# Neutrophil Granulocyte–committed Cells Can Be Driven to Acquire Dendritic Cell Characteristics

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## Summary

Polymorphonuclear granulocytes (PMNs) are thought to fulfill their role in host defense primarily via phagocytosis and release of cytotoxic compounds and to be inefficient in antigen presentation and stimulation of specific T cells. Dendritic cells (DCs), in contrast, are potent antigen-presenting cells with the unique capacity to initiate primary immune responses. We demonstrate here that highly purified lactoferrin-positive immediate precursors of end-stage neutrophilic PMN (PMNp) can be reverted in their functional maturation program and driven to acquire characteristic DC features. Upon culture with the cytokine combination granulocyte/macrophage colony-stimulating factor plus interleukin 4 plus tumor necrosis factor $\alpha$, they develop DC morphology and acquire molecular features characteristic for DCs. These molecular changes include neo-expression of the DC-associated surface molecules cluster of differentiation (CD)1a, CD1b, CD1c, human leukocyte antigen (HLA)-DR, HLA-DQ, CD80, CD86, CD40, CD54, and CD5, and downregulation of CD15 and CD65s. Additional stimulation with CD40 ligand induces also expression of CD83 and upregulates CD80, CD86, and HLA-DR. The neutrophil-derived DCs are potent T cell stimulators in allogeneic, as well as autologous, mixed lymphocyte reactions (MLRs), whereas freshly isolated neutrophils are completely unable to do so. In addition, neutrophil-derived DCs are at least 10,000 times more efficient in presenting soluble antigen to autologous T cells when compared to freshly isolated monocytes. Also, in functional terms, these neutrophil-derived DCs thus closely resemble "classical" DC populations.

Dendritic cells (DCs)[1] comprise a heterogeneous population of professional APCs with particularly potent T cell immunostimulatory capacity (1). Various subtypes can be distinguished that differ in location, stage of maturation, and probably also in their developmental relationship to other hemopoietic cells (2–4).

At least one developmental pathway giving rise to cells with DC characteristics seems to be closely linked to the monocyte/macrophage differentiation system and has recently attracted considerable interest (for review see references 2, 3, 5). DCs of this type, when generated from CD34$^+$ progenitor cells in the presence of GM-CSF and TNF-$\alpha$ undergo a transient stage of development characterized by the expression of CD14 and c-fms (M-CSF receptor) in the absence of CD1. This intermediate stage CD14$^+$CD1$^-$ cell type has bipotential capacity. Stimulation with M-CSF induces acquisition of macrophage characteristics; in the presence of GM-CSF plus TNF-$\alpha$ they give rise to DCs (6, 7).

Similar bipotential capacity has recently also been demonstrated for much further differentiated stages of monocyte/macrophage development, peripheral blood monocytes. Stimulation of blood monocytes with the cytokine combinations GM-CSF plus IL-4 (8, 9) or GM-CSF plus IL-13 (10) was found to induce transition to a DC phenotype in virtually all monocytes; incubation with GM-CSF plus TNF-$\alpha$ in the absence of IL-4 leads to formation of macrophage-like cells (8, 11, 12). Monocytes thought to be committed to macrophage development can thus be driven in one of either two directions. Formation of macrophages with mainly proinflammatory/phagocytic effector cell characteristics, or generation of DC-like cells with professional antigen presenting and limited phagocytic capacity.

Apart from DCs, monocyte/macrophages are also closely related to neutrophil granulocytes. Single cell colony assays demonstrated that both cell types derive from one and the

[1]*Abbreviations used in this paper:* CD, cluster of differentiation; CD40L, CD40 ligand; CML. chronic myeloid leukemia; DC, dendritic cell; HLA, human leukocyte antigen; LF, lactoferrin; md-DC, monocyte-derived DC; MLR, mixed lymphocyte reaction; MPO, myeloperoxidase; PMNp, PMN granulocyte precursor; rh, recombinant human; TT, tetanus toxoid.


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same bipotent progenitor (CFU-GM; references 13, 14) that, upon further differentiation, gives rise to the irreversibly committed monocyte (CFU-M) or granulocyte (CFU-G) lineage (for review see reference 15).

The first morphologically unequivocally identifiable stage of neutrophil granulocyte development is the promyelocyte. This cell type has already a limited growth capacity in semisolid culture systems (16) but can, at least with certain differentiation inducing agents, still be driven to acquire either granulocytic or monocytic features (17, 18).

The later stages of neutrophil differentiation (myelocytes, metamyelocytes, band cells, and end-stage PMNs) are characterized by the presence of specific neutrophilic granules in their cytoplasm and by the expression of the specific granule marker molecule lactoferrin (LF; references 19–21). These late $LF^+$ stages of neutrophil differentiation have so far been considered as being irreversibly fixed in their maturation program towards end-stage PMN development.

Here we show that such immediate granulocyte precursors of end-stage PMN (PMNp) can still be reprogrammed and even be driven to acquire the characteristic phenotypic and functional features of DCs.

## Materials and Methods

### *Media and Reagents*

As a culture medium RPMI 1640 supplemented with 2 mM L-glutamine, 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin was used. Recombinant human (rh)GM-CSF and rhIL-4 were provided by the Novartis Forschungs Institut (Novartis Research Institute, Vienna, Austria). RhTNF-α was a gift of G.R. Adolf (Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria). RhG-CSF was obtained from Hoffmann–La Roche (Basel, Switzerland). Trimeric human cluster of differentiation (CD)40 ligand fusion protein (CD40L) was provided by S.D. Lyman (Immunex Corp., Seattle, WA).

### *Antibodies*

The following murine mAbs used in the study were generated in our laboratory: VIAP (calf intestine alkaline phosphatase specific) nonbinding control, CD1a mAb VIT6b, CD1b mAb 7C4, CD1c mAb 10C3, CD5 mAb 5D7, CD14 mAb VIM13, CD65s mAb VIM2, CD33 mAb 4D3, CD15 mAb VIM16, and CD16 mAb 8B3. The human leukocyte antigen (HLA)-DR mAb L243 was obtained from American Type Culture Collection (Rockville, MD). The mAbs specific for HLA-DQ (clone SK10), CD34 (clone HPCA2), and CD80 (L307) were obtained from Becton Dickinson (San Jose, CA). The myeloperoxidase (MPO)-specific mAb H-43-5, the LF-specific mAb 4C5, the mAb CD117 (c-kit, 95C3), and the mAb CD3 (UCHT-1) were obtained from An der Grub (Bio Forschungs GmbH, Kaumberg, Austria). CD86 mAb (IT2.2) was from PharMingen (San Diego, CA). The CD83 mAb (HB15a) was obtained from Immunotech Inc. (Marseille, France). The mAb specific for CD19 (SJ25-C1) was obtained from Caltag (Burlingame, CA). CD40 mAb (G28-5) was a gift from J.A. Ledbetter (Seattle, WA). Ki-67 mAb (MIB-1) was obtained from Dianova (Hamburg, Germany).

### *Cells*

PBMCs were isolated by standard density gradient centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden) from heparinized whole blood of patients with chronic myeloid leukemia (CML) and of patients with leukocytosis and left shifted differential blood counts due to bacterial infections or G-CSF treatment. CML diagnosis was made on the basis of hematological findings and the presence of the Philadelphia chromosome. Three patients were newly diagnosed, one patient had for 10 d received treatment with hydroxyurea, and one patient was in accelerated phase and previously untreated. Mean leukocyte counts in CML patients were 131,000/μl (range 91,000–153,000). The nonleukemic peripheral blood samples were obtained from two patients with pneumonia, one patient suffering from osteomyelitis and from two donors (one patient with high-grade non-Hodgkin lymphoma in complete remission and one normal donor) who received G-CSF treatment at a dose of 10 μg/kg of body weight on four consecutive days (mean leukocyte count 24,900/μl, range 16,500–28,000).

### *Cell Separation*

PBMCs were washed twice in PBS supplemented with 5 mM EDTA and 0.5% human serum albumin. Subsequently, $VIM16^+$ (CD15) cells were separated by high gradient magnetic sorting using the *VARIO*MACS technique (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). This method has been described in detail elsewhere (22). mAb VIM16 (clone CD15-10C4) was selected for granulocyte isolation from our panel of CD15 mAbs (23) since it selectively binds to normal and malignant granulocytes, but neither to monocytes (23) nor to immature leukemic blast cells, with the exception of very rare cases (W. Knapp, unpublished observation). In brief, PBMCs were incubated with saturating concentrations of biotinylated VIM16 mAb for 15 min on ice, washed in PBS containing 5 mM EDTA and 0.5% human serum albumin, and incubated, thereafter, with colloidal superparamagnetic microbeads conjugated with streptavidin for 10 min at 4°C. Labeled and positively enriched cells were eluted from magnetic columns by removal of columns from the magnetic device. To evaluate the efficiency of the cell separation, aliquots of the positively enriched cell fraction were stained and analyzed by flow cytometry.

Highly purified T cells (purity >97%) were obtained by depletion of all non–T cells by the above described method using mAbs against CD14, CD16, CD19, and HLA class II. Monocytes were enriched essentially as described before (11) by using the biotinylated CD14 mAb VIM13 (purity >95%).

### *Cultivation of Cells*

Isolated PMNp cells were cultured at a cell density of $5 \times 10^5$ cells/ml in standard culture flasks (Costar, Cambridge, MA) in RPMI 1640/10% FCS medium at 37°C in a humidified $CO_2$ containing atmosphere. For induction of cell differentiation, the culture medium was supplemented with rhGM-CSF (550 IU/ml), rhIL-4 (100 IU/ml) and rhTNF-α (50 IU/ml). During culture, cells were not replenished with cytokines. Two PMNp cell samples were cultured in parallel with G-CSF at 1,000 IU/ml. For induction of "terminal" maturation (8, 24), GM-CSF plus IL-4 and TNF-α cultured cells were recovered on day 7, washed, and recultured for a further 2 d with CD40L (200 ng/ml) plus GM-CSF (550 IU/ml) supplemented culture medium.

Monocyte-derived DCs (md-DCs) were generated in the presence of GM-CSF plus IL-4 as described previously (11).

### Morphological Cell Analysis

Freshly isolated VIM16$^+$ cells and cells cultured for 1–13 d were centrifuged onto microscope slides using a centrifuge (Cytospin-2; Shandon Southern Products, Astmoor, UK), stained with May-Grünwald-Giemsa solution and analyzed by light microscopy on a Leitz Aristoplan microscope (Wetzlar, Germany).

### Immunofluorescence Staining Procedures

*Membrane Staining.* For membrane staining, 50 μl of cells ($1 \times 10^7$/ml) were incubated for 15 min at 0–4°C with conjugated FITC, PE, or unconjugated mAb. For staining using unconjugated mAbs, FITC-conjugated $F(ab')_2$ fragments of sheep anti–mouse immunoglobulin antibodies (An der Grub), were used as a second step reagent as described (25).

*Intracellular Staining.* For suspension staining of intracellular antigens, we used the reagent combination Fix & Perm (An der Grub) as described previously (26). In brief, cells are first fixed in fixation medium for 15 min at room temperature and after one washing step, cells are resuspended and mixed with permeabilization medium plus fluorochrome (FITC or PE) -labeled antibody. After a further incubation for 15 min at room temperature, cells are washed again and analyzed.

*Combined Membrane/intracellular Staining.* For combined staining of membrane and intracellular molecules, cells were first stained with FITC-conjugated mAb as described above, and stained thereafter as described for intracellular staining.

*Flow Cytometry.* Flow cytometric analyses were performed using a FACScan® flow cytometer.

### Cell Cycle Analysis

Staining procedures were performed with slight modifications as described previously (27). In brief, cells were centrifuged onto glass slides and fixed with 1% paraformaldehyde in PBS for 2 min at ambient temperature, followed by permeabilization with pure methanol for 10 min at −20°C. For staining, individual slides were incubated either with FITC-conjugated Ki-67 mAb or with the FITC-labeled isotype-matched nonbinding control mAb VIAP for 1 h at room temperature. The fluorescence signal was improved by using FITC-conjugated goat anti–mouse immunoglobulin antibodies (An der Grub) as a second step reagent. After two washes, slides were mounted with glycerol and analyzed on a microscope (Leitz Aristoplan; Wetzlar, Germany).

### Proliferation Assays

A constant number of $10^5$ autologous or allogeneic, highly purified T cells were incubated with graded numbers of irradiated (3,000 rad, $^{137}$Cs source) freshly isolated PMNp or monocytes and cultured PMNp or monocytes. Experiments were performed in 96-well cell culture plates in RPMI 1640 medium supplemented with 10% human serum. Proliferation of T cells was monitored by measuring [methyl-$^3$H]TdR uptake (Amersham, Buckinghamshire, UK) incorporation on day 4 of culture. Cells were harvested 18 h later and radioactivity was determined on a Topcount microplate scintillation counter (Packard Instrument Co., Meriden, CT).

### Tetanus Toxoid Presentation Assay

Presentation of tetanus toxoid (TT; Connaught Laboratories, Willowdale, Ontario, Canada) by neutrophil-derived DCs or monocytes to autologous T cells was performed by coculturing $10^4$ DCs or monocytes with a constant number of $10^5$ highly purified T cells. The cultures were set up in the presence of increasing amounts of TT. Proliferation was measured by [$^3$H]TdR uptake on day 7 of culture. Background counts due to ongoing autologous mixed lymphocyte reaction (MLR) without TT were subtracted.

## Results

### Purification of Neutrophil Granulocytes

Neutrophilic granulocytes were isolated from PBMC fractions of blood samples with high neutrophil counts and

**Table 1.** *Morphological, Cytochemical, and Molecular Characterization of Freshly Isolated VIM16$^+$ Cells*

| Patient No. | Diagnosis | Differential blood count (morphological) | | | | | Esterase (% positive) | Expression of surface/cytoplasmatic molecules (percent positive) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | PMN | BC | MMC | MC | PMC | | VIM16 | MPO | LF | CD14 | CD16 | CD65s | CD34 | CD117 | HLA-DR |
| 1 | CML | 0 | 41 | 34 | 14 | 11 | 0 | 94 | 97 | 62 | 0 | 48 | 97 | 0 | 0 | 1 |
| 2 | CML | 0 | 52 | 26 | 13 | 8 | 0 | 99 | 99 | 57 | 2 | n.t. | 98 | 0 | 0 | 0 |
| 3 | CML | 0 | 97 | 2 | 1 | 0 | 0 | 99 | 99 | 98 | 0 | 92 | 99 | 0 | 0 | 0 |
| 4 | CML | 0 | 90 | 6 | 4 | 0 | 0 | 97 | 98 | 97 | 0 | 90 | 98 | 0 | 0 | 0 |
| 5 | CML | 0 | 92 | 7 | 3 | 0 | 0 | 97 | 100 | 96 | 0 | 75 | 98 | 0 | 0 | 0 |
| 6 | Pn. | 0 | 96 | 3 | 0 | 0 | 0 | 99 | 98 | 98 | 0 | 80 | 99 | 0 | 0 | 0 |
| 7 | Pn. | 2 | 94 | 0 | 0 | 0 | 1 | 95 | 96 | 95 | 1 | 82 | 97 | 0 | 0 | 1 |
| 8 | Om. | 9 | 90 | 1 | 0 | 0 | 0 | 100 | 100 | 99 | 0 | 99 | 99 | 0 | 0 | 0 |
| 9 | N.D. | 0 | 90 | 6 | 2 | 0 | 1 | 99 | 99 | 96 | 2 | 83 | 99 | 0 | n.t. | 1 |
| 10 | NHL | 0 | 91 | 7 | 1 | 0 | 0 | 99 | 99 | 98 | 0 | 84 | 99 | 0 | n.t. | 0 |

*BC*, band cells; *MMC*, metamyelocytes; *MC*, myelocytes; *PMC*, promyelocytes; *CML*, chronic myeloid leukemia; *Pn.*, pneumonia; *Om.* osteomyelitis; *N.D.*, normal donor; *NHL*, non-Hodgkin lymphoma, *n.t.*, not tested.

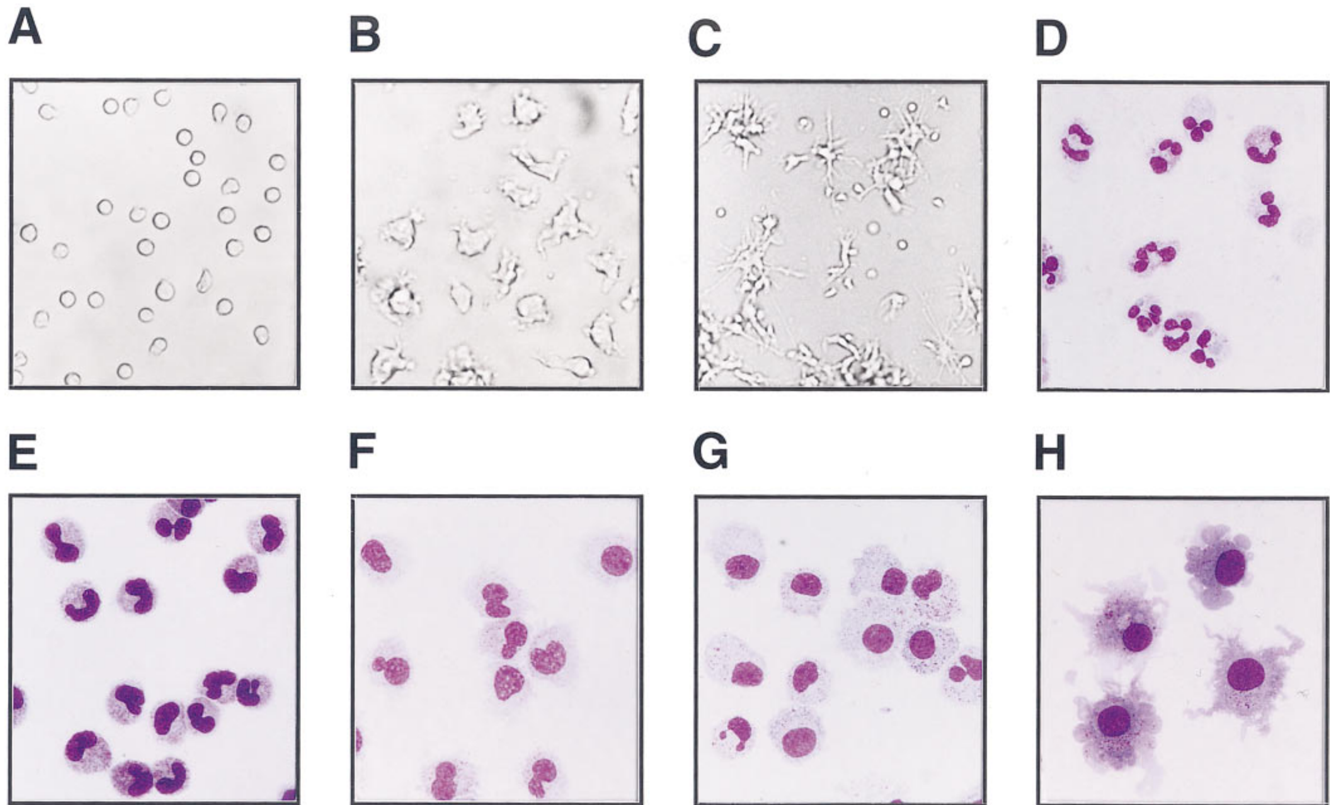


**Figure 1.** Appearance of cell preparations in liquid culture and upon cytochemical staining. Characteristic phase contrast morphology of freshly isolated PMNp showing a homogeneous population of equally sized, round cells (*A*), and after 9 d of culture with medium containing GM-CSF plus IL-4 plus TNF-α, cells uniformly impose with widespread projections and irregular cell forms (*B*). Cells cultured for 7 d with GM-CSF plus IL-4 plus TNF-α and then stimulated with CD40L in the presence of GM-CSF develop long dendrites and form numerous small clusters within 24 h (*C*). Representative photographs of May-Grünwald-Giemsa–stained cytospin preparations: PMNp cultured in G-CSF–supplemented medium for 3 d exhibit polysegmented nuclei typical for end-stage PMNs (*D*). *E* shows freshly isolated PMNp with uniformly rod-shaped nuclei. Between days 3 (*F*) and 6 (*G*), cultured cells develop small nonlobulated round nuclei whereas cell size increases. After a culture period of 9 d, the vast majority of GM-CSF plus IL-4 plus TNF-α–treated cells show widespread cytoplasmic projections (*H*). Most cells contain numerous dense granules (*G* and *H*).

outspoken shifts to the left (five patients with CML, patient No. 1–5; three patients with bacterial infections, patient No. 6–8; two donors treated with G-CSF, patient No. 9 and 10) using a magnetic bead-based positive immunoselection procedure and the mAb VIM16 (CD15, Lewis$^x$ antigen; see Materials and Methods).

The obtained preparations of mAb VIM16-reactive cells were strongly enriched for immediate PMNp and included band cells, metamyelocytes, and myelocytes (Table 1). The achieved purity of neutrophil granulocytes belonging to the maturation compartment of myeloid differentiation is exemplified also by the degree of cells expressing LF, a highly reliable marker molecule for late-stage neutrophil development (20). Of particular importance to our studies, the isolated PMNp preparations contained in all instances no or very few (<2%) monocytes as evaluated by morphology, cytochemistry and CD14 expression (Table 1) and they were virtually free of immature CD34$^+$, CD117$^+$, and/or HLA-DR$^+$ progenitor cells (Table 1). In three of the five CML samples tested (patients No. 3–5) the proportions of LF$^+$ cells were particularly high (97, 96, and 98%, respectively), which makes these samples particularly informative. Also the isolated PMNp samples from nonleukemic patients were highly enriched for LF$^+$ cells (95–99%, the remaining cells were mainly CD3$^+$ T cells, data not shown) and contained almost exclusively band cells (Table 1). Two such cell samples (from patient No. 9 and 10) were cultivated in medium supplemented with G-CSF (1,000 IU/ml) to study their maturation potential. Within

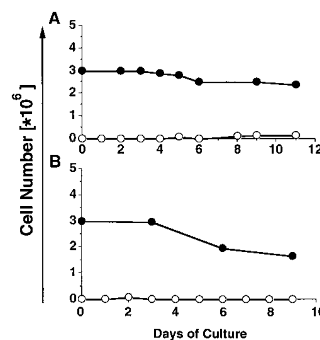


**Figure 2.** Total cell number and number of Ki-67$^+$ cells upon culture of PMNp from CML (*A*) and nonleukemic cell samples (*B*). PMNp were cultivated in RPMI 1640 plus 10% FCS with GM-CSF plus IL-4 plus TNF-α. Total cell number (*black circles*) and number of cells reactive with the FITC-conjugated mAb Ki-67 (*open circles*) were evaluated at indicated time points. The figure shows mean values of independently performed experiments with cell samples from patient No. 3, 4, and 5 (*A*) and patient No. 6, 7, and 8 (*B*).

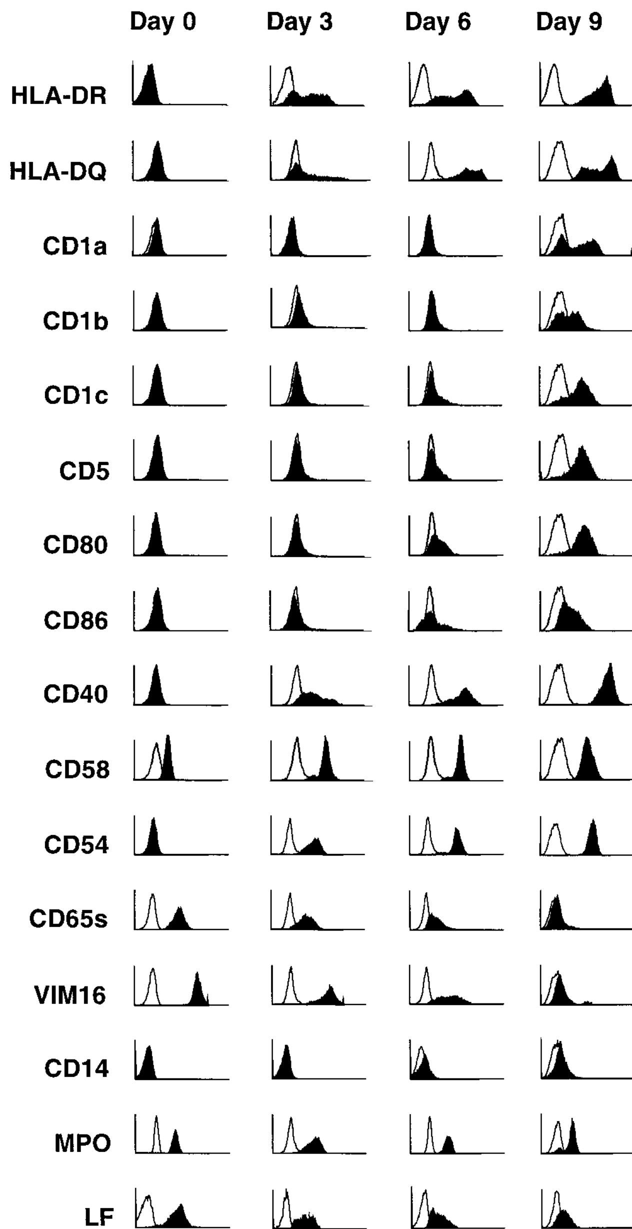


**Figure 3.** Expression kinetics of surface and cytoplasmic molecules upon cultivation of PMNp. The figure shows overlay histogramms of freshly isolated PMNp or PMNp cultured with GM-CSF plus IL-4 plus TNF-α for indicated time periods. Single histogramm profiles of indicated markers are shown of one representative experiment (patient No. 8). *Open profiles*, staining pattern with a nonbinding control antibody; *black profiles*, staining pattern with mAbs of the indicated specificity. *Abscissa*, fluorescence intensity (log 10 scale); *ordinate*, the respective cell number.

2–3 d, these late-stage PMN precursors almost uniformly (>80%) developed polysegmented nuclei typical for end-stage PMNs (Fig. 1 *D*)

### *Morphological Changes and Growth Characteristics during Culture*

Freshly isolated PMNp represented a homogeneous population of equally sized, round cells (Fig. 1 *A*). In

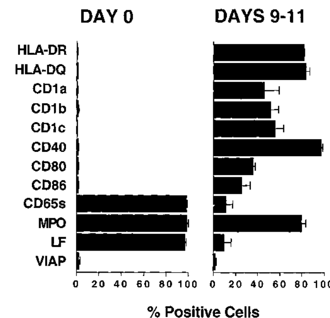


**Figure 4.** Expression pattern of neo-expressed or downregulated differentiation molecules on freshly isolated or cultured PMNp. Expression pattern of differentiation molecules of freshly isolated PMNp (*left*) and after cultivation in GM-CSF plus IL-4 plus TNF-α (*right*) was analyzed by flow cytometry. Binding of mAbs to individual cell samples is represented as percent positive cells (mean values ± SD) calculated from three separate experiments performed with cell samples derived from patient No. 3, 4, and 5.

cytospin preparations, most of their nuclei imposed rod-shaped with plague-shaped chromatin (Fig. 1 *E*). Upon culture in GM-CSF plus IL-4 plus TNF-α supplemented medium, in contrast to G-CSF (Fig. 1 *D*), the isolated PMNp from 10 donors analyzed in this study uniformly grew in size and developed, relative to the cytoplasmic proportion, small nonlobulated round nuclei (Fig. 1 *E*–*H*). The cytoplasm imposed with widespread projections and most cultivated cells contained numerous dense granules (Fig. 1, *G* and *H*). During culture, cells showed little or no tendencies to adhere to the culture plate and formed only a few aggregates (Fig. 1 *B*).

To test for potential mitotic activity, cells were analyzed daily during culture for expression of the proliferation-associated nuclear Ag Ki-67 and incorporation of [methyl-$^3$H]TdR. In PMNp samples obtained from CML patients, only at late stages of culture (from day 8 onwards) and also here only in small proportions of cells (6–8%) Ki-67 expression, known to be present in all phases of the cell cycle with the exception of G0 and early G1, could be detected (Fig. 2). In addition, the number of cells in culture slightly decreased (mean: 16.5% decrease). At variance, only in one of the three tested nonleukemic VIM16$^+$ cell samples, Ki-67 expression (7%) could be detected at day two of culture, correlating with the otherwise undetectable [methyl-$^3$H]TdR uptake (data not shown). In contrast to the leukemic cells, a significant cell loss (40–50%) occurred. The moderate proliferative activity detected in some of the cultured cell samples may represent dividing myelocytes, known to have limited proliferative capacity (16, 28).

Thus, the vast majority of cells recovered and analyzed between days 9 and 11 obviously represent in vitro differentiated nonreplicating cells rather than a replicating subset of input cells.

### *Phenotypic Changes*

*Surface molecules.* Concomitant with the observed morphological changes, profound molecular alterations emerge upon cultivation of purified PMNp. As can be seen from the staining patterns (FACS® profiles) shown in Fig. 3, the first molecular changes to be observed are neo-expression

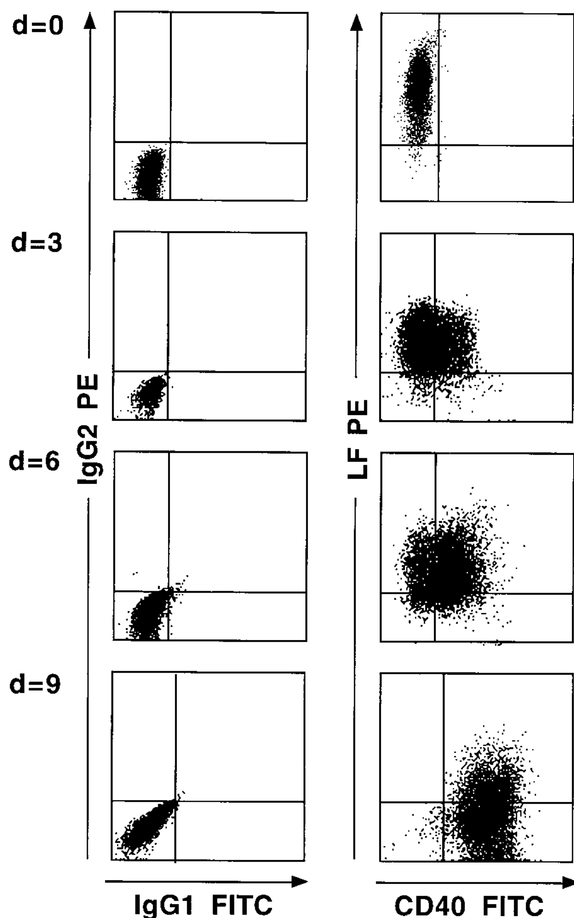


**Figure 5.** PMNp cultured with GM-CSF plus IL-4 plus TNF-α differentiate into CD40-positive cells and loose LF content. PMNp either freshly isolated or cultured were analyzed at the indicated time points. Results are shown as two parameter dot plots. For staining, a directly labeled CD40 mAb (FITC conjugated) and a LF-specific mAb (PE conjugated) were used. As a negative control, nonbinding isotype-matched mAbs were applied. Markers were set according to the fluorescence characteristics of cells incubated with negative control mAbs.

of HLA-DR, HLA-DQ, CD40, and CD54. Expression of HLA-DR and CD40 are detected already on day 1 of culture (3–13% and 30–40% positive cells, respectively) and the number of positive cells constantly increased (day 2 5–40 and 38–75%, respectively). With prolonged culture (days 6–9), several additional DC-related molecules become surface expressed. Among them, all tested members of the CD1 family (CD1a, b, and c), CD80 (B7-1), CD86 (B7-2), and CD5 are observed (Figs. 3 and 4). In parallel, the poly-*N*-acetyllactosamine structures CD65s and CD15 are downregulated (Figs. 3 and 4), as previously reported for GM-CSF plus IL-4 cultured monocytes (11).

*Intracellular Antigens.* Interestingly, upon differentiation of neutrophils during culture, LF content steadily decreased (Fig. 3). This decrease occurred virtually in parallel to a steady increase of CD40 expression (Fig. 3). At an intermediate time point (between days 2 and 6), considerable numbers of cultured cells coexpressed the neutrophil secondary granule protein LF with the costimulatory surface molecule

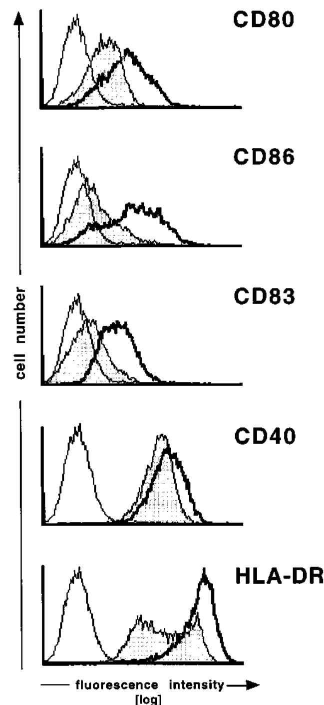


**Figure 6.** CD40L upregulates costimulatory molecules and induces CD83 antigen expression. Expression patterns of PMNp (patient No. 9) on day 9 that were either cultured with GM-CSF plus IL-4 plus TNF-α or PMNp that were replated on day 7 and cultured for a further 2 d in the presence of CD40L plus GM-CSF. Open profiles depict staining pattern with a nonbinding control antibody. *Gray profiles,* PMNp cultured with GM-CSF plus IL-4 plus TNF-α; *open profiles with thick lines,* staining pattern of PMNp cultured with CD40L plus GM-CSF with mAbs of the indicated specificity.

CD40 (Fig. 5). The granulomonocyte-restricted primary granule molecule MPO also decreased upon differentiation, but remained detectable even after prolonged culture period.

### *CD40L-induced Maturation*

The phenotype of cells generated as described above in GM-CSF plus IL-4 and TNF-α–containing medium is reminiscent of immature DCs. We, therefore, attempted to differentiate these cells to mature DCs. For this purpose, GM-CSF plus IL-4 and TNF-α cultured PMNp were harvested on day 7, washed, and then replated in CD40L (200 ng/ml) plus GM-CSF supplemented medium. Under these conditions, PMNp-derived DCs acquired the characteristic features of mature DCs, as has been described before for DCs derived from $CD34^+$ cells (24) and monocytes (8). Surface expression density of HLA-DR homogeneously increased, the costimulatory molecules CD80 and CD86 were significantly upregulated, and clearcut induction of the DC maturation marker CD83 occurred (Fig. 6). When these cells, after 48 h of culture with CD40L plus GM-CSF were washed and replated in medium alone, they remained viable and expression of CD80, CD86, CD83, CD40, and HLA-DR was maintained for at least 4 d (data not shown).

Also in morphological terms, treatment with CD40L plus GM-CSF had a profound impact. Within 24 h, CD40L-treated cells developed long dendrites and formed typical small clusters as has been reported before for DCs derived from $CD34^+$ cells (24; Fig. 1 *C*).

### *Functional Changes*

Potent induction of primary MLR responses is a characteristic functional feature of DCs. We, therefore, compared

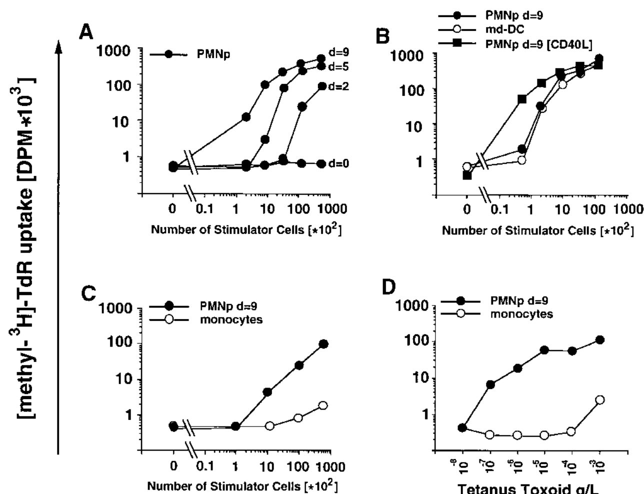


**Figure 7.** T cell stimulatory capacity of PMNp-derived DCs. (*A*) $10^5$ allogeneic highly purified T cells were incubated with graded numbers of irradiated, either freshly isolated PMNp (day 0) or cells cultured with GM-CSF plus IL-4 and TNF-α for 2, 5, and 9 d, respectively. Proliferation of T cells was monitored in all instances by adding [$^3$H]TdR to individual cultures on day 4 of culture followed by measuring incorporated radioactivity 18 h later. Degree of proliferation is indicated as disintegrations per minute $\times 10^3$ on the ordinate. The representative experiment shown here was done with patient No. 3. (*B*) $10^5$ allogeneic highly purified T cells were incubated with graded numbers of GM-CSF plus IL-4 and TNF-α cultured PMNp (PMNp day 9), with md-DCs, or with PMNp that were cultured for 7 d with GM-CSF plus IL-4 and TNF-α and then replated and cultured for 48 h with CD40L plus GM-CSF (PMNp day 9 [CD40L]). The representative experiment shown here was done with patient No. 9.(*C*) $10^5$ autologous highly purified T cells were incubated with graded numbers of cultured PMNp (day 9) or highly purified autologous monocytes. (*D*) Presentation of TT by PMNp cultured for 9 d to autologous T cells was performed by coculturing $10^4$ irradiated PMNp or $10^4$ irradiated autologous monocytes with $10^5$ T cells. The cultures were set up in the presence of increasing amounts of TT. Proliferation of T cells as shown in *B* and *C* was monitored by adding [methyl-$^3$H]TdR to individual cultures on day 6 of culture followed by measuring incorporated radioactivity 18 h later. Background counts due to ongoing autologous MLR without TT were subtracted.

the allostimulatory capacity of both the in vitro–generated cell populations and the freshly isolated cells by using highly purified T cells as responder cells.

Fig. 7 *A* shows a representative experiment comparing freshly isolated PMNp with PMNp cultured in the cytokine cocktail GM-CSF plus IL-4 and TNF-α for different time periods. Freshly isolated PMNp do not induce any allogenic T cell proliferation even at the highest stimulator cell numbers tested (up to $2 \times 10^5$, data not shown). Upon cultivation, the ability of these cells to stimulate T cells constantly increased. A clear [methyl-$^3$H]TdR incorporation into T cells is already detected at higher stimulator cell counts after 2 d of culture. Upon further cultivation and in perfect correlation with increased expression of MHC class II and CD40 molecules (see Fig. 3) and appearance of the costimulatory molecules CD80 and CD86 (approximately days 6–8), cultured cells developed toward highly potent T cell stimulatory cells (Fig. 7 *A*).

As shown in Fig. 7 *B*, the allogenic T cell stimulatory capacity of PMNp cultured for 9 d with GM-CSF plus IL-4 and TNF-α is virtually identical to the stimulatory capacity of md-DCs. Also shown in Fig. 7 is the markedly increased T cell stimulatory capacity of CD40L-treated neutrophil-derived DCs. These cells were first cultured for 7 d with GM-CSF plus IL-4 and TNF-α and then for the last 2 d of the 9-d culture period with CD40L plus GM-CSF. As few as 50 of these CD40L-treated cells were sufficient to induce substantial T cell responses (52,000 disintegrations/min with $10^5$ responder T cells in the experiment shown in Fig. 7 *B*). PMNp cultured for 9 d with GM-CSF plus IL-4 and TNF-α, in contrast to freshly isolated blood monocytes, were also highly effective to stimulate autologous T cells (Fig. 7 *C*).

To test the capacity of cultured PMNp for presentation of soluble antigen, we evaluated these GM-CSF plus IL-4 and TNF-α cultured cells also for their efficiency to present TT to autologous highly purified T cells. As shown in Fig. 7 *D*, cultured PMNp were approximately four logs more efficient APC as compared to autologous monocytes.

## Discussion

Neutrophil PMNs are the most abundant white blood cells and comprise about two thirds of all leukocytes in peripheral blood. In inflammatory conditions, the production of PMNs further increases and also "young forms" representing the immediate precursors of end-stage PMNs (band cells, metamyelocytes, and myelocytes) are released from bone marrow and can be found in blood and tissue (shift to the left; reference 29). Neutrophil granulocytes are the first phagocytes to reach inflamed tissues and play a critical role in phagocytosis and destruction of invading pathogens (30–32). Unlike monocytes/macrophages, the second major phagocyte population, and in contrast to professional antigen-presenting DCs, neutrophil granulocytes are considered as being highly inefficient in presenting antigenic material to T cells and to induce T cell activation. They normally do not express antigen presenting MHC class II molecules and they lack signal 2 providing costimulatory molecules (33, 34).

Here we demonstrate that the immediate PMNp characterized by the presence of specific neutrophilic granules in the cytoplasm and the expression of LF can be drastically shifted in their maturation program. By simply culturing them with selected cytokines (GM-CSF plus IL-4 plus TNF-α), they can be driven to acquire virtually all of the characteristic features of professional APCs.

The combination of these three cytokines seems to be critical. With GM-CSF plus IL-4 alone, shown before to be efficient in driving monocytes to acquire DC characteristics (8, 11), we observed with PMNp in a preliminary series of experiments only limited differentiation along the DC axis (data not shown).

PMNp cultured with GM-CSF plus IL-4 and TNF-α first start to express the MHC class II molecules HLA-DR and HLA-DQ and the accessory molecules CD40 and in-

tercellular adhesion molecule 1 (ICAM-1; CD54). With prolonged culture, several additional APC-related surface molecules are neo-induced. Among them are the costimulatory molecules CD80 (B7-1) and CD86 (B7-2), the DC-related putative antigen-presenting structures CD1a, CD1b, and CD1c (35), and the scavenger receptor family member CD5 known to be expressed by DCs in addition to T cells (11, 36).

Change of medium at day 7 with culturing cells for the last 2 d in the presence of CD40L and GM-CSF induces an even further differentiated phenotype (Fig. 6). Cells cultured in that way exhibit significant CD83 expression and upregulated surface expression of HLA-DR and costimulatory molecules CD80 and CD86. Thus, neutrophil-derived DCs react to CD40L stimulation also, as has been reported before for hemopoietic progenitor cell and monocyte–derived DCs (8, 24).

Concurrent with these molecular alterations, cultured PMNp also acquired functional features highly reminiscent of professional APCs. In contrast to freshly isolated PMNp, which were completely unable to induce a primary MLR response even at high stimulator cell numbers, PMNp cultured with GM-CSF plus IL-4 and TNF-$\alpha$ were potent stimulators of allogeneic (Fig. 7 *A*) as well as autologous T cells (Fig. 7 *C*). In terms of their allostimulatory capacity, PMNp cultured with GM-CSF plus IL-4 plus TNF-$\alpha$ are virtually identical with monocyte-derived DCs (Fig. 7 *B*). Activation of these PMNp-derived DCs with CD40L even further enhances their stimulatory capacity (Fig. 7 *B*). The results of soluble antigen presentation experiments were also particularly striking. PMNp after cultivation were at least 10,000 times more efficient in presenting soluble antigen (TT) to autologous T cells in comparison with freshly isolated autologous monocytes (Fig. 7 *D*)

In parallel to these molecular and functional changes during culture, significant morphological alterations were also evident. PMNp cultured with GM-CSF plus IL-4 plus TNF-$\alpha$ uniformly enlarged and developed relative to the cytoplasmic proportion, small nonlobulated round nuclei. The irregular shaped cells imposed with widespread cytoplasmic projections giving the cells a DC-like appearance. As previously observed in monocyte-derived DCs (8, 11), cultured neutrophils showed few tendencies to adhere to the culture plate and formed only a few aggregates. In contrast, CD40 activation induced extensive aggregate formation, enhanced adhesiveness to plastic, and a remarkable increase of dendrite development (Fig. 1 *C*). A characteristic feature of most cultivated PMNp, not seen before in other in vitro DC differentiation systems, are numerous fine cytoplasmic inclusions (Fig. 1, *G* and *H*). They may represent residual granule structures as indicated also by the observed residual expression of the granule marker molecules MPO and LF.

When taken together, the presented molecular, functional, and morphological findings clearly demonstrate that neutrophil granulocytes have, even at a late stage of maturation, not only maintained the capacity to synthesize and express antigen-presenting MHC class II molecules (33, 34), but can be reprogrammed to acquire virtually all the critical features of DCs.

Such a transition from neutrophil-committed granulocytes to potent APCs is particularly surprising. Neutrophil granulocytes were for a long time thought to fulfill their role as host defense via phagocytosis and release of cytotoxic compounds, but not to actively synthesize proteins or to interact with the T cell system. Recently it was shown that neutrophil granulocytes can synthesize and release immunoregulatory cytokines (37) and can be induced to express MHC class II molecules (33, 34). Our observation that neutrophil granulocytes at very advanced stages of maturation can still be shifted in their developmental program and can be induced to acquire the molecular, functional, and morphological characteristics of DCs, adds to those previous findings a new dimension.

Whether such transition to DC-like cells might, in principle and with better culture conditions, also be possible with fully mature PMNs cannot be said at the moment. Similar to other investigators (38, 39), we were unable to keep end-stage PMNs viable for prolonged periods of time in culture. This precluded us from studying the differentiation potential of PMNs with scrutiny. In a few preliminary experiments we observed, however, induction of MHC class II expression, as previously reported (33, 34) and also weak CD40 expression (data not shown). This might indicate that also fully mature PMNs could potentially be driven to acquire DC characteristics provided that better PMN culture conditions were available. One caveat clearly is, however, that also the in vivo half-life of PMNs is relatively short (1 to 2 d; reference 19) and may not allow substantial alterations of the functional program. Circulating PMNp regularly seen in inflammatory conditions would, therefore, seem to be more likely candidates for such processes to occur.

Concerning the question as to whether neutrophil-committed cells can also in vivo be induced to acquire DC characteristics, we can only speculate. At least in principle, the cytokines used in our in vitro differentiation system should be present at inflammatory sites where monocytes/macrophages, T cells, and potentially mast cells are also accumulating (40–42). Whether these cytokines also quantitatively reach similar levels as used by us in our in vitro system remains, however, to be shown. On the other hand, one also has to envisage that counteractive forces not present in our culture medium may come into action in vivo, which may inhibit DC development. Perhaps of relevance in this respect, are putative differentiation inhibitory factors present in human serum (43–45). A further limitation might be the time period required for DC development from neutrophil granulocytes, which is 6–8 d in our culture system. This clearly exceeds the life time of tissue infiltrating end-stage PMNs (19, 30), but not necessarily so for more immature neutrophils including band cells and metamyelocytes accumulating in blood and tissue in inflammatory disease states (30). During these infection episodes, cytokines are likely to be released that are also present in our culture system.

Independent of these considerations concerning a potential in vivo role, our finding that neutrophils at a late stage

of maturation that are virtually lacking any T cell activating property can, under defined conditions, be driven to acquire the characteristics of professional APCs with potent T cell immunostimulatory capacity, is also of general interest. It clearly demonstrates, that neutrophil commitment is not as irreversible as one has so far been inclined to believe. It, in addition, offers the opportunity to study the molecular basis of (sub)lineage differentiation control. With its two extremes in functional behavior, this model may further help us in the future to gain better insight into the molecular processes involved in the development and regulation of accessory cell function.

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