

## Efficient Presentation of Soluble Antigen by Cultured Human Dendritic Cells Is Maintained by Granulocyte/Macrophage Colony-stimulating Factor Plus Interleukin 4 and Downregulated by Tumor Necrosis Factor $\alpha$

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### Summary

Using granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin 4 we have established dendritic cell (DC) lines from blood mononuclear cells that maintain the antigen capturing and processing capacity characteristic of immature dendritic cells in vivo. These cells have typical dendritic morphology, express high levels of major histocompatibility complex (MHC) class I and class II molecules, CD1, Fc $\gamma$ RII, CD40, B7, CD44, and ICAM-1, and lack CD14. Cultured DCs are highly stimulatory in mixed leukocyte reaction (MLR) and are also capable of triggering cord blood naive T cells. Most strikingly, these DCs are as efficient as antigen-specific B cells in presenting tetanus toxoid (TT) to specific T cell clones. Their efficiency of antigen presentation can be further enhanced by specific antibodies via FcR-mediated antigen uptake. Incubation of these cultured DCs with tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) or soluble CD40 ligand (CD40L) for 24 h results in an increased surface expression of MHC class I and class II molecules, B7, and ICAM-1 and in the appearance of the CD44 exon 9 splice variant (CD44-v9); by contrast, Fc $\gamma$ RII is markedly and sometimes completely downregulated. The functional consequences of the short contact with TNF- $\alpha$  are an increased T cell stimulatory capacity in MLR, but a 10-fold decrease in presentation of soluble TT and a 100-fold decrease in presentation of TT-immunoglobulin G complexes.

Dendritic cells (DCs)<sup>1</sup> play a critical role in antigen presentation in vivo (1). They exist in two stages of maturation. As immature cells, DCs are scattered throughout the body in nonlymphoid organs, where they appear to exert a sentinel function. They pick up and process antigen and subsequently move to the T-dependent areas of secondary lymphoid organs. During this process of maturation, they lose antigen-capturing capacity and become mature immunostimulatory DCs that trigger naive T cells recirculating through these areas (2, 3).

A similar maturation process occurs spontaneously when Langerhans cells (LCs, which represent immature DCs in skin) are cultured in vitro (4, 5). Under these conditions LCs rapidly lose the capacity to pick up and process soluble antigen, but acquire high T cell costimulatory capacity. Thus, both in vivo and in vitro studies suggest that antigen capture/pro-

cessing and immunostimulation are the property of DCs at different stages of maturation.

The mechanism of antigen uptake determines the efficiency of presentation of soluble antigens on class II molecules (6). The pinocytic activity of DCs has been reported to be at least as high as that of other APCs (7). In addition, splenic DCs and LCs express Fc $\gamma$ RII, which is lost when these cells mature in vitro (4, 8). Although DCs are able to present soluble antigen (5, 9–11), their efficiency has not been compared to that of other APCs. A general consensus has emerged that DCs may actually be rather inefficient in presenting soluble antigens and it has been argued that, for this reason, efficient presentation in vivo may be an exclusive property of antigen-specific B cells (12).

A common progenitor for granulocytes, macrophages, and DCs has been identified in mouse bone marrow (13) as a MHC class II negative cell that can develop into the three different myeloid pathways under the aegis of GM-CSF. Proliferating precursors of DCs that can be expanded into DC lines in vitro with GM-CSF are present both in mouse bone marrow (14) and peripheral blood (15). To date, human DC lines have

<sup>1</sup> Abbreviations used in this paper: CD40L, CD40 ligand; DCs, dendritic cells; Ii, invariant chain; LCs, Langerhans cells; TT, tetanus toxoid.

been generated only from CD34<sup>+</sup> precursors isolated from cord blood (16) or bone marrow (17) using a combination of GM-CSF and TNF- $\alpha$ .

There are several reasons for wishing to establish *in vitro* cultures of immature DCs. First, to exploit their antigen-presenting capacity; second to compare them with other APCs for this function; third, to identify the signals that modulate antigen capturing and presenting function. Here we describe a method to culture DCs from human peripheral blood, such that the phenotypic and functional characteristics of immature DCs are retained. These cells are indeed as efficient as antigen-specific B cells and can use Fc $\gamma$ RII to further increase uptake of antigen in antigen-antibody complexes. Maturation of these cells can be induced by TNF- $\alpha$ , resulting in upregulation of their capacity to stimulate naive allogeneic T cells and downregulation of their capacity to present soluble antigen.

## Materials and Methods

**Media and Reagents.** The medium used throughout was RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% pyruvate, 50  $\mu$ g/ml kanamycin,  $5 \times 10^{-5}$  M 2-ME (Gibco Laboratories, Grand Island, NY) and 10% FCS (Hyclone Laboratories, Inc., Logan, UT). Tetanus toxoid (TT) was purchased from Connaught Laboratories, Ltd. (Willowdale, Ontario, Canada). Human anti-TT antibodies (all IgG) were purified by chromatography on protein A-Sepharose from concentrated culture supernatants of EBV-B cell clones (18). Human recombinant IL-2, IL-4, and GM-CSF were produced in our laboratory by PCR cloning and expression in the myeloma expression system described by Traunecker et al. (19). The concentration of IL-4 and GM-CSF were determined using commercial ELISA assays. Purified human recombinant GM-CSF and TNF- $\alpha$  were a generous gift of Dr. Manfred Brockhaus (Hoffmann-La Roche, Basel, Switzerland). A soluble chimeric fusion protein between the mouse CD8  $\alpha$ -chain and the human CD40 ligand (CD40L) was a generous gift of Dr. Peter Lane (Basel Institute for Immunology) (20).

**Culture of DCs from Peripheral Blood.** PBMC were isolated on lymphoprep cushions (LSM; Organon Teknika Corp., Rockville, MD) resuspended in RPMI-10% FCS, and allowed to adhere to 6-well plates (Costar Corp., Cambridge, MA). After 2 h at 37°C the nonadherent cells were removed and the adherent cells were detached by incubation with Mg<sup>2+</sup> and Ca<sup>2+</sup> free PBS containing 0.5 mM EDTA at 37°C as described (21). In some experiments, PBMCs were separated on multistep Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden) and the light density fraction from the 42.5–50% interface was recovered and depleted of CD19<sup>+</sup> B and CD2<sup>+</sup> T lymphocytes using magnetic beads (Dynal, Oslo, Norway). The adherent or light density fractions were cultured at  $3 \times 10^5$ /ml in RPMI-10% FCS supplemented with 50 ng/ml GM-CSF and 1,000 U/ml IL-4.

**FACS<sup>®</sup> Analysis.** Cell staining was performed using mouse monoclonal antibodies followed by FITC- or PE-conjugated affinity purified, isotype-specific goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL). The following mAbs were used: L243 (IgG2a, anti-DR), W6/32 (IgG2a, anti-MHC class I); 32.2 (IgG1, anti-Fc $\gamma$ RI), AT10 (IgG1, anti-Fc $\gamma$ RII), OKT3 (IgG2a, anti-CD3) (all from the American Type Culture Collection, Rockville, Maryland); SPVL3 (IgG2a, anti-DQ, provided by Dr. H. Spits, DNAX, Palo Alto, CA); B7.21 (IgG1, anti-DP,

provided by Dr. J. Townsdales, ICRF, London, UK); BU45 [IgG1, anti-invariant chain (Ii); provided by Dr. N. Koch, Institute of Immunology and Genetics, DKF2, Heidelberg, Germany]; NA1/34 (IgG2a, anti-CD1a), WM25 (IgG1, anti-CD1b), IOC3 (IgG1, anti-CD1c) (all provided by Dr. F. Calabi, Hammersmith Hospital, London, UK); B7.24 (IgG2a, anti-B7; provided by Dr. Mark Deboer, Immunogenetics, Ghent, Belgium); RR1/1 (IgG1, anti-ICAM-1), TS1/22 (IgG, anti-LFA1), TS2/9 (IgG1, anti-LFA3) (all provided by Dr. T. Springer, Harvard Medical School, Boston, MA); Leu 11a (IgG1, anti-CD16), Leu M3 (IgG2b, anti-CD14), Leu M5 (IgG2b, anti-CD11c), Leu M9 (IgG1, anti-CD33), Leu 11c (IgG1, anti-Fc $\gamma$ RIII) (Becton Dickinson & Co., Mountain View, CA); 25.34 (IgG, anti-CD44), 11.24 (IgG1, anti-CD44-v9) (all provided by Dr. C. Mackay, Basel Institute for Immunology); G28.5 (IgG1, anti-CD40, provided by Dr. A. E. Clark, University of Washington, Seattle, WA); HD37 (IgG1, anti-CD19; Boehringer Mannheim, Mannheim, Germany). The samples were analyzed on a FACScan<sup>®</sup> (Becton Dickinson) using propidium iodide to exclude dead cells.

**MLR.**  $1.5 \times 10^5$  responding cells either from allogeneic adult PBMCs or cord blood (allogeneic MLR) or autologous PBMCs (autologous MLR) were cultured in 96 flat-bottom microplates (Costar Corp.) with different numbers of irradiated (3,000 rad from a <sup>137</sup>Cs source) stimulator cells (DC or PBMC). Thymidine incorporation was measured on day 5 by a 16-h pulse with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well, sp act, 5 Ci/mMol; Amersham Life Science, Buckingham, UK).

**Antigen Presentation Assays.** TT-specific EBV-B cell clones and TT-specific T cell clones were isolated and maintained as previously described (18). T cell clone AS11.15 recognizes a TT determinant corresponding to residues 947–967 in association with DP4. To measure the efficiency of presentation of a soluble antigen,  $2 \times 10^4$  TT-specific T cells were cultured with  $5 \times 10^3$  cultured DCs (3,000 rad),  $2 \times 10^4$  irradiated autologous EBV-B cells (6,000 rad) or  $10^5$  autologous PBMCs (3,000 rad) in the presence of different concentrations of TT in 200  $\mu$ l RPMI-10% FCS in flat-bottom microplates. The cultures were set up in the presence or absence of a fixed concentration of a mixture of six different anti-TT IgG antibodies (0.5  $\mu$ g/ml each). [<sup>3</sup>H]Thymidine incorporation was measured after 48 h. TT and anti-TT antibodies were allowed to react for 1 h before the addition of APCs and T cells. In some experiments, total PBMCs ( $1.5 \times 10^5$ ) or polyclonal short-term TT-specific T cell lines ( $2 \times 10^4$ ) were used as a source of TT-specific cells. [<sup>3</sup>H]Thymidine incorporation was measured on days 5 and 2, respectively.

## Results

**Culture Conditions for the Generation of DCs with Antigen-presenting Capacity.** Adherent cells or the light density Percoll fraction from PBMCs were depleted of T and B cells and cultured in RPMI-FCS supplemented with various combinations of GM-CSF, IL-4, and TNF- $\alpha$ . The cell yields, surface phenotype and functional properties of cells grown with different cytokine combinations are shown in Table 1. It is evident that a combination of GM-CSF and IL-4 provided the best conditions for the generation of cells with the characteristic phenotype and functional properties of DCs (high expression of CD1, class II and B7, and high stimulatory capacity in allogeneic and autologous MLR). Furthermore cells from GM-CSF + IL-4-dependent cultures were the most efficient at presenting soluble antigen TT to specific T cell

clones. Cells grown with a combination of GM-CSF and TNF- $\alpha$  (16) were inferior to those obtained with GM-CSF + IL-4, especially for presentation of soluble antigen. We therefore used DCs from GM-CSF + IL-4-dependent culture in subsequent experiments.

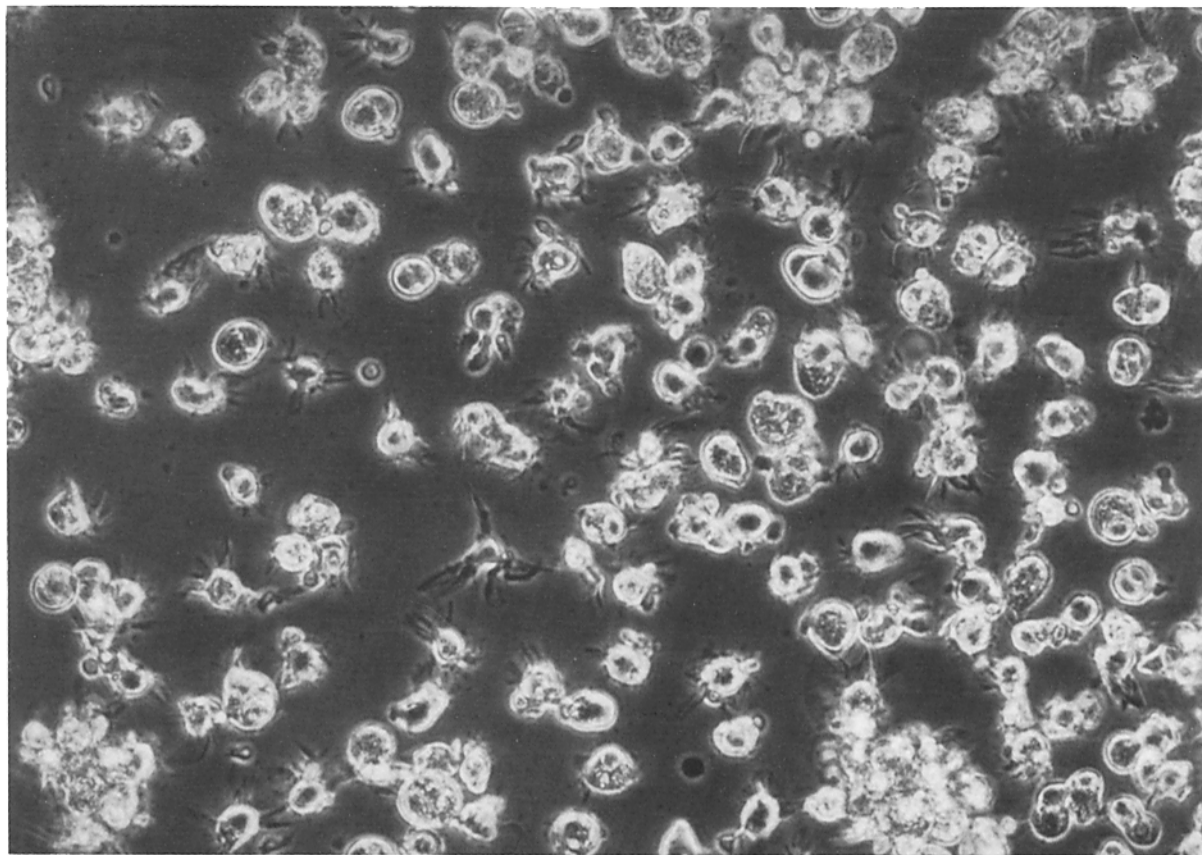
In a typical experiment, after  $\sim 7$  d of culture with GM-CSF + IL-4, 50–80% of the cells appear as loosely adherent clumps or isolated floating cells with the typical dendritic morphology (see an example in Fig. 1) and motility, as assessed by time lapse videorecording (data not shown). Analysis of surface markers (Table 1 and Fig. 2) showed that the large cells were homogeneous and expressed high levels of MHC class I and class II molecules, CD1a, CD1b and CD1c, Fc $\gamma$ RII, ICAM-1, CD11b, CD11c, CD40, B7, and CD33. CD14 was either low or negative in different preparations. Furthermore, DCs were positive for Ii, LFA-1, LFA-3, and CD44 and negative for Fc $\gamma$ RI and Fc $\gamma$ RIII (see also Table 2). Anti-CD3 and anti-CD19 antibodies were always used as control and found negative. Cell growth rapidly slowed down after the first 3–4 wk, but viable cells could be maintained in culture with occasional feeding for up to 3 mo.

**Stimulatory Capacity of DCs.** Cultured DCs were compared with PBMCs for their capacity to stimulate alloreactive T cells. Different numbers of DCs or PBMCs from the same donor were cultured with a fixed number of allogeneic

T cells. Fig. 3 *a* shows that as few as 50 DCs could trigger a substantial response; on a per cell basis, DCs were 300-fold more effective than PBMCs in stimulating adult T cells. It is interesting to note that only DCs but not PBMCs could trigger cord blood T cells (Fig. 3 *b*). The observation that cord blood T cells, which are entirely naive, could be stimulated only by DCs underlines the specialized role of DCs for T cell priming.

**Efficient Presentation of Soluble Antigen and Antigen–Antibody Complexes by Immature DCs.** To evaluate the capacity of DCs to present a soluble antigen, we compared DCs, PBMCs, and antigen-specific B cells for their capacity to present TT to a TT-specific T cell clone. To evaluate the possible effect of Fc $\gamma$ R in enhancing capture of antigen–antibody complexes, the cultures were set up in the presence or absence of a fixed concentration of anti-TT IgG antibodies.

As evident from Fig. 4, the efficiency of presentation, as measured from the TT concentration necessary to give 50% of maximum response, varies with the type of APC. DCs (Fig. 4 *a*) were the most effective APCs, since they could present TT at a concentration of  $10^{-10}$  M, while PBMCs (Fig. 4 *b*) and nonspecific B cells (Fig. 4 *c*) required antigen concentrations higher than  $10^{-8}$  M. Furthermore, in the presence of anti-TT antibodies, the efficiency of DCs increased at least 100-fold and a significant proliferative response was



**Figure 1.** Typical appearance of DC cultures on day 15.

**Table 1.** Cell Yield, Surface Phenotype, Stimulatory, and Antigen-presenting Capacity of Light Density Mononuclear Cells Cultured with Various Cytokine Combinations

	Alone	GM-CSF	IL-4	TNF- $\alpha$	GM-CSF TNF- $\alpha$	GM-CSF IL-4
Cell yield (%) <sup>*</sup>						
Day 4	23	68	23	43	57	84
Day 8	6	114	25	46	74	99
Day 20	2	152	14	64	90	125
Surface markers						
CD1a	—	—	—	—	±	+
CD1b	—	±	—	—	±	+
CD1c	—	—	—	—	±	+
DR	++	++	++	++	++	++
DQ	—	—	+	—	+	+
Class I	++	++	++	++	++	++
Fc $\gamma$ RII	++	+	++	++	+	+
B7	—	±	—	+	+	+
CD40	—	++	+	+	++	++
CD11c	+	++	++	+	++	++
ICAM-1	+	++	+	++	++	++
CD14	+	±	±	+	—	—
Stimulation and antigen presentation <sup>†</sup>						
Allogeneic MLR <sup>‡</sup>						
Maximum response	nd	32 ± 2 <sup>†</sup>	40.9 ± 2.7	33.4 ± 1.7	53.1 ± 3	68.1 ± 2.2
No. cells for 50% response		6,000	4,000	12,000	3,000	600
Autologous MLR <sup>‡</sup>						
Maximum response	nd	<1	<1	<1	<1	10.1 ± 2
TT presentation <sup>§</sup>						
Maximum response	nd	89.4 ± 4.5	nd	nd	8.2 ± 2.1	134.6 ± 4
ng/ml TT 50% response		100			>10 <sup>4</sup>	10

\* Cell yield at days 4, 8, and 20 expressed as percent of input cells.

† The various cell populations were tested on day 8 for their capacity to stimulate in allogeneic and autologous MLR. The maximum response and the number of cells required for stimulating 50% of the maximum response are shown.

§ 5 × 10<sup>3</sup> cells from the various cell populations were tested on day 10 for their capacity to present different concentrations of TT to a TT-specific T cell clone. The maximum response and the concentration of TT required for stimulating 50% of the maximum response are shown.

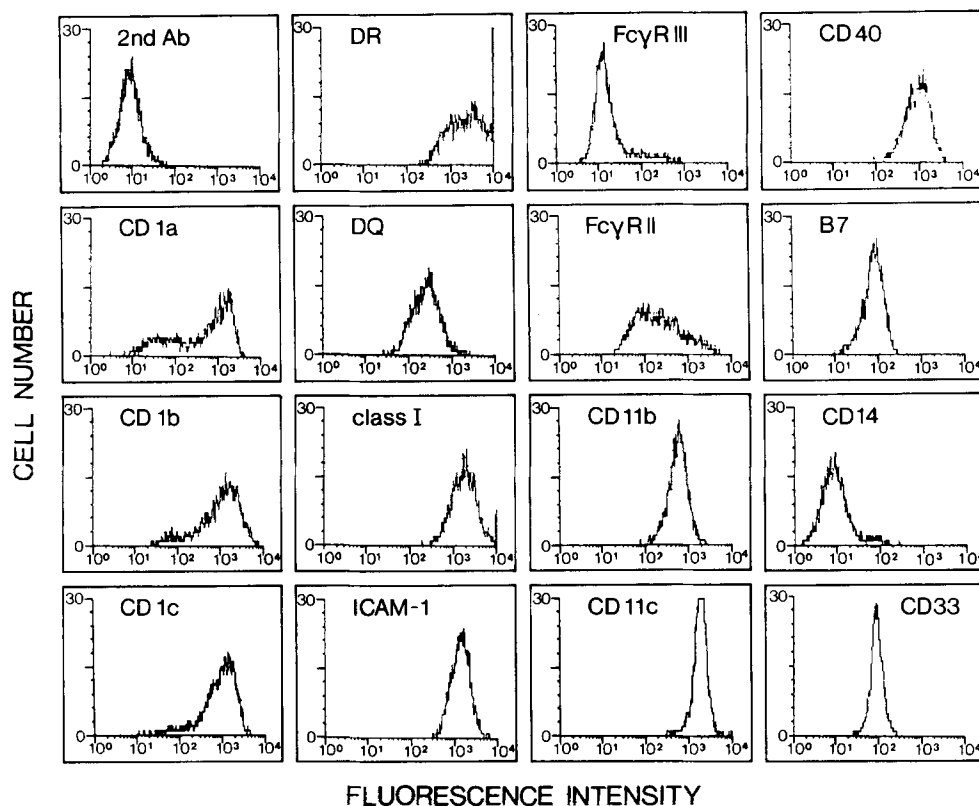
† cpm × 10<sup>-3</sup> ± SD.

obtained at TT concentrations of 10<sup>-14</sup> M. When compared with PBMCs, DCs were at least 100-fold more efficient, both in the absence and in the presence of anti-TT antibody (Fig. 4 *b*). Finally, DCs were as efficient as some antigen-specific B cells (Fig. 4 *c*) and, in the presence of soluble antibodies, appeared to be the most efficient APC for soluble antigens.

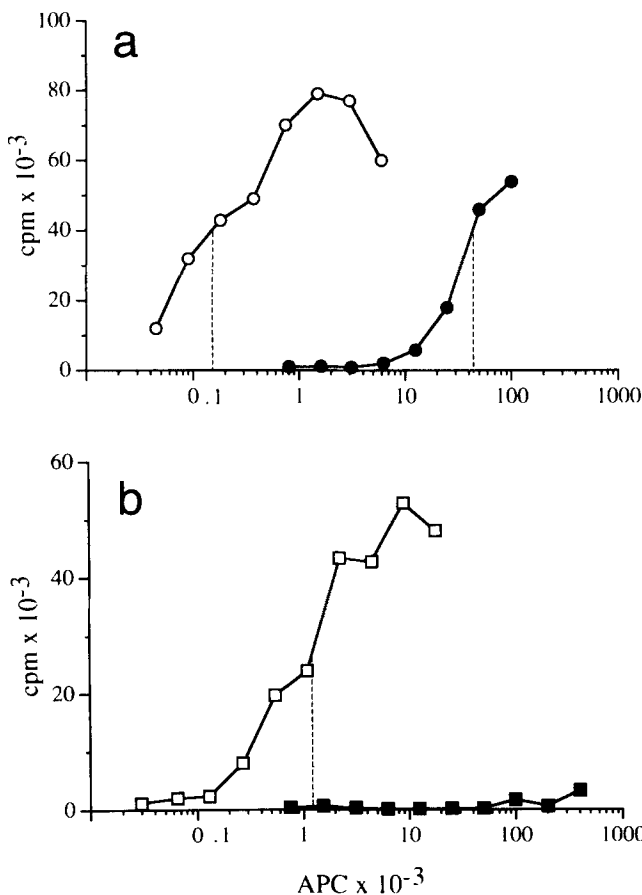
We also compared DCs and PBMCs for their capacity to present TT to autologous peripheral blood T cells and to polyclonal short-term TT-specific lines. As evident from Fig.

5, *a* and *b*, DCs were again more powerful than PBMCs, both in terms of maximum response and amount of TT required. The shape of the dose-response curve may be due to the presence of T cells with different sensitivities to antigen and thus may reflect the ability of DCs to stimulate a higher number of specific T cells.

*Modulation of Surface Phenotype and Antigen-presenting Function by CD40L and TNF- $\alpha$ .* The above results indicated that DCs obtained from GM-CSF + IL-4 cultures shared many prop-



**Figure 2.** Surface phenotype of DCs at day 8 of culture. The histograms show fluorescence values on gated large cells. The amplification was set at a very low value ( $\times 400$ ) to allow quantitative measurement of the extremely bright cells. The first panel shows the control staining with isotype-matched irrelevant antibody.

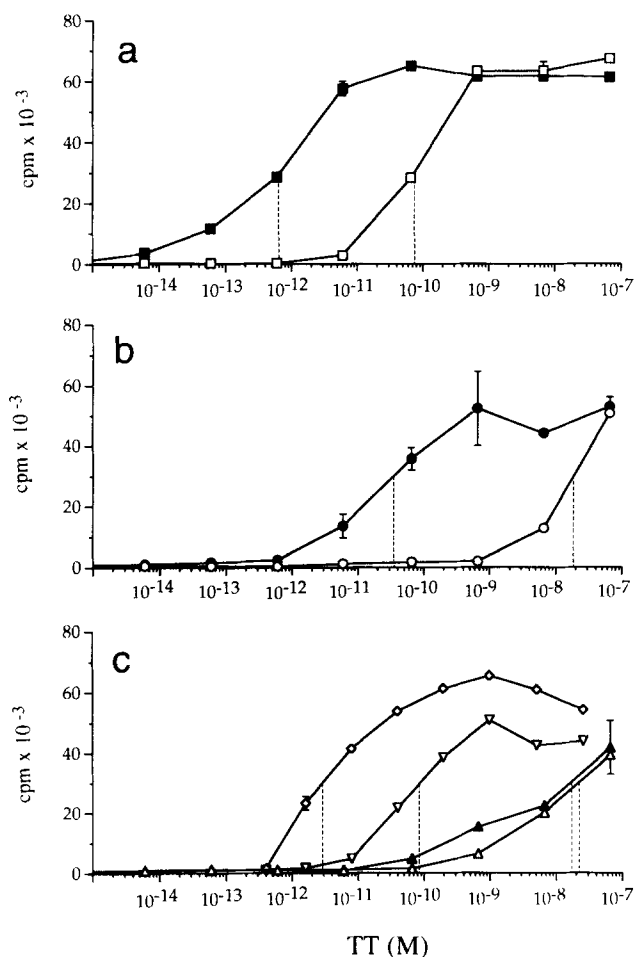


erties with immature DCs such as LCs, at least with respect to surface phenotype and presentation of soluble antigen. We therefore asked whether these cells might be induced to mature in culture and whether this maturation would affect antigen-presenting capacity.

We tested the effect of  $\text{TNF-}\alpha$  and CD40L, which represent two effector molecules of natural and acquired immunity. As shown in Table 2, DCs underwent a rapid change in surface phenotype upon incubation with  $\text{TNF-}\alpha$  or CD40L. By 24 h, surface MHC class I and class II molecules increased two- to threefold, while Ii expression was reduced by approximately half. ICAM-1 expression also increased, a fact that was presumably responsible for the spontaneous cell aggregation observed. B7 and CD40 were also upregulated, whereas  $\text{Fc}\gamma\text{R II}$  was rapidly and sometimes completely downregulated. Interestingly, treatment with  $\text{TNF-}\alpha$  increased CD44 expression and induced the appearance of a new splice variant carrying exon 9 (22).

Some of these changes resemble those occurring *in vivo* when LCs move from skin to lymph nodes (4). We therefore asked what consequence these changes might have on the capacity of DCs to stimulate T cells and to present soluble

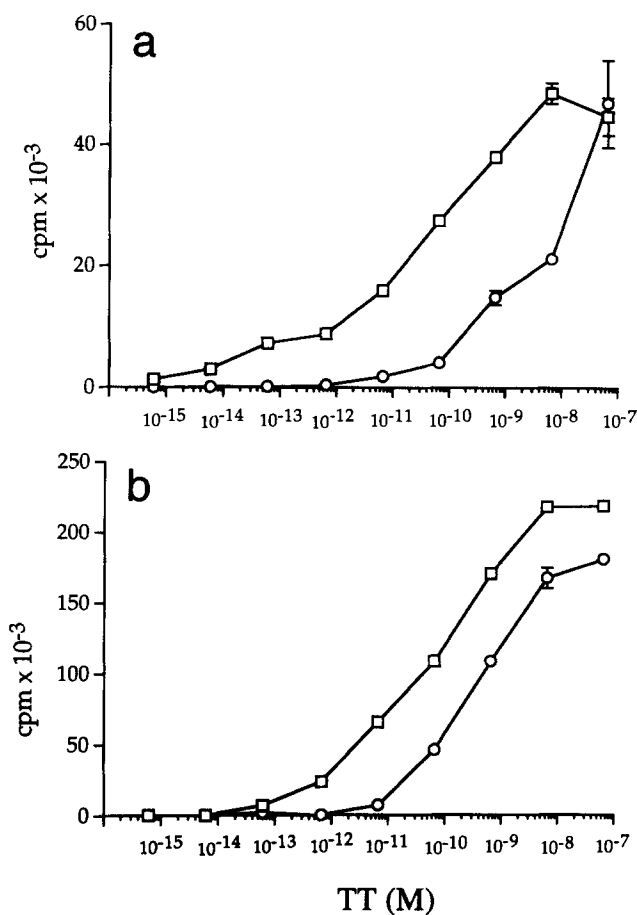
**Figure 3.** Cultured DCs are highly stimulatory in MLR and are the only cells capable of triggering cord blood T cells. Adult peripheral blood (a) or cord blood mononuclear (b) cells were cultured with different numbers of allogeneic PBMCs (●, ■) or DCs (○, □) from the same donor. The proliferative response was measured on day 5.



**Figure 4.** Cultured DCs are the most efficient APCs for presentation of soluble antigen as such or complexed with IgG antibodies. The three panels represent the proliferative response of the same T cell clone to different concentrations of TT in the presence or absence of a fixed concentration of anti-TT IgG antibodies and different irradiated APCs. (a):  $5 \times 10^3$  DCs in the absence ( $\square$ ) or presence ( $\blacksquare$ ) of anti-TT antibody. (b):  $10^5$  PBMCs in the absence ( $\circ$ ) or presence ( $\bullet$ ) of anti-TT antibody. (c):  $2 \times 10^4$  EBV-B cells from two TT-specific clones ( $\diamond$ ,  $\nabla$ ) in the absence of anti-TT antibody or a nonspecific polyclonal line in the absence ( $\Delta$ ) or presence ( $\blacktriangle$ ) of anti-TT antibody. Proliferative response was measured on day 2.

antigen. Pretreatment with TNF- $\alpha$  or CD40L increased two- to threefold the stimulatory capacity in MLR (data not shown), a result that is consistent with the expression of higher levels of MHC products, adhesion, and costimulatory molecules. Remarkably, the same DCs showed a reduced capacity to present TT after treatment with TNF- $\alpha$  (Fig. 6 a), being about 10-fold less efficient. Moreover, the enhanced presentation of immune complexes was completely abrogated, concomitant with a downregulation of Fc $\gamma$ RII (Fig. 6 b).

TNF- $\alpha$  did not have cytotoxic effect on GM-CSF + IL-4-dependent DCs and actually, in some cases, slightly enhanced cell viability. However, the downregulation of Fc $\gamma$ RII and the decrease in antigen-presenting capacity were irreversible, since they were still present even several days after TNF- $\alpha$  had been removed (data not shown).



**Figure 5.** Presentation of TT to polyclonal T cells. Proliferative response of peripheral blood T cells (a) or polyclonal short-term TT-specific T cell lines (b) to different concentrations of TT presented by autologous mononuclear cells (O) or autologous DCs ( $\square$ ). Proliferative response was measured on days 5 and d 2, respectively. The backgrounds of the response without TT (autologous MLR) were subtracted.

## Discussion

The availability of immature DCs is instrumental for studying the mechanisms of antigen capture and processing by these cells, as well as to identify signals that modulate this function. In this study we have shown that it is possible to grow *in vitro* human cell lines with many of the characteristics of immature DCs. The two most striking findings are the highly efficient presentation of soluble antigen by these cell lines, and their rapid response to TNF- $\alpha$  leading to up-regulation of adhesion and costimulatory molecules and down-regulation of antigen-capturing and -processing capacity.

Our DC lines differ from those described by Caux et al. (16) in two important aspects: the use of adult PBMCs (23) rather than cord blood precursors and the use of IL-4 rather than TNF- $\alpha$ . Indeed adult adherent cells grown with GM-CSF + TNF- $\alpha$  have lower stimulatory capacity and are unable to present soluble antigen (Table 1), a fact that can be explained by the capacity of TNF- $\alpha$  to regulate antigen-presenting function (Fig. 6). Our DC lines were generated from adult peripheral blood and require IL-4 in addition to

**Table 2.** Phenotypic Changes of DCs Cultured for 24 h with  $TNF-\alpha$  or CD40L

	GM-CSF+IL4	+ $TNF-\alpha$ *	+ CD40L†
Second Ab	285	33	30
CD1a	369	492	428
CD1b	200	423	270
CD1c	295	454	377
DR	1,555	2,938	2,117
DQ	549	1,416	1,210
DP	129	281	187
Ii	245	185	126
MHC class I	991	2,044	1,798
Fc $\gamma$ RII	1,684	737	862
B7	84	163	127
CD40	673	1,612	742
CD11c	534	414	481
ICAM-1	228	620	457
LFA-1	550	448	517
LFA-3	219	290	252
CD44	845	1,235	1,274
CD44-v9	62	147	98
CD14	75	40	40

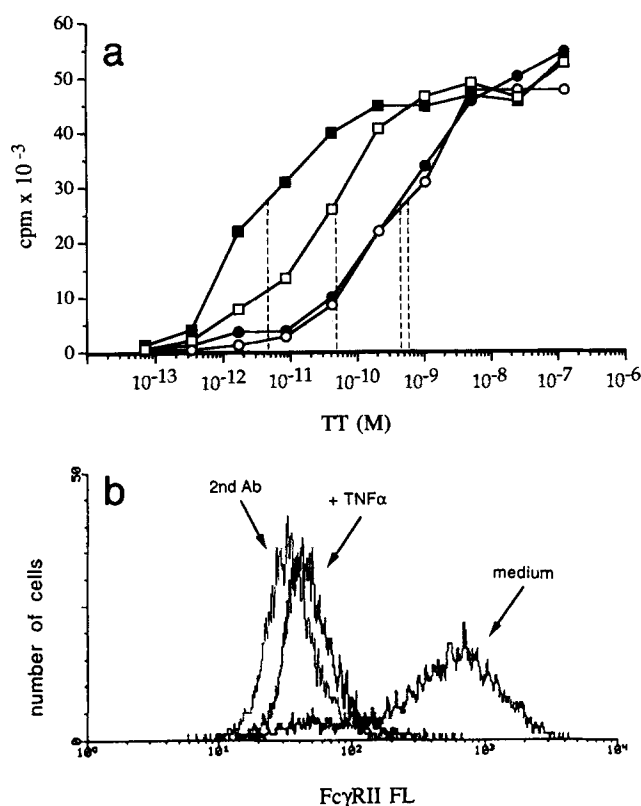
\* DCs were incubated for 24 h with 10 ng/ml  $TNF-\alpha$ .

† DCs were incubated for 24 h with CD40L-mouse CD8 at a saturating concentration as determined by staining with anti-mouse CD8.

§ Mean fluorescence intensity. Comparable results were obtained in four separate experiments.

GM-CSF to maintain the immature, antigen presentation competent state. These conditions have been previously shown to increase CD1 expression on adherent cells (21, 24). It is not clear what role IL-4 may play, but it is interesting to speculate it may antagonize the effect of  $TNF-\alpha$  and other maturation-inducing signals. Whether IL-4 or other cytokines may play a physiological role in maintaining the immature DC pool in vivo is a matter of speculation.

The identification of GM-CSF/IL-4-expanded cells as DCs was based on three well-established and accepted criteria (25, 26): first, their typical morphology and motility; second, their surface phenotype, with high expression of CD1, MHC class I and class II, Ii, Fc $\gamma$ RII, B7, CD40, ICAM-1, LFA-3, and CD11c; and third, their high stimulatory capacity for naive T cells (27–29). In this regard, it is worth noting that only DCs but not PBMCs could activate cord blood T cells. This finding reinforces the notion that DCs are the only cells capable of triggering naive T cells and is an apparent contrast with a report that adult CD45RA<sup>+</sup> naive T cells can be stimulated by allogeneic PBMCs (30). It is possible, however, that



**Figure 6.** Treatment of cultured DCs with  $TNF-\alpha$  downregulates Fc $\gamma$ RII and decreases antigen-capturing and -presenting capacity. (a) Proliferative response of a TT-specific T cell clone to different concentrations of TT in the presence of  $5 \times 10^3$  irradiated DCs that have been pretreated with  $TNF-\alpha$  (○, ●) or left untreated (□, ■). The culture were set up in the absence (○, □) or in the presence (●, ■) of anti-TT IgG antibodies as in Fig. 4. (b) surface expression of Fc $\gamma$ RII on DCs that had been pretreated overnight with  $TNF-\alpha$  or left in growth medium.

either cord blood cells have higher requirements for costimulation than adult T cells, or that the alloreactive response by adult CD45RA<sup>+</sup> T cells may involve memory cells that have reverted to the CD45RA<sup>+</sup> phenotype, but retain the capacity to respond to nonprofessional or semiprofessional APCs (31, 32).

Presentation of soluble antigen by DCs has been reported to be rather inefficient in the sense that relatively high concentrations of antigen were required ( $10^{-6}$  and  $10^{-7}$  M), comparable to those required by other nonantigen-specific APCs (4, 9–11). In contrast, we found that DCs cultured with GM-CSF + IL-4 can present TT at concentrations of  $10^{-10}$  M and are therefore 100–300-fold more efficient than nonspecific B cells or PBMCs. These DCs are actually comparable to antigen-specific B cells, which can use membrane Ig for antigen capture. Furthermore, in the presence of immune complexes, DCs become even more efficient than antigen-specific B cells, being able to present TT at the extraordinary low concentration of  $10^{-12}$  M. This is the most efficient presentation of soluble antigen reported to date.

This highly efficient presentation of soluble antigen depends on the preservation of the immature phenotype, since

it is lost when DCs are induced to mature by TNF- $\alpha$ . It is thus possible that the antigen-presenting capacity of DCs has been previously underestimated because the immature Fc $\gamma$ R<sup>+</sup> cells may have been lost or induced to mature during the isolation procedure.

Several mechanisms may contribute to the efficient antigen presentation of DCs: first, their capacity for clustering T cells in an antigen-independent fashion (33); second, the expression of high levels of MHC molecules, allowing presentation of more T cell determinants; third, the high expression of adhesion and costimulatory molecules and the low surface charge (34, 35), which may lower the number of determinants required for T cell activation (36, 37); and fourth, the high level of fluid-phase pinocytosis (7) and the expression of functional Fc $\gamma$ R (11, 38).

A striking finding is the response of DCs to TNF- $\alpha$  and CD40L. Within 24 h, surface expression of MHC class II, ICAM-1, LFA-3, CD40, and B7 increases two- to threefold, whereas expression of Ii and Fc $\gamma$ RII decreases. The functional consequences are an increased T cell stimulatory capacity in MLR, but a 10-fold decrease in presentation of soluble TT and a 100-fold decrease in presentation of TT-IgG complexes. The effect on class II molecules and Ii is of particular interest. Preliminary experiments indicate that the increase in surface class II expression is not accompanied by an increase in class II biosynthesis, suggesting an effect of TNF- $\alpha$  at the post-translational level. Indeed, staining for intracellular class II molecules revealed that TNF- $\alpha$  induces a rapid disappearance of class II containing structures that are prominent in immature DCs and a redistribution of class II molecules towards the cell periphery (Sallusto, F., unpublished results).

Thus, there are differences in the mechanism that lead to down-regulation of antigen presenting capacity in cultured DCs and in fresh LCs. Whereas in both cases Fc $\gamma$ RII is down-regulated, a downregulation of class II synthesis is observed only in LCs (39, 40). Further work is required to identify the level of this regulation and the signals involved (41, 42).

It is interesting to discuss our results in the context of the well known maturation pathway of LCs. It has been shown that LCs form a reservoir of immature DCs that, upon antigenic stimulation, resume their migratory behavior and move to the draining lymph nodes, where they arrive as mature DCs (1, 43). A similar maturation process is known to occur spontaneously when these cells are cultured in vitro (4). On the basis of our results it is interesting to hypothesize that TNF- $\alpha$  may play a physiological role in vivo in the induction of migration and maturation (44, 45). Local production of TNF- $\alpha$  at sites of encounter with "infectious" antigen (46) may induce maturation of DCs and their migration from tissues into secondary lymphoid organs. It is interesting to note that TNF- $\alpha$  induces the appearance of a CD44 isoform carrying the v9 exon, which may be involved in controlling migratory behavior (47). The role of CD40L may be limited to a later stage, when DCs localize in T-dependent areas of lymph nodes.

Whereas local production of TNF- $\alpha$  may play a physiological role in regulating antigen presentation by DCs, high systemic levels of TNF- $\alpha$ , such as in malignancies or chronic inflammatory diseases, may be detrimental (48). Too much TNF- $\alpha$  may cause generalized immunosuppression by inducing all DCs to mature and lose the capacity to present new incoming antigens.

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