. The *in vitro* generated mature DCs grown in the presence of TNF- $\alpha$  or CD40L with or without IL-10 seem to have a similar phenotype, but they differ in their T-cell stimulatory function. We therefore additionally analyzed cytokine production and expression of various chemokines by the different cell populations.

**Cytokine Production by Cultured Cells.** The T-cell stimulatory capacity of DCs is regulated by the expression of MHC, costimulatory and adhesion molecules on the cell surface, and the production of cytokines like IL-12, IL-15, IL-18, TNF- $\alpha$ , or IL-6 that are important for the induction of Th1 responses and proliferation of naive T-cells (14, 49–57). Cytokine secretion by generated cells *in vitro* was analyzed using commercially available two-site sandwich ELISAs. As shown in Table 2, IL-10 treatment of cell cultures containing GM-CSF and IL-4 resulted in decreased levels of cytokine production of the cells. However, when the cells were grown in the presence of sCD40L or TNF- $\alpha$  together with GM-CSF, IL-4, and IL-10, the

supernatants contained similar levels of IL-15, IL-18, and TNF- $\alpha$  as compared with the cultures without IL-10. In contrast to these results, sCD40L or TNF- $\alpha$  could not overcome the inhibition of the IL-6 by IL-10. Analysis of IL-12 (p40) expression using RT-PCR (Fig. 2) demonstrated that IL-10 down-regulated the IL-12 expression in all cell populations, even in the presence of sCD40L or TNF- $\alpha$ , similar to a previous report where IL-10 inhibited IL-12 production of CD40 ligand-activated immature DCs (27). In line with these RT-PCR results, the presence of IL-10 inhibited completely the IL-12 production (p70 heterodimer) in the cultures independent of the stimuli used for activation. sCD40L or TNF- $\alpha$  lead to very low levels of bioactive IL-12 secretion, consistent with previous reports (58, 59). Higher levels of IL-12 were detectable after LPS stimulation.

**Expression of Chemokines.** DCs express a broad spectrum of inflammatory (MIP-1 $\alpha$  and MCP-2) and constitutive chemokines like DC-CK1, TARC, and ELC (an EBI1-ligand chemokine) as well as the corresponding receptors that underline the ability of DCs to attract other cells as well as to regulate their own function and migratory capacity (18–24, 60).

As shown in Fig. 6 using RT-PCR, DC-CK1 mRNA was found as expected in immature DCs generated with GM-CSF and IL-4. As demonstrated above (Fig. 1), addition of IL-10 to these cultures

Table 2 Cytokine secretion by cells cultured with various cytokine combinations

Cytokines <sup>a</sup>	IL-18 (pg/ml)	TNF- $\alpha$ (pg/ml)	IL-15 (pg/ml)	IL-6 (pg/ml)	IL-12 (pg/ml)
GM-CSF/IL-4	25.7	110.5	2.5	79.6	2.4
GM-CSF/IL-4/IL-10	16.6	16.9	ND <sup>b</sup>	16.4	<0.1
GM-CSF/IL-4/TNF- $\alpha$	42.4	1954.5	6.8	529	28
GM-CSF/IL-4/TNF- $\alpha$ /IL-10	54.7	1979.1	9.1	166	0.2
GM-CSF/IL-4/sCD40L	68.7	1992	7.8	705.6	42
GM-CSF/IL-4/sCD40L/IL-10	61.7	1900.5	6.9	175.6	0.2
GM-CSF/IL-4/LPS	80.7	649.9	7.2	898.4	136
GM-CSF/IL-4/LPS/IL-10	24.4	60	ND	93.3	0.3

<sup>a</sup> PBMCs were cultured with the indicated combinations of cytokines for 7 days. The cytokine concentrations in the culture supernatants were analyzed on day 7 using a commercially available ELISA. The assay was conducted in duplicate, and results of a representative experiment are shown.

<sup>b</sup> ND, not detectable.

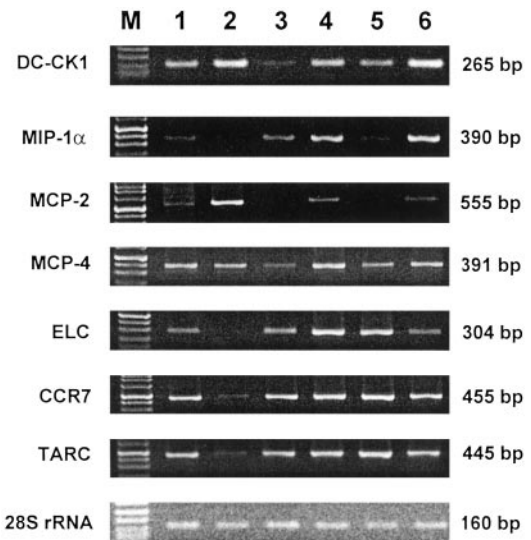


Fig. 6. Expression of chemokines and CCR7 by *in vitro*-generated DC populations. PBMCs were cultured with various combinations of cytokines for 7 days (Lane 1, GM-CSF/IL-4; Lane 2, GM-CSF/IL-4/IL-10; Lane 3, GM-CSF/IL-4/TNF- $\alpha$ ; Lane 4, GM-CSF/IL-4/TNF- $\alpha$ /IL-10; Lane 5, GM-CSF/IL-4/sCD40L; Lane 6, GM-CSF/IL-4/sCD40L/IL-10). Total RNA was isolated from generated cells, and DC-CK1, MIP-1 $\alpha$ , MCP-2, MCP-4, ELC, TARC, and CCR7 expression was examined by semiquantitative RT-PCR. PCR products were run on a 3% agarose gel and visualized by ethidium bromide staining.

prevented the generation of DCs from adherent monocytes, and the cells retained the monocyte/macrophage phenotype. Interestingly, these cells nevertheless expressed DC-CK1 (Fig. 6), in contrast to freshly purified peripheral blood monocytes (data not shown; Ref. 14). Incubation of the cells with CD40L or TNF- $\alpha$  resulted in down-regulation of the DC-CK1 mRNA in DCs. This effect of sCD40L and TNF- $\alpha$  was inhibited by addition of IL-10.

As demonstrated in Fig. 6, all DCs generated in the presence of sCD40L or TNF- $\alpha$  with or without IL-10 expressed ELC, TARC, and the CCR7 receptor. Interestingly, maturing DCs generated in the medium containing IL-10 expressed higher levels of the inflammatory chemokines MIP-1 $\alpha$  and MCP-2.

Using subtractive cDNA libraries generated from DCs and peripheral blood monocytes, we recently detected a selective MCP-4 mRNA (61) expression in DCs.<sup>4</sup> As demonstrated in Fig. 6, IL-10 up-regulated the expression of MCP-4 by DCs, even in the presence of sCD40L and TNF- $\alpha$ . We also found MCP-4 transcripts in cells incubated with GM-CSF, IL-4, and IL-10, whereas we were not able to detect MCP-4 transcripts in freshly isolated blood monocytes (data not shown).

## DISCUSSION

DCs are critical in the function of the immune system, for they are the primary APCs in the initiation of T-lymphocyte responses, and there are probably many different DC subpopulations that may have distinct effects on the generation of primary immune responses (1–3). Generation and maturation of DCs are regulated by various extracellular stimuli, including bacterial products, T-cell interaction, adhesion and costimulatory molecules, cytokines, and chemokines, and these events lead to phenotypical and functional changes.

Previous studies demonstrated that DCs can develop *in vitro* from CD14<sup>+</sup> blood monocytes cultured with GM-CSF and IL-4. These cells have the phenotypic and functional characteristics of immature DCs and can be further induced to mature by activation with TNF- $\alpha$ , by CD40 ligation, monocyte-conditioned medium, LPS, or IL-1 (10–14). Several recent reports demonstrated that IL-10 can inhibit the differentiation and function of immature DCs generated from peripheral blood monocytes, whereas mature DCs are resistant to its action (25–32). Monocytes are recognized as an alternative source of DC precursors. They migrate to the sites of inflammation attracted by cytokines and chemokines released. Upon contact with cytokines like GM-CSF, IL-4, TNF- $\alpha$ , LPS, T cells, or even IL-10, they may differentiate into DCs. Therefore, in contrast to the *in vitro* culture conditions, the differentiation of monocytes toward DCs *in vivo* is influenced by a combined action of different cytokines.

In our study, we therefore analyzed the combined influence of various stimuli like TNF- $\alpha$ , sCD40L, LPS, and IL-10 on the generation and function of DCs from adherent peripheral blood monocytes using GM-CSF and IL-4 by addition of these cytokines at the time of culture initiation. In line with previous results, we show that IL-10 prevents the differentiation of DCs when IL-10, GM-CSF, and IL-4 are added to the medium on the first day of the culture initiation. However, the simultaneous addition of TNF- $\alpha$  or soluble CD40 ligand, together with IL-10, resulted in generation of CD83-positive DCs that displayed the phenotype of mature DCs and expressed high levels of costimulatory and MHC molecules, suggesting that TNF- $\alpha$  or CD40 ligation can antagonize the inhibitory effect of IL-10 on DC development. Interestingly, LPS could not antagonize the inhibitory effects of IL-10 on DC development, suggesting that bacterial and T cell-induced activation of DCs follows different pathways.

The inhibitory effect on the DC generation correlated with the level of the IL-10R expression. Addition of IL-10 to the cultures containing GM-CSF and IL-4 with or without LPS resulted in the up-regulation of the IL-10R on the cell surface. These results might explain why mature DCs, in contrast to immature DCs, are resistant to the action of IL-10.

Our results are complementary to the recent report by Buelens *et al.* (27). Using immature DCs after a 7-day culture with GM-CSF and IL-4, they demonstrated that CD40 ligation can, in contrast to LPS, induce maturation of DCs in the presence of IL-10 characterized by expression of CD83 and up-regulation of costimulatory molecules.

In addition to the phenotypic analysis, we investigated the functional properties of the various cell populations generated with or without IL-10 in terms of RelB induction, cytokine production, expression of chemokines, and initiation of antigen-specific CTL responses.

The transcription factor RelB is a member of nuclear factor- $\kappa$ B/Rel family. In several reports, it was demonstrated that RelB is essential for the development of myeloid-related DCs and their antigen-presenting function (45–48). In line with the results from the phenotypic studies, IL-10 had no effect on mRNA or nuclear protein expression of RelB in the presence of TNF- $\alpha$  or sCD40L, whereas LPS failed to overcome the inhibition mediated by IL-10 (Fig. 3), confirming the important role of RelB expression for the development and function of DCs.

Functional analyses of the phenotypically mature DCs generated with TNF- $\alpha$  or sCD40L revealed that these cells were potent stimulatory cells, even in the presence of IL-10, and induced primary antigen-specific CTLs that were able to lyse tumor cells expressing the corresponding tumor-associated antigen, thus demonstrating that sCD40L or TNF- $\alpha$  can antagonize the suppression of antigen-specific T-cell responses mediated by IL-10. However, the addition of IL-10 to the cultures resulted in a reduced capacity of these DCs to stimulate allogeneic T cells in a MLR and to induce antigen-specific MHC class I-restricted cytotoxic T cells directed against viral and tumor-associated antigens as compared with cultures without IL-10 (Figs. 4 and 5; Table 1).

This observation is of special interest, because IL-10 has been shown to be produced and secreted by tumor cells and may therefore be involved in the defective DC function observed in cancer patients. Furthermore, the production of IL-10 by tumor cells was demonstrated to be one of the mechanisms by which tumor cells can escape from immunological recognition and destruction (38–40).

Analysis of cytokine secretion in the cell cultures demonstrated that DCs grown with IL-10 and TNF- $\alpha$  or soluble CD40L expressed lower levels of IL-6 and IL-12 as compared with cells generated without IL-10. The reduced capacity to secrete IL-6, a factor known to costimulate the proliferation of naive Th cells (57), and the reduced IL-12 expression may be one possible explanation for their reduced functional APC properties, because we could not detect significant differences in the production of IL-15, IL-18, or TNF- $\alpha$ , and other factors may also contribute to this phenomenon.

DCs generated *in vitro* using GM-CSF, IL-4, and TNF- $\alpha$  or sCD40L with or without IL-10 expressed TARC and ELC and the CCR7 receptor, which was shown to be important for the migration of DCs from tissues to the lymphatic vessels and nodes and initiation of T-cell activation (18, 22–24). The presence of IL-10 resulted in up-regulation of the inflammatory chemokines MIP-1 $\alpha$ , MCP-2, and MCP-4, which are responsible for attracting cells involved in inflammatory reactions and are produced at early time points of DC activation, suggesting that these DCs have a less mature phenotype, although they express CD83 and high levels of costimulatory molecules.

<sup>4</sup> Manuscript in preparation.



DC-CK-1 is a recently identified chemokine that is selectively expressed in DCs with preferential chemotactic activity for naive T cells (14, 58). Surprisingly, DC-CK1 mRNA expression was up-regulated in mature DCs treated with IL-10 and down-regulated in the absence of IL-10 (Fig. 6). In cells incubated with GM-CSF, IL-4, and IL-10, displaying the phenotype of monocytes/macrophages and lacking any stimulatory capacity as analyzed by MLR and CTL induction, a high level of DC-CK-1 expression was detected by RT-PCR. Expression of DC-CK-1 could not be found in purified, freshly isolated CD14-positive peripheral blood monocytes (14). Thus, monocytes or macrophages can express DC-CK-1 upon contact with IL-10 and attract naive T cells without their further activation, which might lead to anergy.

In conclusion, phenotypically mature DCs generated *in vitro* in the presence of IL-10 and TNF- $\alpha$  or sCD40L seem to have an intermediate activation status that is modulated by the effect of IL-10. These data support the concept of the regulatory function mediated by IL-10 during inflammatory reactions and induction of immune responses. Furthermore, our results demonstrate that, depending on stimuli provided by T cells or bacterial components during DC development, the presence of IL-10 does not necessarily result in induction of T-cell anergy, and sCD40L or TNF- $\alpha$  can prevent the IL-10-mediated inhibition of antigen-specific CTL induction by DCs. These results might be important for development of efficient immunotherapies designed to overcome the tumor-mediated immunosuppression because IL-10 was shown to be an important factor suppressing APC function in cancer patients.

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