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Generation of dendritic cells from bone marrow progenitors using GM-CSF, TNF-α, and additional cytokines: antagonistic effects of IL-4 and IFN-γ and selective involvement of TNF-α receptor-1

F. LARDON,* H.-W. SNOECK,* Z. N. BERNEMAN,* V. F. I. VAN TENDELOO,* G. NIJS,* M. LENJOU,* E. HENCKAERTS,* C. J. BOECKXTAENS,† P. VANDENABEELE,‡ L. L. KESTENS,§ D. R. VAN BOCKSTAELE* & G. L. E. E. VANHAM§ **Laboratory of Experimental Haematology, University of Antwerp, †Department of Cardiac Surgery, Antwerp University Hospital, ‡Laboratory of Molecular Biology, University of Ghent, Flanders Interuniversity Institute for Biotechnology, and §Laboratory of Immunology, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium*

SUMMARY

We report the generation of dendritic cells (DC) starting from $CD34^+$ bone marrow (BM) progenitor cells, using a two-stage culture system in which, besides granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor-α (TNF-α), stem-cell factor (SCF) was added during the first 5 days, while interleukin-4 (IL-4) and/or interferon-γ (IFN-γ) were added during the secondary culture period of 9 days. Addition of IL-4 favoured the outgrowth of $CD1a^+$, $HLA\text{-}DR^+$, $CD4^+$, $CD40^+$, $CD80^+$ but $CD14^-$ cells with dendritic morphology and strong antigen-presenting capacity. Addition of IFN-γ selectively induced HLA-DR and CD86 but did not up-regulate CD1a expression or antigen-presenting capacity of the differentiated cells. An antagonism between IL-4 and IFN-γ could further be confirmed in that, as compared with IL-4 alone, the simultaneous addition of IL-4 and IFN-γ to GM-CSF plus TNF-α during maturation reduced both the phenotypical (CD1a, CD4, CD40) and functional characteristics of DC. Using receptor-specific TNF-α mutants, we investigated the relative involvement of TNF-α receptors R1 and R2 in the generation of DC. The induction of CD1a and HLA-DR, as well as the increase in allostimulatory capacity were dependent on TNF-R1 triggering, whereas triggering through TNF-R2 had no measurable effect. We conclude first, that the expansion of DC from BM progenitors could most effectively be enhanced in a two-stage culture assay using SCF, GM-CSF, TNF-α and IL-4; second, that the effect of TNF-α in DC generation involves signalling via the TNF-R1 receptor; and third, that IFN-γ counteracts some of the effects of IL-4 in DC generation.

INTRODUCTION

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the human immune system.[1] The study of their ontogeny, phenotype and function has been difficult, because DC are scarce in any organ or tissue. Recently, many authors have shown that DC can be generated from precursors in liquid culture either directly from bone marrow (BM) progenitor cells or from peripheral blood monocytes. In both cases granulocyte–macrophage colony-stimulating factor (GM-CSF) is required, but is not sufficient in the human system (in contrast to the murine system).[1–4] Addition of tumour necrosis factor-α (TNF-α) is mandatory and further supplementation with interleukin-4 (IL-4) is favourable for DC generation. The combination of the latter cytokines has a dual effect. First, IL-4 suppresses differentiation into monocytes/macrophages, which are characterized by expression of CD14 and low antigen-presenting but high phagocytic capacity. Second, TNF-α enhances the DC maturation pathway; most characteristic are the dendritic morphology, the expression of high levels of major histocompatibility complex (MHC)-, adhesion- and costimulatory molecules (e.g. HLA-DR, CD40, CD80, CD86) and the low or absent phagocytic but strong antigen-presenting capacity.[5–7] One class of DC, namely the Langerhans' cells, have a high CD1a expression, like most *in vitro*-generated DC.[2]

However, even a combination of GM-CSF, stem cell factor (SCF), TNF-α and IL-4 is unable to induce pure DC out of BM progenitor cells.[7,8] One of the reasons could be the requirement of other or additional cytokines for full DC maturation. In this context we wondered whether interferon-γ (IFN-γ) could be important, since it is known to induce both HLA-DR and B7 on myeloid cells.[9] In addition, it is

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Correspondence: Dr F. Lardon, Laboratory of Experimental Haematology, University of Antwerp (UIA/UZA), Wilrijkstraat 10, B-2650 Edegem, Belgium.

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interesting to study the relationship between IL-4 and IFN-γ, since both cytokines play an important and opposing role in the regulation of the immune response.[10]

Finally, we wanted to determine the effects of TNF-α and study its interaction with either one of the TNF-α receptors (CD120a, p55 or TNF-R1, and CD120b, p75 or TNF-R2).[11–16]

MATERIALS AND METHODS

Source of cells

DC were cultured starting from normal human BM progenitor cells. BM samples were aspirated by sternal puncture from haematologically normal patients undergoing cardiac surgery after informed consent. Mononuclear cells were separated on a Lymphocyte Separation Medium density gradient (ICN Biomedicals Inc., Costa Mesa, CA).

Progenitor labelling and cell sorting

Mononuclear BM cells were indirectly stained using supernatant of the 43A1 hybridoma (anti-CD34, kindly donated by Dr H-J. Bühring, University of Tübingen, Germany[17]) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulins (DAKO, Glostrup, Denmark).

The CD34-labelled cells were sorted on a FACStar^{PLUS} cell sorter (Becton Dickinson, Erembodegem, Belgium). Sort windows were set to include cells with low side scatter and with positive green fluorescence ($CD34^+$). Purities of >95% were routinely obtained.

Cytokines

Recombinant human (rh)GM-CSF [specific activity (SA) approximately 10^7 U/mg], rhTNF-α (SA $>10^8$ U/mg), rhSCF (SA $>10^5$ U/mg) and rhIFN-γ (SA $>2\times10^7$ U/mg) were purchased from Boehringer Mannheim GmbH (Penzberg, Germany). Recombinant hIL-4 (SA$=2{\cdot}41\times10^7$ U/mg) was obtained from Genzyme (Cambridge, MA).

TNF-α mutant R32WS86T was used as an agonist of TNF-R1, and mutant D143NA145R as an agonist of TNF-R2.[14,15]

Culture procedures

The $CD34^+$ cells were cultured at 37°, 5% CO_2 and 7·5% O_2, in Iscove's modified Dulbecco's medium (IMDM), containing 10% fetal calf serum (FCS), 1% bovine serum albumin (BSA) (Sigma, Bornem, Belgium) and recombinant growth factors. These consisted of GM-CSF (500 ng/ml), TNF-α (2·5 ng/ml) and SCF (50 ng/ml) during a primary liquid assay of 5 days, and of GM-CSF (500 ng/ml), TNF-α (2·5 ng/ml) and IL-4 (1000 U/ml) and/or IFN-γ (1000 U/ml) during the next 9 days.

For experiments examining the role of TNF-α mutants, either no TNF-α, rhTNF-α, or one of the mutants specific for TNF-R1 or TNF-R2 were used, at a final concentration ranging from 2·5 to 50 ng/ml. TNF-α (mutants) were combined with GM-CSF plus SCF during the first 5 days and with GM-CSF plus IL-4 during the last 9 days of the culture.

After a total culture period of 14 days, the morphology of the cells was observed using an inverted microscope. Cells were harvested for flow cytometric analysis and study of antigen-presenting capacity.

Flow cytometric analysis of phenotype

The following monoclonal antibodies (mAb) were used: CD1a-FITC (Ortho Diagnostic Systems, Beerse, Belgium), CD1a-PE (Caltag Laboratories, San Francisco, CA), CD14-PE, HLA-DR-PE, CD4-PE, CD80-PE (Becton Dickinson), CD40-FITC (BioSource, Zoersel, Belgium), CD86-PE (Pharmingen, San Diego, CA), CD13-FITC (DAKO). Non-reactive antibodies of the same isotype were used as controls: mouse IgG1-FITC, IgG1-PE, and IgG2a-PE (Becton Dickinson).

Immunophenotyping was performed as follows: $2{\cdot}5\times10^5$ cells were incubated with the mAb for 30 min at 4° and washed twice. Stained cells were analysed on a FACScan analytical flow cytometer (Becton Dickinson).

Antigen-presenting capacity

Responder cells were either peripheral blood mononuclear cells (PBMC) or purified resting $CD4^+$ T cells. Blood was always obtained from the same healthy donor and PBMC were prepared by density gradient separation. The $CD4^+$ T cells were purified from PBMC, using a positive selection by CD4 Dynabeads (Dynal, Oslo, Norway), followed by detachment (DETACH-a-BEAD, Dynal). To purify further the resting fraction of the T cells, a negative selection with anti-HLA-DR-coated Dynabeads was performed. $CD4^+$ HLA-DR^- T cells were resuspended at 10^6/ml in RPMI-1640 (Gibco, Paisley, UK) and 5% pooled human serum.

Stimulator cells were resuspended at 10^5/ml and irradiated (6000 Rad). Allostimulation was set up in quadruplicate using 10^5 responder cells and either 10^4, 3×10^3, 10^3, or 3×10^2 stimulator cells in 200 μl of complete medium. As negative controls 10^5 responders or 10^4 stimulators were cultured in medium alone. The microcultures were incubated in a humidified CO_2 incubator at 37°. At day 5, each well was pulsed with 0·4 μCi [^{3}H]thymidine (Amersham, Buckinghamshire, UK) and harvested on glass filter paper 8 hr later. Isotope incorporation was measured by liquid scintillation counting. Negative controls never showed any significant proliferation.

Statistics

In all experiments, Student's *t*-test for paired samples was used. All data are presented as mean±SD.

RESULTS

Expansion and morphology of cultured cells

Sorted $CD34^+$ BM cells were cultured in the presence of SCF+GM-CSF+TNF-α. After 5 days, cells were cultured for another 9 days in the presence of either GM-CSF+TNF-α, GM-CSF+TNF-α+IL-4, GM-CSF+TNF-α+IFN-γ, or GM-CSF+TNF-α+IL-4+IFN-γ.

Figure 1 depicts cells cultured for 14 days in the presence of GM-CSF+TNF-α+IL-4, as seen under an inverted microscope. Both floating round or elongated cells and numerous small aggregates can be distinguished. Higher magnification revealed that many of the cells and the peripheral cells of the aggregates displayed a veiled or dendritic appearance. These clusters of dendritically shaped cells were already present after 7 days of culture, and were abundant on day 14. Strikingly, in all cultures without IL-4, these clusters were much less abundant or even absent.

A mean 10·8±6·3-fold increase in the total number of

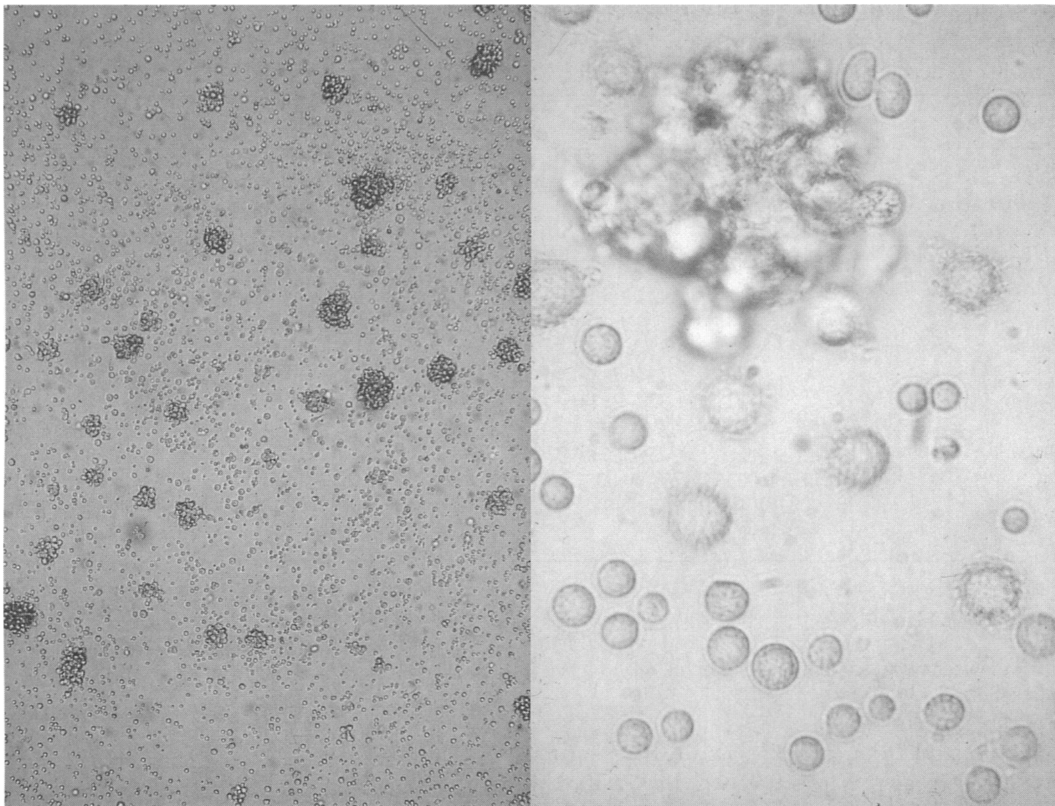

Figure 1. Microphotographs of sorted $CD34^+$ bone marrow progenitors cultured for a primary liquid assay of 5 days in the presence of SCF+GM-CSF+TNF-α and for a secondary liquid assay of 9 days in the presence of GM-CSF+TNF-α+IL-4, as seen under an inverted microscope (left, ×40; right, ×400 magnification). Note the numerous small aggregates (left) and cells with a veiled or dendritic appearance (right).

nucleated cells was observed after the secondary culture in GM-CSF+TNF-α. In cultures supplemented with GM-CSF+TNF-α+IL-4, a mean $10{\cdot}4 \pm 5{\cdot}1$-fold increase was observed, while this was $5{\cdot}9 \pm 3{\cdot}1$-fold and $7{\cdot}2 \pm 4{\cdot}1$-fold after culture in GM-CSF+TNF-α+IFN-γ or in GM-CSF+TNF-α+IL-4+IFN-γ, respectively.

Phenotypic analysis

Immunofluorescence studies were performed in nine different BM-derived cultures. To characterize the phenotype of the cultured cell populations, two-colour flow cytometric analysis was performed using CD1a in combination with CD14, HLA-DR, CD4, CD80, CD86, or CD40. The results of single-colour analyses are summarized in Table 1.

Less than 10% of the cells cultured in GM-CSF+TNF-α expressed the Langerhans' cell-associated marker CD1a and the adhesion/costimulatory molecules CD40, CD80 and CD86. The markers CD4 and CD14 were expressed on 5–20%, whereas HLA-DR was present on 20–60% of the differentiated cells.

The number of $CD1a^+$ cells in cultures supplemented with GM-CSF+TNF-α was significantly increased after addition of IL-4. While addition of IFN-γ to GM-CSF+TNF-α did not significantly alter CD1a expression, addition of IFN-γ to GM-CSF+TNF-α+IL-4 resulted in a lower proportion of $CD1a^+$ cells.

HLA-DR and CD86 were selectively upregulated by addition of IFN-γ to GM-CSF+TNF-α. The number of $CD14^+$ cells tended to be down-regulated by both IL-4 and IFN-γ, although the reduction was only significant when IL-4 was present. In contrast, CD40 and CD80 were up-regulated by both cytokines, but there was no clear-cut additive effect of the simultaneous presence of IL-4 and IFN-γ: the latter even partly inhibited the IL-4-induced increase of CD40. The expression of CD4 was selectively up-regulated by IL-4, whereas IFN-γ tended to reduce CD4 expression.

Thus, the IL-4-induced increase in the number of $CD1a^+$, $CD4^+$ and $CD40^+$ cells was counteracted by IFN-γ.

Next, we evaluated the expression of the different antigens within the $CD1a^+$ subset of the cultured cells (Table 2).

$CD1a^+$ cells generated in GM-CSF+TNF-α co-expressed HLA-DR to almost 100% and were also mainly (>40%) positive for CD4, CD40, CD80, CD86 and CD14. Addition of IL-4 significantly increased the total percentage of $CD1a^+$ cells (Table 1), but dramatically reduced both their proportional CD14 and CD86 expression (Table 2). Expression of CD80 and HLA-DR within the $CD1a^+$ subset was also slightly reduced by IL-4, but the level of CD40 was strongly increased to almost 100%. Addition of IFN-γ to GM-CSF+TNF-α induced no proportional changes within the $CD1a^+$ cells, implying that the increase of HLA-DR and costimulatory markers CD40, CD80 and CD86 mainly occur in $CD1a^-$ cells. Simultaneous addition of IL-4 and IFN-γ reduced CD14 expression (to the same extent as IL-4 alone). Remarkably, the proportion of $CD4^+$ cells within the $CD1a^+$

Table 1. Expression of surface markers on cells cultured for a primary liquid assay of 5 days in the presence of SCF+GM-CSF+TNF-α and for a secondary liquid assay of 9 days in the presence of cytokine combinations as tabulated (1 to 4). Results are expressed as mean % of positive fluorescing cells ±SD

	CD1a⁺	HLA-DR⁺	CD14⁺	CD4⁺	CD80⁺	CD40⁺	CD86⁺
1 GM-CSF+TNF-α	4·9 ±3·8	39·2±19·0	13·8±8·0	11·3±9·6	5·8±3·9	7·6±8·3	5·2±3·6
2 GM-CSF+TNF-α+IL-4	26·0±12·7	46·7±15·1	4·1±4·1	29·2±19·7	14·4±8·3	32·9±13·4	6·5±2·7
3 GM-CSF+TNFα+IFN-γ	4·4±2·3	71·3±18·7	6·4±7·7	6·5±4·8	12·7±9·5	18·4±9·7	21·1±14·7
4 GM-CSF+TNF-α+IL-4+IFN-γ	12·7±11·2	71·7±14·3	3·2±5·0	12·4±10·1	16·5±10·6	20·5±11·4	17·9±8·9
Δ [1,2] (+IL-4)	+21·1±9·8 *P*<0·0001	NS	−9·7±9·5 *P*<0·01	+17·9±13·5 *P*<0·005	+8·6±6·9 *P*<0·01	+25·3±12·3 *P*<0·0001	NS
Δ [1,3] (+IFN-γ)	NS	+32·2±13·8 *P*<0·0001	NS	NS	+6·9±7·7 *P*<0·025	+10·8±8·0 *P*<0·0025	+15·9±12·0 *P*<0·0025
Δ [1,4] (+IL-4+IFN-γ)	+7·8±9·1 *P*<0·025	+32·5±21·0 *P*<0·001	−10·6±10·2 *P*<0·01	NS	+10·7±8·4 *P*<0·01	+12·9±9·2 *P*<0·0025	+12·7±6·3 *P*<0·001
Δ [2,4] (IL-4+IFN-γ)	−13·2±9·3 *P*<0·0025	+25·0±21·6 *P*<0·005	NS	−16·8±14·6 *P*<0·01	NS	−12·4±9·5 *P*<0·0025	+11·4±7·4 *P*<0·001
Δ [3,4] (IFN-γ+IL-4)	+9·3±9·2 *P*<0·025	NS	NS	+5·9±7·1 *P*<0·025	NS	NS	NS

Differences (Δ) between GM-CSF+TNF-α and GM-CSF+TNF-α+IL-4 (Δ [1,2]), between GM-CSF+TNF-α and GM-CSF+TNF-α+IFN-γ (Δ [1,3]), between GM-CSF+TNF-α and GM-CSF+TNF-α+IL-4+IFN-γ (Δ [1,4]), between GM-CSF+TNF-α+IL-4 and GM-CSF+TNF-α+IL-4+IFN-γ (Δ [2,4]), and between GM-CSF+TNF-α+IFN-γ and GM-CSF+TNF-α+IFN-γ+IL-4 (Δ [3,4]) were statistically validated using Student's *t*-test for paired samples ($n=9$, only significant Δ are mentioned, together with their respective *P*-value/NS = not significant, $P>0·05$).

subset was somewhat reduced by both IL-4 and IFN-γ alone, but both cytokines together down-regulated this marker significantly.

Thus, the various combinations of cytokines induced partly distinct, but always heterogeneous cell phenotypes. The most obvious effect of IL-4 was the generation of CD1a⁺CD14⁻ cells, with a proportionally high CD40 but relatively low B7 (CD80 and CD86) expression. IFN-γ, on the other hand, increased the total percentage of HLA-DR-, CD40- and B7-expressing cells, but these markers were mainly expressed on the CD1a⁻ subset.

Analysis of antigen-presenting capacity

The cultured cell populations containing DC, derived from nine different BM samples, were examined for their capacity to stimulate peripheral blood-derived allogeneic CD4⁺ T lymphocytes or PBMC in a one-way mixed lymphocyte reaction (MLR). The results are presented in Table 3.

APC derived from GM-CSF+TNF-α-treated cultures induced limited proliferation in allogeneic PBMC, but stimulated purified T cells significantly more. Generation of APC in the additional presence of IL-4 most dramatically enhanced their allostimulatory capacity. However, addition of IFN-γ to GM-CSF and TNF-α did not alter the stimulatory activity. Addition of IFN-γ to cultures supplemented with GM-CSF+TNF-α+IL-4 resulted in a significant reduction of the allostimulatory response.

Thus, like in the phenotypical analysis, an antagonism was seen between IL-4 and IFN-γ in the alteration of antigen-presenting capacity.

Effects of TNF-α mutants

Using specific TNF-α mutants with selective affinity for either of the two TNF-α receptors, we investigated the relative involvement of the two TNF-α receptors in the generation of DC.

No significant changes in cell numbers were observed. A mean 11·2±5·9-fold expansion was seen after culture in GM-CSF+IL-4+rhTNF-α, while this was 13·8±5·8-fold and 12·9±9·2-fold after culture in GM-CSF+IL-4+TNF-R1 or TNF-R2, respectively.

Figure 2 (a) represents the data from phenotypic analysis (CD1a and HLA-DR) of cells cultured in the presence of GM-CSF+SCF+IL-4 alone, or supplemented with either rhTNF-α, or one of the TNF-α mutants. Figure 2 (b) represents the response of purified CD4⁺ T cells to allostimulation with these various APC ($n=8$).

Culturing progenitor cells in GM-CSF+IL-4 (without TNF-α) induced CD1a and HLA-DR expression (Fig. 2 a), approximately to the same extent as the combination GM-CSF+TNF-α (without IL-4) (Table 1). Substitution of rhTNF-α by the TNF-R1-specific mutant induced an identical phenotype, but the TNF-R2-specific mutant was inactive in this respect.

As could be expected from the analysis of surface markers, GM-CSF+IL-4-treated cells displayed some allostimulatory capacity towards CD4⁺ T cells. This APC-activity was enhanced by rhTNF-α and, to the same extent, by the mutant specific for TNF-R1, whereas the TNF-R2-specific mutant was completely inactive. Both the effect of the TNF-R1-specific mutant and that of rhTNF-α on the phenotype and function were already optimal at a concentration of 2·5 ng/ml. Even a 20-fold higher concentration of the TNF-R2-specific mutant had no influence on phenotype or allostimulatory capacity.

DISCUSSION

Dendritic cell generation from BM progenitor cells requires the co-ordinated action of several cytokines. In the present study, we investigated the specific contribution of TNF-α and

Table 2. Expression of surface markers within the CD1a$^+$ subset, on cells cultured for a primary liquid assay of 5 days in the presence of SCF+GM-CSF+TNF-α and for a secondary liquid assay of 9 days in the presence of cytokine combinations as tabulated (1 to 4). Results are expressed as mean % of positive fluorescing cells within the CD1a$^+$ subset ±SD

	[CD1a$^+$] HLA-DR$^+$	[CD1a$^+$] CD14$^+$	[CD1a$^+$] CD4$^+$	[CD1a$^+$] CD80$^+$	[CD1a$^+$] CD40$^+$	[CD1a$^+$] CD86$^+$
1 GM-CSF+TNF-α	93·1±8·4	42·7±29·1	76·0±20·7	58·2±14·1	59·4±21·2	60·0±22·4
2 GM-CSF+TNF-α+IL-4	83·6±13·9	10·4±11·8	64·8±18·8	43·8±16·2	90·7±3·3	17·9±13·0
3 GM-CSF+TNF-α+IFN-γ	94·4±6·4	25·1±24·0	62·3±22·5	70·2±20·8	58·0±28·1	65·6±21·8
4 GM-CSF+TNF-α+IL-4+IFN-γ	90·8±8·2	14·7±16·2	53·5±21·2	68·8±15·8	60·6±18·9	40·1±12·2
Δ [1,2] (+IL-4)	−9·5±11·8 $P<0·05$	−32·4±25·5 $P<0·01$	NS	−14·5±19·3 $P<0·05$	+31·3±20·9 $P<0·01$	−42·1±22·8 $P<0·005$
Δ [1,3] (+IFN-γ)	NS	NS	NS	NS	NS	NS
Δ [1,4] (+IL-4+IFN-γ)	NS	−28·0±33·4 $P<0·025$	−22·5±30·6 $P<0·05$	NS	NS	NS
Δ [2,4] (IL-4 +IFN-γ)	NS	NS	NS	+25·0±15·1 $P<0·0025$	−30·1±15·5 $P<0·005$	+22·2±20·3 $P<0·025$
Δ [3,4] (IFN-γ+IL-4)	NS	NS	NS	NS	NS	+25·6±14·5 $P<0·005$

Differences (Δ) between GM-CSF+TNF-α and GM-CSF+TNF-α+IL-4 (Δ [1,2]), between GM-CSF+TNF-α and GM-CSF+TNF-α+IFN-γ (Δ [1,3]), between GM-CSF+TNF-α and GM-CSF+TNF-α+IL-4+IFN-γ (Δ [1,4]), between GM-CSF+TNF-α+IL-4 and GM-CSF+TNF-α+IL-4+IFN-γ (Δ [2,4]), and between GM-CSF+TNF-α+IFN-γ and GM-CSF+TNF-α+IL-4+IFN-γ (Δ [3,4]) were statistically validated using Student's *t*-test for paired samples ($n=9$), only significant Δ are mentioned, together with their respective *P*-value/NS=not significant $P>0·05$).

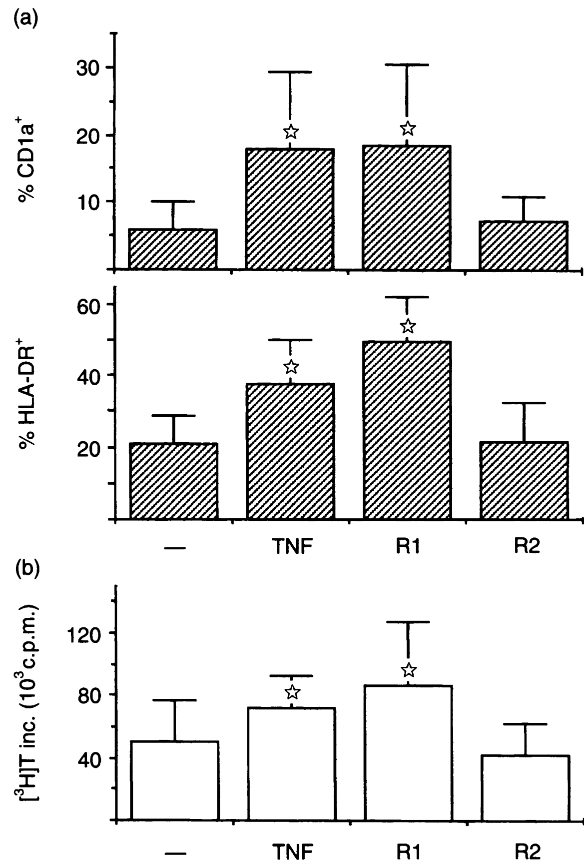

Figure 2. Phenotypical expression of CD1a and HLA-DR (a) and allostimulatory capacity of 10^4 stimulator cells towards 10^5 allogeneic CD4+ T cells (b) of cells cultured for a primary liquid assay of 5 days in the presence of SCF+GM-CSF and for a secondary liquid assay of 9 days in the presence of GM-CSF+IL-4. Both in the primary and the secondary culture phase, either no TNF-α (—), rhTNF-α (TNF), the TNF-R1-specific mutant (R1), or the TNF-R2-specific mutant (R2) was added. Results in (a) are expressed as mean percentage of positive fluorescing cells ±SD ($n=8$). Results in (b) are expressed as mean [^{3}H]TdR incorporation (in 10^3 c.p.m.)±SD, as measured by liquid scintillation counting ($n=5$). *$P<0·05$.

its receptor-specific mutants, as well as the effects of IL-4 and/or IFN-γ in the induction of phenotypic and functional characteristics of DC. Our data clearly indicate that GM-CSF has to be supplemented with both IL-4 and TNF-α to induce optimal expression of CD1a, CD4, CD40 and CD80, as well as optimal allostimulatory capacity. IFN-γ cannot substitute for IL-4 and, in combination with the latter even has a rather negative effect on some aspects of DC generation from progenitor cells, including a lower expression of CD1a and CD40 and a lower allostimulatory capacity. The R2-specific TNF-α mutant was completely inactive, in that it showed neither positive nor negative effects on DC generation over a 20-fold concentration range.

Earlier studies have already shown that the combination of GM-CSF+TNF-α (± SCF) was able to induce DC generation in a number of CD34$^+$ cells, but it was also demonstrated that monocyte-like cells, expressing CD14 and displaying high phagocytic but low antigen-presenting capacity were generated in these cultures.[5–8,18–20] Addition of IL-4 is known to reduce

Table 3. Allostimulatory capacity of cells cultured for a primary liquid assay of 5 days in the presence of SCF+GM-CSF+TNF-α and for a secondary liquid assay of 9 days in the presence of cytokine combinations as tabulated [1 to 4]. Results are expressed as mean [^{3}H]-thymidine incorporation (in 10^3 c.p.m.) ±SD, as measured liquid scintillation counting. Responder cells were either peripheral blood mononuclear cells (PBMC) or $CD4^+$ T cells ($CD4^+T$). The tabulated data are the results from allostimulation using 10^5 responder cells and either 10^4 or 3×10^3 stimulator cells

	PBMC (10^4 stimulator cells)	PBMC (3×10^3 stimulator cells)	$CD4^+T$ (10^4 stimulator cells)	$CD4^+T$ (3×10^3 stimulator cells)
1 GM-CSF+TNF-α	2·6±2·9	1·3±1·2	20·3±15·8	11·3±12·8
2 GM-CSF+TNF-α+IL-4	14·5±13·4	5·6±6·8	42·4±18·7	31·0±17·1
3 GM-CSF+TNF-α+IFN-γ	2·0±1·3	1·3±0·9	23·7±13·5	13·5±12·2
4 GM-CSF+TNF-α+IL-4+IFN-γ	4·9±6·6	2·2±2·5	36·2±21·0	22·1±18·7
Δ [1,2] (+IL-4)	+11·9±13·4 $P<0·025$	+4·3±5·9 $P<0·05$	+22·2±15·7 $P<0·0025$	+19·6±15·3 $P<0·0025$
Δ [1,3] (+IFN-γ)	NS	NS	NS	NS
Δ [1,4] (+IL-4+IFN-γ)	NS	NS	+15·9±12·6 $P<0·005$	+10·9±14·6 $P<0·05$
Δ [2,4] (IL-4+IFN-γ)	−9·5±7·9 $P<0·005$	−3·4±4·8 $P<0·05$	−6·2±9·9 $P<0·05$	−8·7±12·6 $P<0·05$
Δ [3,4] (IFN-γ+IL-4)	NS	NS	+12·6±10·1 $P<0·005$	+8·7±12·2 $P<0·05$

Differences (Δ) between GM-CSF+TNF-α and GM-CSF+TNF-α+IL-4 (Δ [1,2]), between GM-CSF+TNF-α and GM-CSF+TNF-α+IFN-γ (Δ [1,3]), between GM-CSF+TNF-α and GM-CSF+TNF-α+IL-4+IFN-γ (Δ [1,4]), between GM-CSF+TNF-α+IL-4 and GM-CSF+TNF-α+IL-4+IFN-γ (Δ [2,4]), and between GM-CSF+TNF-α+IFN-γ and GM-CSF+TNF-α+IFN-γ+IL-4 (Δ [3,4]) were statistically validated using Student's *t*-test for paired samples ($n=9$, only significant Δ are mentioned, together with their respective *P*-value /NS = not significant $P>0·05$).

the monocytic 'contamination' (as evidenced by the lower number of $CD14^+$ cells) and to further increase the proportion of $CD1a^+$ cells, with high antigen-presenting capacity.[7] In our study, we wanted to know whether supplementation of IFN-γ, alone or in combination with IL-4, to GM-CSF+TNF-α treated progenitor cells would improve DC generation. IFN-γ has been known for a long time for its ability to induce or increase HLA-DR expression on various cell types, to increase B7 molecules in monocytes and to increase antigen-presenting capacity.[21,22] In contrast, it was recently shown that IFN-γ selectively decreased CD80 expression, but not CD40 or CD86 expression, on cultured epidermal Langerhans' cells and reduced their antigen-presenting capacity.[23] The effect of IFN-γ thus seems to vary according to the cell type or maturation stage. In fact, its influence on DC generation in our system was also mixed. Addition of IFN-γ to GM-CSF and TNF-α significantly enhanced HLA-DR and CD86 expression as well as CD40 and CD80. Although it has been repeatedly shown that all of these molecules, and especially the first two, are essential in the MLR induction by myeloid APC,[1] IFN-γ actually did not up-regulate the allostimulatory capacity as compared to GM-CSF+TNF-α-treated APC. One tentative explanation for this paradox is that the percentage of CD1a was not increased and that within this subset the proportional expression of costimulatory molecules was not enhanced by IFN-γ.

Other possible reasons for the lack of beneficial effect of IFN-γ on DC generation include: an increased tendency of APC towards apoptosis[24] and a lowered IL-12 production (because this cytokine is triggered through CD40, the expression of which is lower on $CD1a^+$ cells generated in the presence of IFN-γ as compared to those treated with IL-4).[25]

It is very interesting to note the antagonism of IL-4 and IFN-γ on the function and phenotype of cultured DC. These cytokines often act as natural antagonists of each other's action on cells implicated in the immune response, such as B cells, T cells and macrophages. This antagonism is also important in the reciprocal regulation of the immune response by subclasses of T-helper cells, i.e. Th1 and Th2.[10,26,27] In the present study we have shown that also precursors of DC are susceptible for the antagonistic action of IL-4 and IFN-γ: the effects of IL-4 on both phenotypical expression of CD1a, CD4 and CD40 and on antigen-presenting capacity are counteracted by INF-γ.

Our results using receptor-selective TNF-α mutants suggest that the induction of CD1a and HLA-DR, as well as the increase in allostimulatory capacity, was dependent on TNF-R1 triggering, whereas triggering through TNF-R2 had no measurable effect. It has been proposed that the two TNF-α receptors are each responsible for distinct TNF activities, although a redundancy or antagonism in their functions cannot be excluded.[11] In haematopoiesis, it was shown that the inhibitory effects of TNF-α on primitive BM progenitors were mediated through both TNF receptors, whereas bidirectional effects, seen on more mature cells, were exclusively TNF-R1 dependent.[28] In the present study, we have shown that, starting from BM progenitors, the generation of $CD1a^+$ $HLA\text{-}DR^+$ cells with a high allostimulatory capacity involves signalling via TNF-R1 only. It is therefore most likely that TNF-R1 is the sole mediator of TNF-α-induced signalling for DC maturation. As far as phenotype and antigen-presenting capacity are concerned, TNF-R2 has no measurable effect on the generation and maturation of dendritic lineage cells. Whether this is also true for other features of DC is a subject for further research.

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