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### TNF Skews Monocyte Differentiation from Macrophages to Dendritic Cells <sup>1</sup>

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# TNF Skews Monocyte Differentiation from Macrophages to Dendritic Cells<sup>1</sup>

Pascale Chomarat,<sup>2,3</sup> Carole Dantin,<sup>2</sup> Lynda Bennett, Jacques Banchereau, and A. Karolina Palucka<sup>4</sup>

Monocytes represent a large pool of circulating precursors of APCs, both macrophages and dendritic cells (DCs). It is thus important to identify the mechanisms by which microenvironment regulates monocyte differentiation. We have previously shown that, upon contact with resting stromal cells such as fibroblasts, monocytes differentiate into macrophages in an IL-6/M-CSF-dependent fashion. Yet, in the inflamed tissue, monocytes need to yield DCs for the adaptive immunity to be induced. Inasmuch as TNF and IL-1 are present at the site of inflammation, we tested their capacity to modulate monocyte differentiation into either macrophages or DCs. TNF, but not IL-1, induce monocytes to become DCs despite the presence of fibroblasts. TNF-induced DCs contain Langerin-positive cells and are able to induce allogenic T cell proliferation. Then, TNF was found to decrease the expression and internalization of the M-CSF receptor, thus overriding the IL-6/M-CSF pathway. Thus, TNF facilitates the induction of adaptive immunity by promoting DC differentiation not only from CD34<sup>+</sup> progenitors but also from CD14<sup>+</sup> blood precursors. *The Journal of Immunology*, 2003, 171: 2262–2269.

Monocytes represent a large pool of circulating precursors that can differentiate into macrophages (M $\phi$ )<sup>5</sup> or dendritic cells (DCs) (1–4). M $\phi$ , scavengers of the immune system, are essential to eliminate the burden of pathogens. We have shown previously that in a resting stromal microenvironment, resembling a noninflammatory situation, monocytes differentiate into M $\phi$  in a process regulated by fibroblast-derived IL-6 (5). IL-6 up-regulates expression of the M-CSF receptor and facilitates M-CSF internalization, resulting in predominant M $\phi$  phenotype. However, M $\phi$  are not sufficient for the development of pathogen-specific protective immunity. This can only be initiated by DCs, the inducers of the immune response. The inflammatory reaction occurring upon pathogen invasion, “danger” signal, leads to DC activation, migration, and maturation, culminating in the induction of immunity and pathogen elimination (3, 4, 6).

Tissue injury can cause dramatic changes throughout the microenvironment (7), including release of inflammatory mediators (cytokines, chemokines) (8) and cellular infiltrates of activated leukocytes, neutrophils, and mast cells. Therefore, by determining precursor differentiation, the cytokines in the local environment shape protective immune responses. It is thus important to understand the regulation of monocyte differentiation. Whereas IL-6 acts

as a major acute inflammation cytokine (9, 10), two other cytokines play a very important role in the inflammatory process, i.e., IL-1 (11) and TNF (12, 13). Both cytokines are implicated in acute and chronic inflammation, likewise pathogen-mediated, for example sepsis, and autoimmune, for example rheumatoid arthritis (9, 14). They share several functional properties, as both are able to induce IL-6, up-regulate the expression of adhesion molecules, and increase the synthesis of acute phase proteins (12, 13, 15). Yet, we show here that TNF, but not IL-1, is able to redirect the differentiation of monocytes toward DCs by offsetting the IL-6/M-CSF pathway. Therefore, TNF is essential for the induction of adaptive immunity.

## Materials and Methods

### Cell cultures

Mononuclear cells were isolated from peripheral blood of healthy volunteers by Ficoll-Paque density gradient centrifugation. Monocytes were purified by depletion using mAbs and Dyna beads, routinely resulting in >90% purity. Monocytes were seeded at  $1 \times 10^6$  cells/well in six-well plates in 3 ml of RPMI 1640 medium supplemented with 10% FBS (Life Technologies, Rockville, MD), 2 mM L-glutamine, 200 IU/ml penicillin, and 200  $\mu$ g/ml streptomycin (Sigma-Aldrich, St. Louis, MO). Normal skin fibroblasts (CD1074SK; American Type Culture Collection, Manassas, VA) were added to the six-well plates at a density of  $2.5 \times 10^5$  cells/well, unless otherwise indicated. Cultures were fed with cytokines every 3 days. Recombinant human cytokines (R&D Systems, Minneapolis, MN) were as follows: IL-1 $\alpha$  (50 ng/ml), IL-1 $\beta$  (25 ng/ml), IL-6 (200 ng/ml) and TNF (20–100 ng/ml unless otherwise indicated, batch-related variability), IL-4 (25 ng/ml), and GM-CSF (100 ng/ml, Leukine; Immunex, Seattle, WA). Neutralizing TNF, IL-6, and IL-6R mAbs were purchased from R&D Systems.

### Allogeneic MLR assay

After 5 days of culture, the CD1<sup>+</sup> cells generated from unstimulated or TNF-treated monocyte/fibroblast cocultures were FACS sorted (FACS-Vantage; BD Biosciences, Mountain View, CA) and used as stimulators for purified naive (CD45RA<sup>+</sup>CD45RO<sup>-</sup>) allogenic CD4<sup>+</sup> T cells. Graded doses of stimulator cells were seeded with  $1 \times 10^5$  naive CD4<sup>+</sup> T cells in round-bottom microtest tissue culture plates in complete RPMI 1640 with 10% human AB serum (Gemini Bio-Products, Woodland, CA). After 4 days of incubation, cells were pulsed overnight with 1  $\mu$ Ci of [<sup>3</sup>H]thymidine to determine T cell proliferation.

Baylor Institute for Immunology Research, Dallas, TX 75204

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<sup>2</sup> P.C. and C.D. are equal contributors to this study.

<sup>3</sup> Current address: Institut de Recherche, SERVIER, 125 chemin de Ronde, 78290 Croissy sur Seine, France. E-mail address: pascale.chomarat@fr.netgrs.com

<sup>4</sup> Address correspondence and reprint requests to Dr. A. Karolina Palucka (E-mail address: karolinp@baylorhealth.edu) or Dr. Jacques Banchereau (E-mail address: jacquesb@baylorhealth.edu), Baylor Institute for Immunology Research, 3434 Live Oak, Dallas, TX 75204 TX.

<sup>5</sup> Abbreviations used in this paper: M $\phi$ , macrophage; DC, dendritic cell; LC, Langerhans cell; RA, rheumatoid arthritis.

*Cytokine detection*

Cytokines were detected in cell-free culture supernatants using commercial quantitative sandwich immunoassay kits: IL-6 (BD PharMingen, San Diego, CA) and M-CSF (R&D Systems).

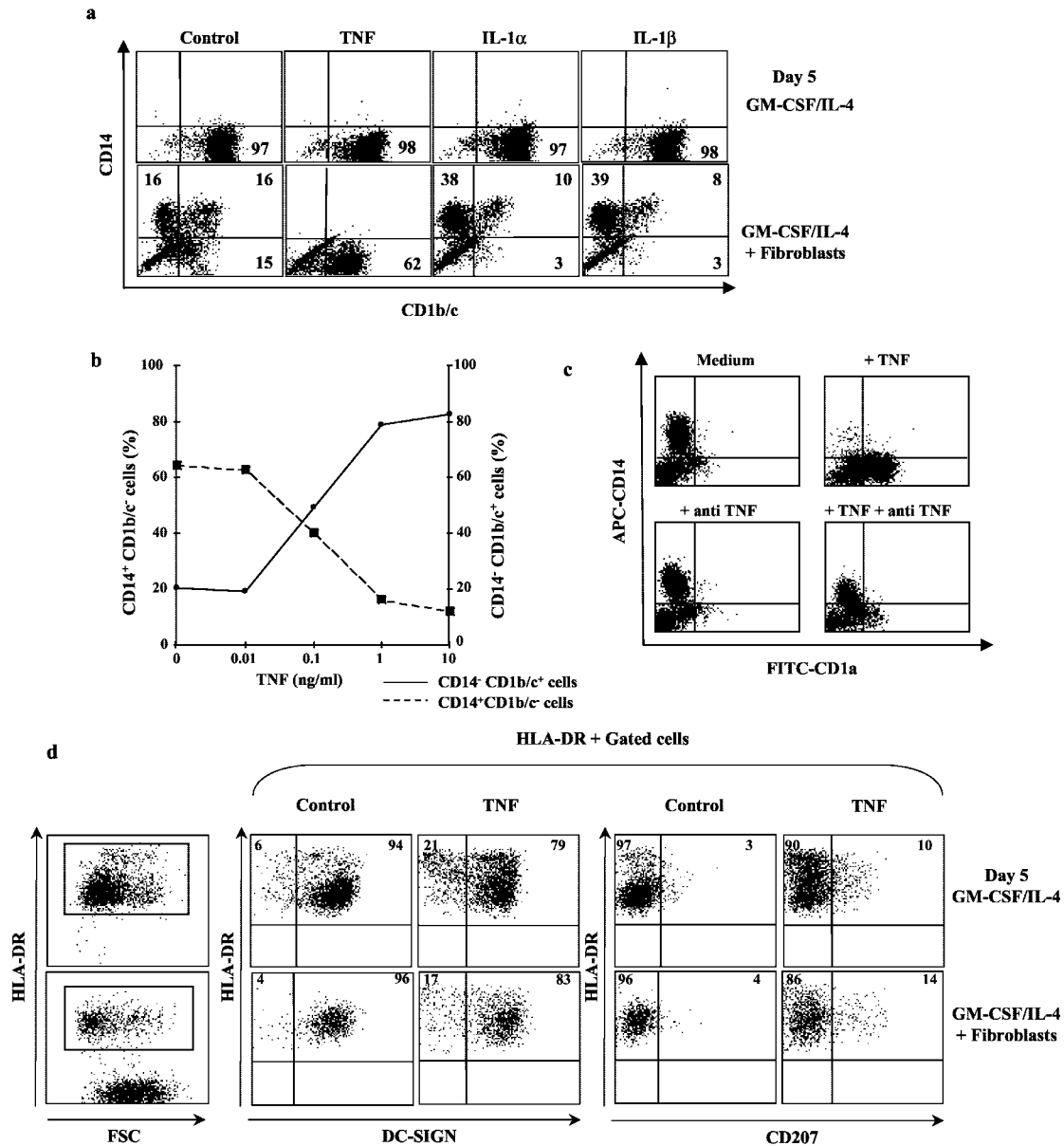
*FACS analysis*

At indicated time points, cells were processed for double staining (30 min at 4°C) using FITC-CD1a (recently reclassified as a CD1b/c mAb; BioSource International, Camarillo, CA) and PE-CD14 or PE-CD83 (BD Biosciences) mAbs. Surface M-CSF receptors were detected using a rat anti-M-CSFR mAb 3-4A4 (Santa Cruz Biotechnology, Santa Cruz, CA) revealed in a second step by FITC-labeled goat F(ab')<sub>2</sub> anti-rat Ig (dilution

1/25; BioSource International). For the kinetic studies, cells were double stained with PE-HLA-DR (BD Biosciences) to allow the analysis of M-CSF receptors on myeloid cells.

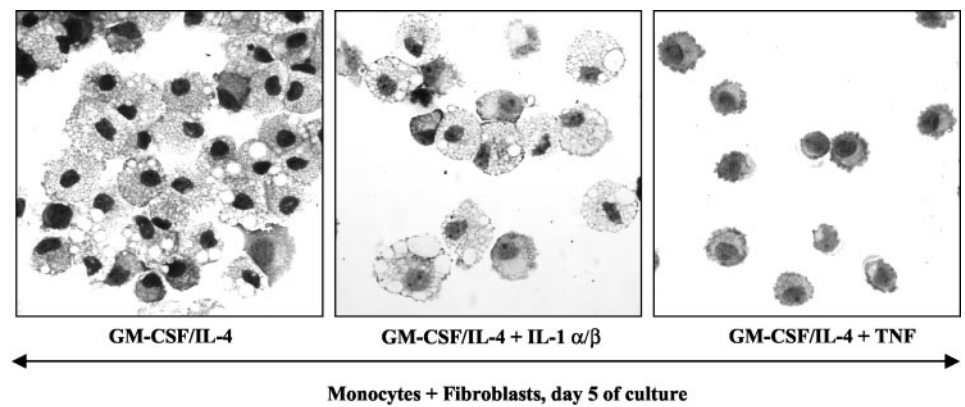
*Real-time RT-PCR*

Monocytes were incubated with GM-CSF and IL-4 for 5 days with or without IL-6 (200 ng/ml), TNF (100 ng/ml), or a combination. Cells were harvested at day 5 and RNA was extracted using an RNeasy kit from Qiagen (Valencia, CA). Real-time PCR was conducted using an Applied Biosystems Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Probes and primers for the *IL-6R* and *M-CSFR* genes were



**FIGURE 1.** TNF skews monocyte differentiation toward DCs in fibroblast/monocyte cocultures. Monocytes were cultured for 5 days in GM-CSF and IL-4 with or without stromal cells (ratio 4:1). *a*, IL-1 $\alpha$  (50 ng/ml), IL-1 $\beta$  (25 ng/ml), and TNF (30 ng/ml) were added at the onset of the cultures and differentiation was monitored using expression of CD1b/c and CD14. The percentages of each myeloid cell subset in the total cell population are indicated. Double-negative cells are fibroblasts. *b*, Dose-response curve. TNF was added to cocultures at increasing concentrations. Percentages (vertical axis) of CD14<sup>+</sup>CD1b/c<sup>-</sup> (---) and CD14<sup>-</sup>CD1b/c<sup>+</sup> (—) cells gated on HLA-DR expression at day 5 of culture. DC differentiation was observed already at the dose of 10 ng/ml. However, because of batch-to-batch variation TNF was used at 100 ng/ml in the majority of experiments unless otherwise indicated. *c*, Skewing of monocyte differentiation toward DCs is dependent on added TNF. Monocytes are cultured without (upper left plot) or with (upper right plot) TNF. TNF-neutralizing mAbs are added at day 0 (lower plots). Monocyte differentiation is determined by flow cytometry at day 5. *d*, Phenotypic characterization. Day 5 HLA-DR<sup>+</sup> cells are gated on forward scatter (FSC)/side scatter plots to distinguish monocytes from fibroblasts (left panel). Surface expression of DC-SIGN (middle panel) and Langerin (CD207, right panel) by HLA-DR<sup>+</sup> cells.

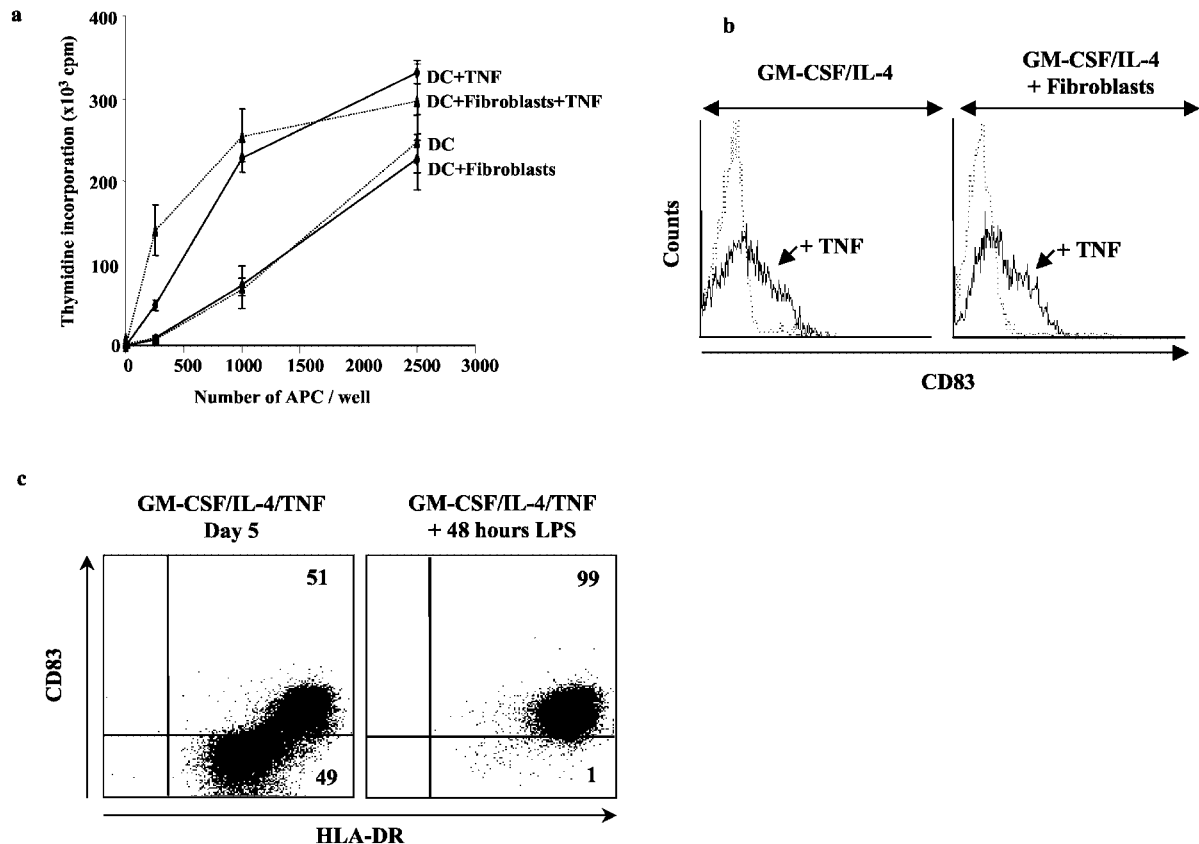
**FIGURE 2.** TNF, but not IL-1, skews monocyte differentiation toward DCs in fibroblast/monocyte cocultures. Giemsa staining of cells retrieved from cultures supplemented with GM-CSF and IL-4 supplemented or not with TNF or IL-1. Note an abundant highly vacuolated lightly stained cytoplasm and a centrally positioned nucleus in cells cultured without TNF addition (*left and middle panels*). Cells from cultures with TNF (*right panel*) display a homogeneous smooth intensely stained cytoplasm, cytoplasmic protrusions, and a laterally positioned nucleus, consistent with DC morphology.



designed using the computer software Primer Express (Applied Biosystems). Duplicate target and endogenous control reactions were set up in separate tubes. Reactions were prepared using the TaqMan One Step RT-PCR Master Mix and the human 18S RNA Pre-Developed Assay Reagent kit (Applied Biosystems) according to the manufacturer's instructions. Primers and probes were used at final concentrations of 900 and 50 nM, respectively. The Sequence Detector was programmed for an initial step of 30 min at 48°C and 10 min at 95°C, followed by 40 thermal cycles of 15 s at 95°C and 1 min at 60°C. Fold expression was calculated using both the Relative Standard Curve and the Comparative  $C_T$  methods, where 18S ribosomal RNA was used as the endogenous control for normalization (Applied Biosystems TaqMan User Bulletin 2).

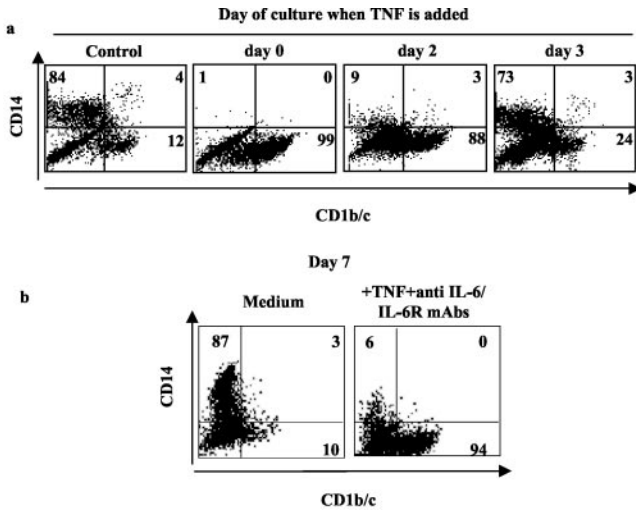
### Confocal analysis

Day 5 DCs were incubated for 24 h in GM-CSF and IL-4 with or without 200 ng/ml IL-6, 100 ng/ml TNF, or a combination. Cells were then collected and incubated for 30 min at 37°C in medium alone or with M-CSF (50 ng/ml). After ice-cold washes, cells were stained for surface M-CSFR expression and analyzed by flow cytometry. For immunofluorescence and confocal microscopy, DCs were adhered to polylysine-coated glass slides for 4 h at 4°C. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.05% saponin in PBS (pH 7.4). M-CSFR was detected using a non-blocking rat mAb 3-4A4 revealed by FITC-conjugated goat F(ab')<sub>2</sub> anti-rat Ig (BioSource International). Slides were mounted using a fluoromount



**FIGURE 3.** TNF skews monocyte differentiation toward partially mature DCs. *a*, MLR: day 5 DCs and monocyte/fibroblast cocultures treated or not with TNF (100 ng/ml) were sorted using CD1b/c marker and incubated at graded doses with allogenic naive (CD45RA<sup>+</sup>CD45RO<sup>-</sup>) CD4<sup>+</sup> T cells. Thymidine incorporation is expressed as mean  $\pm$  SD of triplicates. Results are representative of three separate experiments. *b*, Monocytes were cultured for 5 days with or without fibroblasts in GM-CSF and IL-4 in the presence or not of TNF. At day 5, the cells were stained with FITC-CD1b/c and PE-CD14 and then sorted. CD14<sup>-</sup>CD1b/c<sup>+</sup> cells were then restained with PE-CD83 and analyzed by flow cytometry. Each plot shows CD83 expression in cultures without adding TNF (dotted line) and TNF-stimulated cultures (solid line). *c*, Flow cytometry plots of HLA-DR (ordinate) and CD83 (vertical axis) expression in DCs generated by culturing monocytes with GM-CSF/IL-4 and TNF (day 5, *left plot*) and further exposed for 48 h to LPS 100 ng/ml (day 7, *right plot*).





**FIGURE 4.** TNF converts early M $\phi$  into DCs in stromal cell/monocyte cocultures. *a*, Monocytes were cultured for 5 days in GM-CSF and IL-4 with normal skin fibroblasts. TNF (10 ng/ml) was added from day 0 to day 4 cocultures. At day 5, differentiation was monitored using expression of CD1b/c and CD14. Similar results were obtained when the differentiation was measured at day 7. *b*, Monocytes were cultured in GM-CSF and IL-4 with normal skin fibroblasts. From days 3 to 5, cultures were pulsed with TNF (100 ng/ml) and blocking IL-6 and IL-6R mAbs (15  $\mu$ g/ml) for 3 consecutive days. Forty-eight hours after the last pulse, cells were stained for CD1b/c and CD14 expression. Results are representative of three to six separate experiments.

medium containing 7-aminoactinomycin D (Sigma-Aldrich). Confocal laser scanning microscopy was performed with a confocal microscope (TCS-SP; Leica, Brussels, Belgium) equipped with  $\times 63$  oil objective.

#### Statistical analysis

A paired Student *t* test was used to determine the statistical significance of the data. In all cases, values were log transformed before statistical analysis. A value of  $p < 0.05$  was chosen for rejection of the null hypothesis.

## Results

### Adding TNF to monocytes cultured with fibroblasts promotes monocyte differentiation toward DCs

Under “steady-state” conditions fibroblasts skew monocyte differentiation toward M $\phi$  (5). We tested whether an inflammatory environment that contains IL-1 and/or TNF would alter such a differentiation pathway. Adding TNF at the onset of the monocyte/fibroblast cocultures in the presence of GM-CSF/IL-4 resulted in the generation of cells with a DC phenotype. Thus, the analysis of 10 independent experiments revealed that  $>95\%$  of HLA-DR<sup>+</sup> cells generated in the presence of TNF express CD1 but not CD14. In contrast,  $>90\%$  of HLA-DR<sup>+</sup> cells generated without adding TNF express CD14 (Fig. 1*a*). Dose-response analysis revealed that

TNF-mediated skewing of monocyte differentiation toward DCs could be observed already at a concentration of 1 ng/ml TNF and reached a peak at 10 ng/ml (Fig. 1*b*). The observed effect on monocyte differentiation was TNF dependent rather than due to contamination such as LPS, as it could be prevented by the addition of neutralizing TNF Ab (Fig. 1*c*). Monocyte/fibroblast cocultures in the presence of TNF yield DCs that express surface CD1 (Fig. 1*a*) and DC-SIGN ( $43 \pm 9\%$  of HLA-DR<sup>+</sup> DC-SIGN<sup>+</sup> cells,  $n = 9$ ) (Fig. 1*d*). However, a discrete fraction of cells displays surface staining with Ab recognizing Langerin ( $9 \pm 4\%$  of HLA-DR<sup>+</sup> CD207<sup>+</sup> cells,  $n = 9$ ; Fig. 1*d*).

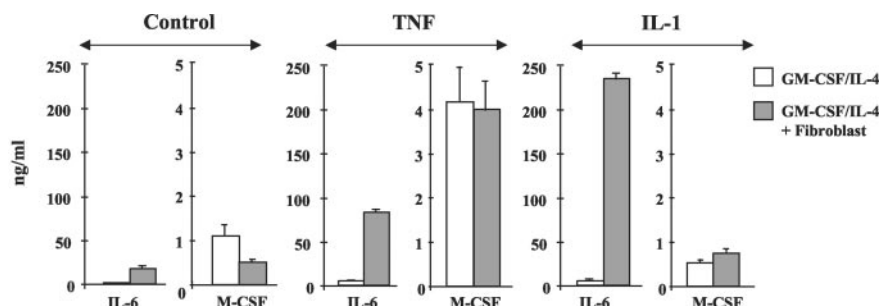
Contrary to TNF, adding either IL-1 $\alpha$  or IL-1 $\beta$  to monocyte/fibroblast cocultures did not promote DC differentiation since hardly any CD1<sup>+</sup> cells could be seen (Fig. 1*a*). Rather, M $\phi$  differentiation prevailed as the cells retained high CD14 expression and displayed M $\phi$  morphology (Fig. 2). Indeed, the analysis of Giemsa-stained cells sorted from cultures supplemented with GM-CSF and IL-4 (with or without IL-1) revealed an abundant highly vacuolated, lightly stained, cytoplasm and a centrally positioned nucleus, a phenotype consistent with M $\phi$  differentiation (Fig. 2). On the contrary, CD1<sup>+</sup> cells isolated from cultures performed with TNF display a homogenous smooth intensely stained cytoplasm, cytoplasmic protrusions, and a laterally positioned nucleus, a morphology typical of DCs. Thus, TNF and IL-1 differentially modulate monocyte differentiation.

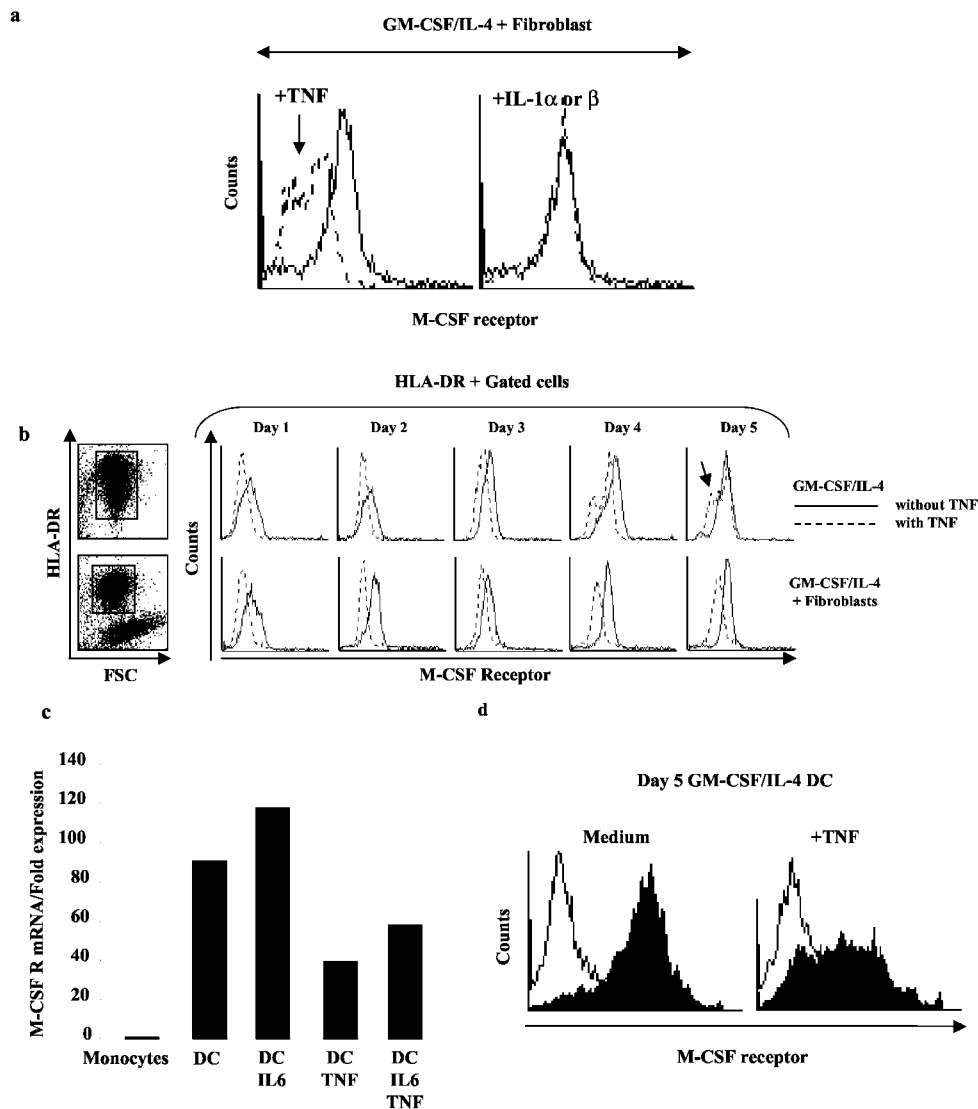
### TNF-induced cells display DC function

Next, we determined whether the cells generated in monocyte/fibroblast cocultures in the presence of TNF were able to prime naive T cells, a property of DCs. To this end, CD1<sup>+</sup> cells from monocyte/fibroblast cocultures or from control cocultures without fibroblasts were sorted and used as stimulators in allogenic MLR with naive CD4<sup>+</sup>CD45RA<sup>+</sup> T cells. As shown in Fig. 3*a*, CD1<sup>+</sup> cells sorted from day 5 TNF-treated cocultures with or without fibroblasts induce a strong proliferation of naive allogenic CD4<sup>+</sup> T cells consistent with DC differentiation. Furthermore, fibroblasts do not inhibit the function of differentiated DCs as cells retrieved from cocultures with fibroblasts are as potent as DCs generated in the absence of fibroblasts (Fig. 3*a*). When compared with CD1<sup>+</sup> cells isolated from non-TNF cocultures, DCs generated in the presence of TNF induce higher T cell proliferation, a finding consistent with the TNF-induced maturation.

Indeed, TNF is a well-established DC maturation factor. However, DCs generated in TNF-stimulated monocyte/fibroblast cocultures were only partially mature as  $\sim 50\%$  of the cells expressed increased levels of CD83 (Fig. 3*b*), CD86, and, to a lesser extent, CD80 (data not shown). TNF-induced DCs were not blocked in their maturation capacity and could undergo further maturation when further cultured with CD40 ligand, TNF, or LPS (Fig. 3*c* and

**FIGURE 5.** TNF increases IL-6 and M-CSF levels. Monocytes were cultured for 5 days with or without fibroblasts in GM-CSF and IL-4 with or without TNF (100 ng/ml) or IL-1 $\alpha\beta$  (50 ng/ml). IL-6 and M-CSF levels were tested in day 5 supernatants. Results are expressed as mean  $\pm$  SD of three independent experiments.





**FIGURE 6.** TNF down-regulates surface M-CSFR expression. Monocytes were cultured for 5 days with or without fibroblasts in GM-CSF and IL-4 with or without TNF (100 ng/ml) or IL-1 $\alpha$  (50 ng/ml). *a*, Cells were double stained for M-CSFR and HLA-DR. Expression of M-CSFR is shown on HLA-DR<sup>+</sup> cells. Results are representative of three independent experiments. *b*, Kinetics of M-CSFR expression. Each day of culture, cells were double stained for M-CSFR and HLA-DR. Expression of M-CSFR receptors is shown on HLA-DR<sup>+</sup> cells. *c*, Expression of *M-CSFR* genes on TNF-treated DCs. Monocytes were cultured for 5 days in GM-CSF and IL-4 with or without TNF (100 ng/ml) and/or IL-6. At day 5, cells were harvested, RNA was extracted, and gene expression was quantified by real-time RT-PCR. *d*, Expression of M-CSFR protein on TNF-treated DCs. Monocytes were cultured for 5 days in GM-CSF and IL-4, washed, and cultured without cytokines (*left*) or with TNF (*right*) for 24 h.

data not shown). Thus, TNF promotes monocyte differentiation toward partially mature DCs.

#### *TNF converts activated monocytes/early M $\phi$ into DCs*

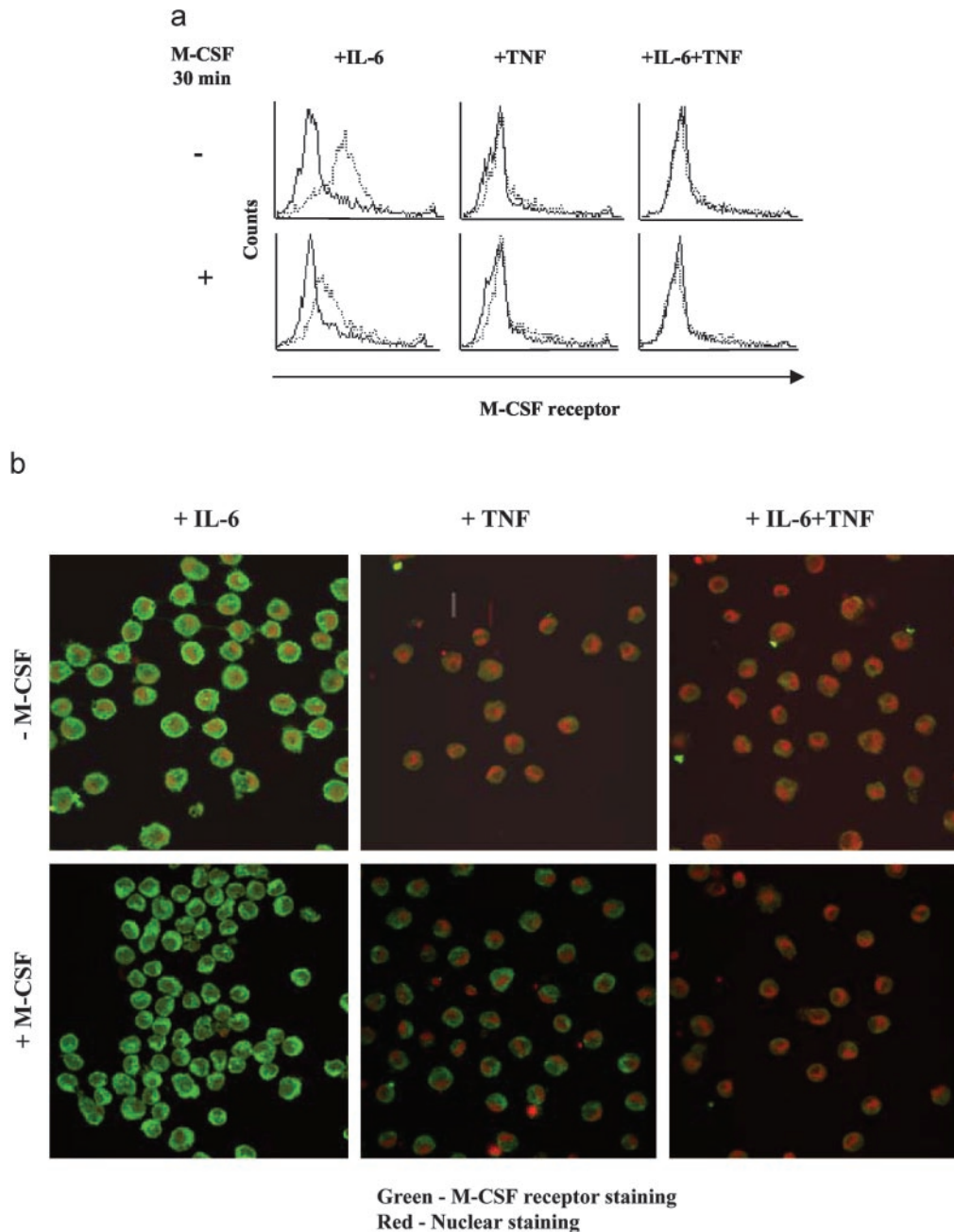
M $\phi$  generated by culturing monocytes with M-CSF can convert into DCs upon exposure to GM-CSF and IL-4, even at late stages of differentiation (16). Furthermore, as shown above, monocyte/fibroblast cocultures made in the presence of GM-CSF and IL-4 yield CD14<sup>high</sup> cells displaying M $\phi$  morphology (Fig. 2). Therefore, we next determined whether delayed addition of TNF could lead to conversion of activated monocytes/early M $\phi$  into DCs. As shown in Fig. 4*a*, adding TNF to monocyte/fibroblast cultures at day 1 or day 2 resulted in the generation of DCs. However, when TNF was added at day 3 or later, the cells retained CD14 expression and did not acquire CD1. This resistance to TNF was not due to a loss of TNFRs (data not shown).

We therefore wondered whether the resistance to TNF reflected an irreversible commitment to M $\phi$  differentiation. Alternatively,

the high levels of fibroblast-derived IL-6 found in day 3 cultures may predominate over TNF and maintain the CD14<sup>high</sup> phenotype. Thus, day 3 monocyte/fibroblast cocultures were pulsed daily (for 3 days) with TNF along with Abs neutralizing IL-6/IL-6R until the cells were analyzed at day 7. As shown in Fig. 4*b*, blocking IL-6 reversed resistance to TNF as determined by the disappearance of CD14<sup>+</sup> cells and the presence of CD14<sup>-</sup>CD1<sup>+</sup> DCs that constituted >90% of the HLA-DR<sup>+</sup> population in monocyte/fibroblast cocultures. These results demonstrate the importance of the IL-6/TNF balance in determining the differentiation of monocytes into either DCs or M $\phi$ .

#### *TNF offsets the IL-6/M-CSF pathway that promotes M $\phi$ differentiation*

Since blocking IL-6 facilitated TNF-induced monocyte/M $\phi$  differentiation into DCs, we surmised that TNF might interfere in the monocyte response to IL-6/M-CSF. To this end, levels of IL-6 and M-CSF were measured in culture supernatants. As shown in Fig. 5,



**FIGURE 7.** Inhibition of M-CSFR internalization by TNF. Day 5 DCs were incubated in GM-CSF and IL-4 with or without IL-6 (200 ng/ml), TNF (100 ng/ml), or a combination for 24 h. Cells were then exposed or not to M-CSF (50 ng/ml), washed, and stained for M-CSFR for flow cytometry (*a*) and confocal analysis (*b*). Confocal photographs represent sections of cells from each culture condition labeled for M-CSFR (green) and nuclei (red). Results are representative of three independent experiments.

in three experiments, monocyte/fibroblast cocultures spiked with TNF contained high levels of IL-6 (average 4-fold increase as compared with GM-CSF/IL-4 cocultures,  $p < 0.0001$ ) and of M-CSF (average 8-fold increase,  $p < 0.0001$ ; Fig. 5). Supernatants of cultures spiked with IL-1 contained very high levels of IL-6 (up to 250 ng/ml, average 12-fold increase as compared with control cultures,  $n = 3$ ) but only low levels of M-CSF (Fig. 5). These results suggest that TNF-driven DC differentiation may result from the ability of TNF to prevent the utilization of IL-6 and/or M-CSF. Conversely, IL-1 may promote M $\phi$  differentiation by increasing the levels of IL-6 and facilitating the utilization of M-CSF.

Therefore, we investigated whether the apparent lack of cytokine utilization in TNF cultures was due to down-regulation of

M-CSF receptors. Adding TNF, but not IL-1 $\alpha$  or IL-1 $\beta$ , at the onset of monocyte/fibroblast cocultures made with GM-CSF/IL-4 resulted in down-regulation of M-CSFR protein expression (Fig. 6*a*). Such down-regulation was independent of the presence of stromal cells (Fig. 6*b*). The analysis of the kinetics of M-CSFR expression showed that moderate down-regulation of its expression could be observed already at the early stages of culture (days 1 and 2; Fig. 6*b*).

The down-regulation of M-CSFR expression was confirmed at the gene transcription level (Fig. 6*c*). In these experiments, M-CSFR mRNA expression (by real-time PCR) in monocytes was analyzed either directly after isolation from blood or after 5 days of culture with GM-CSF and IL-4 with and without the addition of

IL-6 and/or TNF at the onset of culture (without fibroblasts). *M-CSFR mRNA* expression in cultured cells was normalized for comparison to the expression in resting, i.e., uncultured, monocytes. Adding TNF to GM-CSF/IL-4 DC cultures resulted in >2-fold decrease in the *M-CSFR mRNA* expression (Fig. 6c), while the expression of *IL-6-R $\alpha$*  chain was not affected (data not shown). The effect of TNF was prevailing even in the presence of exogenous IL-6 (Fig. 6c). These results suggest that TNF acts in the early stages of monocyte differentiation and renders monocytes unresponsive to IL-6/M-CSF via down-regulation of M-CSFR expression consistent with earlier observations in the mouse (17, 18).

Furthermore, TNF down-regulates the expression of M-CSFR on already differentiated DCs (Fig. 6d). Indeed, when monocytes cultured for 5 days with GM-CSF and IL-4 are exposed for 24 h to TNF a considerable decrease of surface M-CSFR expression could be observed (Fig. 6d). Even though M-CSFR expression may be barely detectable by Ab staining and flow cytometry, a very low number (below detection limit by flow cytometry) number of receptor molecules present on the cell surface might suffice for the internalization of the receptor-ligand complex (12). To address this, day 5 DCs generated with GM-CSF and IL-4 were cultured for 24 h with either IL-6 and/or TNF, washed, and subsequently exposed to M-CSF for 30 min. M-CSFR expression on the cell surface was monitored by flow cytometry and its internalization was monitored by intracytoplasmic staining and confocal microscopy. As shown in Fig. 7, only IL-6-treated DCs displayed intense intracytoplasmic staining, suggesting receptor-ligand internalization. Contrary to IL-6, TNF-induced down-regulation of M-CSFR expression was accompanied by the inhibition of receptor internalization. Thus, TNF stabilizes the phenotype of already differentiated DCs and acts through down-regulation of M-CSFR expression and internalization.

## Discussion

Our studies demonstrate the importance of IL-6 and TNF in the regulation of monocyte differentiation into M $\phi$  or DCs. Our previous study has shown that monocytes cultured with fibroblasts and GM-CSF/IL-4 yield predominantly M $\phi$  in response to fibroblast-derived IL-6 (5). The current study shows that adding TNF at the onset of monocyte/fibroblast cocultures skews monocyte differentiation toward DCs. TNF overcomes IL-6-driven M $\phi$  differentiation by down-regulating the expression and internalization of the M-CSFR on activated monocytes. Although the inhibitory effect of TNF on the M-CSFR expression has been described earlier in murine M $\phi$  (17), our data show the consequence of this process, i.e., skewing of human monocyte differentiation into DCs at the expense of M $\phi$ .

Skewing of monocyte differentiation toward DCs is specific to TNF and could not be obtained by replacing TNF with IL-1. Interestingly, both TNF and IL-1 are essential for migration of DCs from tissue to secondary lymphoid organs as demonstrated in vitro in human skin cultures (19, 20) as well as in vivo in mice upon topical (19) and/or systemic administration of TNF and/or IL-1 (21). Yet, our results show the distinct role of each cytokine in DC differentiation from blood precursors. The lack of DCs in monocyte/fibroblast cocultures with IL-1 might be explained by continuous M-CSF internalization through sustained or up-regulated expression of the M-CSFR as shown previously in human monocytes (22) as well as in mice (18). Alternatively, endogenous M-CSF and/or exogenous GM-CSF and IL-4 can result in the increased expression of the IL-1R antagonist (23, 24). The IL-1R antagonist is likely to counteract the biological effects of IL-1 (11, 15) including its potential activity in skewing monocyte differentiation toward DCs.

We have previously shown that monocytes yield DCs with Langerhans cell (LCs) properties when cultured with GM-CSF and IL-15 (25). In this study, we show that in the presence of fibroblasts and TNF, monocytes yield CD1a<sup>+</sup>DC-SIGN<sup>+</sup> cells as well as a subset of cells expressing surface Langerin, a marker of LCs. TNF has been shown to up-regulate Langerin expression on the surface of immature LCs obtained by culturing monocytes with GM-CSF and IL-4 supplemented with TGF- $\beta$  (26). Whether the acquisition of Langerin expression in monocyte/fibroblast cocultures represents a direct effect of TNF or whether it is mediated by TNF-induced cytokines remains to be determined. Indeed, dermal fibroblasts express cytoplasmic IL-15, which upon long-term (over 3 days) exposure to TNF can be relocated to the cell surface (27). This membrane-bound form of IL-15, particularly when presented to neighboring cells in the complex with IL-15R $\alpha$  (28), may display a potent biological activity as demonstrated for example by sustained T cell proliferation (27).

Our results show that DCs generated in the presence of GM-CSF and TNF are "semimature" because only a fraction of cells express CD83 and they can undergo full maturation upon subsequent exposure to soluble CD40 ligand or LPS. The role of TNF in DC maturation is not entirely clear and the extent of TNF-induced DC maturation is likely to be dependent on the presence of additional signals. Thus, while TNF maintains the viability of freshly isolated murine LCs, GM-CSF is necessary for their functional maturation (29). Furthermore, in vitro-generated human LCs exposed to TNF, in the presence of TGF- $\beta$ , up-regulate surface Langerin and CCR7 expression and migrate in response to CCR7 ligands (26). Such TNF-dependent acquisition of CCR7 expression provides an explanation for the presence of immature LCs in the draining lymph node of the skin area involved by dermatopathic lymphadenitis (26). However, the classical DC maturation markers, DC-lysosome-associated membrane glycoprotein and costimulatory molecules expression, are absent and the cells display low T cell stimulatory activity, findings consistent with only partial maturation (26, 30). Finally, in a murine model of experimental autoimmune encephalitis, TNF-induced semimature DCs lead to Ag-specific protection upon repeated injection (31). In this murine model, whose relevance to our current study may be limited, TNF-induced semimature DCs led to the generation of Ag-specific IL-10-producing T cells in vivo (31). Yet, studies in TNF-deficient mice exposed to recombinant adenovirus reveal impaired virus-specific T cell proliferation and impaired maturation of DCs in draining lymph nodes as compared with wild-type mice (32). In vitro, TNF was required to mature DCs efficiently during virus-mediated stimulation. Furthermore, adoptive transfer of primed, mature DCs results in restored T cell responses and specific humoral responses (32). Thus, by virtue of modulation of DC maturation TNF may play a crucial role in the induction of tolerance or immunity.

The demonstration that TNF promotes DC differentiation in the presence of stromal cells may contribute to our understanding of pathophysiological steps occurring in rheumatoid arthritis (RA), one of the most common autoimmune diseases in humans. Indeed, TNF plays a key role in the pathogenesis of RA and the development of anti-TNF therapy represents a success of immunology (12, 13, 33). TNF, initially thought to originate from in situ M $\phi$ , can be produced locally (likely at high levels) by mast cells (34, 35). Mast cells are also able to release high amounts of IL-4 (36). Given the fact that GM-CSF can be released by a wide variety of cells, including mast cells and synovial fibroblasts (37, 38), all cytokines used in our model may be present at the joint and result in the skewing of newly arriving monocytes into DCs. Indeed, the infiltration of RA synovium with DCs has been demonstrated (39, 40).



Furthermore, consistent with the concept that RA synovium represents an ectopic lymphoid tissue, a distinct localization of both immature and mature DCs can be observed (40). In lieu of our results, by promoting DC differentiation, TNF may facilitate autoantigen presentation and perpetuation of the disease.

Thus, TNF appears as a major factor promoting DC differentiation in tissues. By skewing monocyte differentiation toward DCs and by inducing a partial DC activation/maturation sufficient for the cells to migrate, TNF may facilitate the induction of adaptive immunity and host protection. IL-6, by skewing monocytes newly arriving at the site of tissue damage toward M $\phi$ , may favor innate defense mechanisms and Ag degradation. Our results thus synergize with earlier studies demonstrating the crucial role of TNF in skewing hemopoietic progenitor differentiation toward DCs (41, 42) and show that TNF can act not only on hemopoietic progenitors but also on blood precursors.

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