2 Differentiation of Human Monocytes In Vitro: A Model of Macrophage Ontogeny

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2.1 Introduction

Monocytes are the common "precursor" of the different types of macrophages which are distributed ubiquitous in all tissues. Monocytes and granulocytes both originate from committed progenitor cells in the bone marrow (colony-forming unit granulocyte-macrophage, CFU-GM). Glycoprotein hormones termed colony-stimulating factors [CSF; macrophage CSF (M-CSF), granulocyte-macrophage CSF (GM-CSF)] or interleukin-3 (IL-3; multi-CSF) regulate the differentiation of this stem cell into monoblasts which then differentiate into promonocytes (Metcalf 1991). The promonocyte divides and gives rise to monocytes which

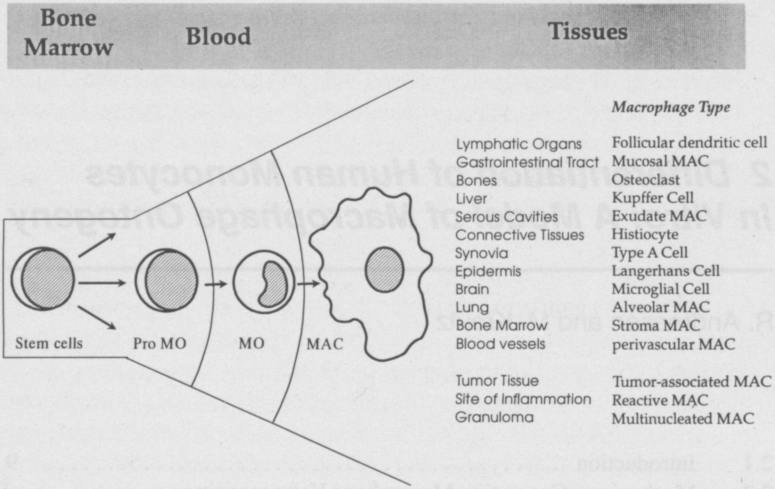

Fig. 1. Schematic description of human macrophage ontogeny

are released into the periphery and circulate for about 2–3 days in the blood stream. Then, upon the action of so far uncharacterized signals, the monocyte leaves the circulation and migrates into tissues and body cavities where it matures into the different types of macrophages, for example, alveolar macrophages in the lung, Kupffer's cells in the liver, and osteoclasts in the bone. Apart from a continuous reconstitution of the various organ subpopulations monocytes also enter infected or malignant tissues as part of the inflammatory response of the host defense system. Here they transform to different forms of reactive histiocytes present, for example, in inflammatory lesions and rejected organ transplants.

Of particular interest and of special importance to the immune surveillance is the ontogeny of tumor-associated macrophages (Mantovani et al. 1992). Here a pivotal role of macrophages becomes evident which is determined by the monocyte to macrophage differentiation process: This results in the generation either of cytotoxic killer cells or of "helper" macrophages appearing as the physiological constituents of the tumor stroma supporting invasive tumor growth and metastasis. A schematic representation of the monocyte differentiation process is shown in

Fig. 1. The signals controlling this second step in the differentiation process are unknown. In addition to this classical pathway of macrophage generation, macrophages can divide in tissues and thereby also renew their population. The signals controlling macrophage proliferation are not defined but M-CSF seems to play a crucial role. In addition, monocytes and macrophages are important effector cells. They produce a variety of cytokines [e.g., IL-1, IL-6, IL-8, IL-10, tumor necrosis factor-α (TNF-α), CSFs, and numerous other factors such as enzyme or reactive oxygen metabolites (Nathan 1987). Other important functions are cytotoxicity against tumor cells, phagocytosis of micro-organisms, and antigen presentation (Johnston and Zucker-Franklin 1988; Unanue and Allen 1987). Most of these functions are dependent on the differentiation and activation stage of monocytes/macrophages.

2.2 Mechanisms Generating Macrophage Heterogeneity: Monocyte Subpopulations and/or Microenvironment

Every tissue has its own type of macrophage; because of the similar morphology and their origin from common precursor cells they are summarized as the "mononuclear phagocyte system". Besides the similarity there are many differences between tissue macrophages (Dougherty and McBride 1984). The question is: how is this heterogeneity generated? One possible explanation is the existence of monocyte subpopulations in the blood dependent on clonal variation of myeloid progenitor cells. Different monocyte subpopulations have been described by several groups. Passlick et al. (1989) discriminate between two populations by the expression of the antigens CD14 and CD16 and their different cytokine production (Ziegler-Heitbrock et al. 1992). Subpopulations have also been determined by size and functional activity (Arenson et al. 1980; Wang et al. 1992), HLA-DR expression (Raff et al. 1980), Fc receptor expression (Zembala et al. 1984), and peroxidase activity (Akiyama et al. 1983). However, these differences are more likely explained by different maturation stages of blood monocytes, depending on the circulation time in the blood stream.

Another way of generating different types of macrophages is the dependence on signals in the microenvironment of the tissue. In vitro it has been shown that different types of macrophages are generated from

monocytes depending on the culture conditions (Munn and Cheung 1990; Ruppert and Peters 1991; Kreutz et al. 1992). Most likely both mechanisms are responsible for macrophage heterogeneity (Rutherford et al. 1993).

2.3 Cell Lines as Model for Monocyte Differentiation

A model system for monocyte maturation is the differentiation of monocytic cell lines. Differentiation of the promyelocytic leukemia cell line HL-60 is induced by 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (Bar-Shavit et al. 1983), retinoic acid (Breitmann et al. 1980), dimethylsulfoxide (Collins et al. 1979), deprivation of essential amino acids (Nichols and Weinberg 1989), phorbolester (Cassileth et al. 1981), TNF-α (Weinberg and Larrick 1987), or combinations of these factors (Trinchieri et al. 1987).

Another cell line, the monoblast leukemia line U937 is induced to differentiate in the presence of phorbolester (Liu and Wu 1992), $1,25(OH)_2D_3$ (Dodd et al. 1983), interferon-γ (Ralph et al. 1983), or combination of those factors. The differentiation process is followed by the expression of antigens (e.g., CD14), morphology, adherence, or functions such as phagocytosis or lysozyme secretion. These markers are, however, specific for normal monocytes, and therefore this differentiation is comparable to that of monoblasts/promonocytes into monocytes rather than the terminal differentiation of monocytes into macrophages.

2.4 Monocyte Differentiation Induced by Serum

The in vitro differentiation of human blood monocytes might serve as a model for the in vivo maturation process of emigrating monocytes. Monocytes which are cultured for 7 days in the presence of human serum differentiate into macrophages (Musson 1983; Andreesen et al. 1983a). In many respects, for example, morphology and functional activity, these cells resemble reactive histiocytes and steady state tissue macrophages. Figure 2a shows the morphology of freshly isolated mononuclear cells and Fig. 2b mononuclear cells cultured for 7 days in

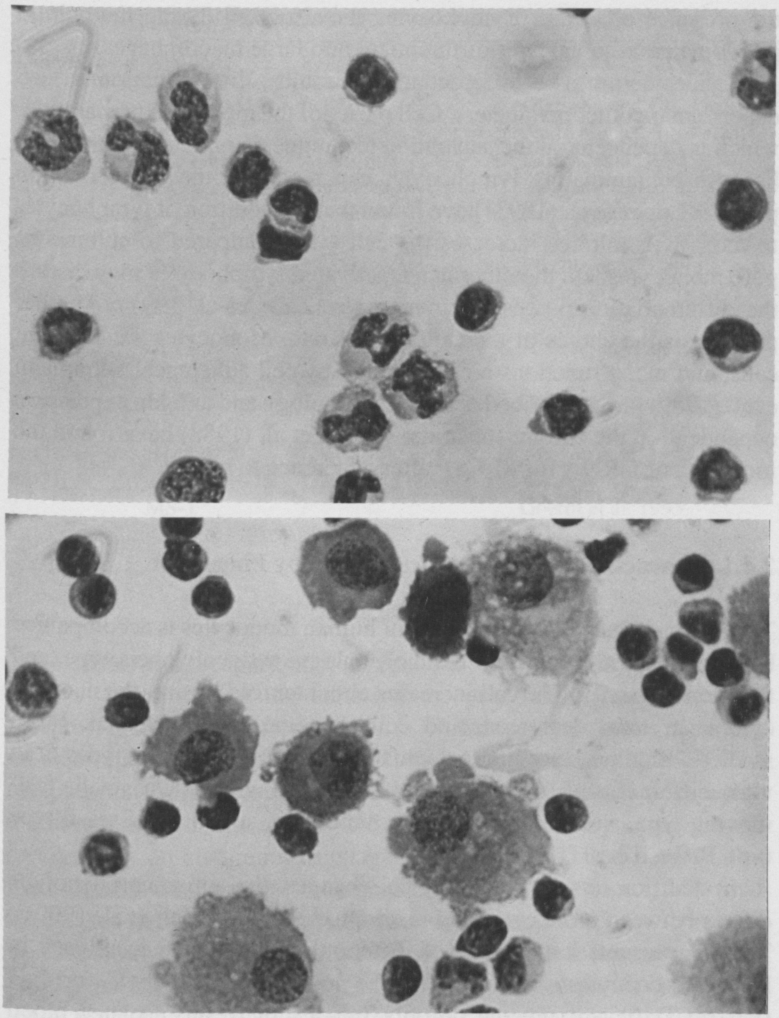

Fig. 2a,b. Human mononuclear cells (600). **a** Freshly isolated from peripheral blood. **b** After 7-day culture with 2% AB blood group (hydrophobic Teflon foils)

the presence of serum. Lymphocytes remain small during the culture period whereas monocytes differentiate into large macrophages.

Besides serum as a differentiation stimulus, differentiation is also dependent on other parameters. Cell purity of the monocyte preparation, which is dependent on the separation technique, is one important factor because contaminating lymphocytes can modulate the differentiation process. Lopez et al. (1993) have found that the addition of lymphocytes to monocyte cultures increases the cell yield compared to cultures of pure monocytes. On the other hand, activated lymphocytes may disturb the differentiation process of monocytes (Zaiss et al. 1991). Another problem is the choice of the culture substrate. Monocytes are adherent cells, and many functions are modulated by cell adherence. Schumann et al. (1989) have described distinct morphology and antigen expression dependent on the culture substrate; Haskill et al. (1988) have found the induction of mRNA for M-CSF after adherence to plastic.

2.4.1 Characterization of Differentiation by Phenotype

The serum-induced differentiation of human monocytes is accompanied by characteristic changes in the morphology, antigenic phenotype, and functional activity. The cell increases about tenfold in size, the nucleus: cytoplasm ratio decreases, and cells become multinucleated. From studies with time-lapse microcinematography three different types of in vitro differentiated macrophages can be distinguished: a small, fast-moving type, an elongated, slow-moving type, and a round sessil type (von Briesen et al. 1992).

In addition to the morphological changes, the antigenic phenotype differs between monocytes and macrophages (Andreesen et al. 1990a). Table 1 presents a summary of differentiation-associated antigens in human monocytes/macrophages. The low-affinity receptor for IgG (FcIII, CD16) is expressed on only 2%–5% of freshly isolated blood monocytes but is found consistently on in vitro differentiated macrophages and resident liver and spleen macrophages (Clarkson and Ory 1988; Andreesen et al. 1990a). Endoglin, an arginine/glycine/aspartic acid (RGD) containing surface antigen, is also absent from peripheral blood monocytes but is detectable on monocyte-derived macrophages and interstitial macrophages in the red pulp of the spleen (Lastres et al.

Table 1. Maturation-associated antigens on human macrophages

Antigen	Antibody	Function	Reference
CD16	e.g., GRM1	FcRIII	Clarkson and Ory 1988, Andreesen et al. 1990a
Endoglin	44G4, 8E11	Adhesion?	Lastres et al. 1992
CD51	13C2, 23C6	Vitronectin receptor (α-chain)	Krissansen et al. 1990, Andreesen et al. 1990a
gp175	–	Mannose receptor	Ezekowitz and Stahl 1988
gp86	25F9	?	Zwadlo et al. 1985
gp64	MAX.1	?	Andreesen et al. 1986, 1988a
gp200	MAX.2	?	Andreesen et al. 1986, 1988a
gp68	MAX.3	?	Andreesen et al. 1986, 1988a
gp116/46/38	B148.4	?	Anegon et al. 1993
CD71	e.g., MEM-75	Transferrin receptor	Andreesen et al. 1990a
?	MS-1	?	Goerdt et al. 1993

1992). RGD is a recognition motif for adhesion receptors of the integrin family. A member of the adhesion receptor family, the vitronectin receptor, is also absent from the surface of monocytes but is expressed on macrophages (Andreesen et al. 1990a; Krissansen et al. 1990). This receptor may be involved in apoptosis induction. The mannose receptor, a lectin that mediates uptake and killing of micro-organisms, is expressed only on mature macrophages (Ezekowitz and Stahl 1988). This may explain the fact that macrophages are better effector cells than monocytes in the phagocytosis of micro-organisms.

An unknown 86-kDa protein is recognized by the mouse monoclonal 25F9. Again, monocytes do not express this antigen, but it is found on tissue macrophages such as Kupffer's cells, alveolar macrophages, and monocyte-derived macrophages (Zwadlo et al. 1985). Another set of unknown proteins are recognized by the antibodies of the MAX series. These antigens are absent from monocytes and are expressed on exudate-macrophages from pleural and peritoneal cavity (Andreesen et al. 1988a) and on in vitro differentiated macrophages (Andreesen et al. 1986). The MS-1 antigen is also found on in vitro differentiated mono-

cytes/macrophages and on dendritic perivascular macrophages in situ (Goerdt et al. 1994). The opposite regulation is found for the human antigen B18.4, which is highly expressed on monocytes but is lost during the differentiation into macrophages (Anegon et al. 1993). All these antigens are expressed after in vitro differentiation of monocytes with serum and can therefore serve as maturation markers. CD4, an antigen which is involved in infection with the human immunodeficiency virus (HIV), also seems to be expressed at higher density on macrophages; this may explain the finding that macrophages are better targets for HIV than are monocytes (own unpublished results). Other antigens such as the CD14 molecule are found on monocytes as well as on macrophages. The transferrin receptor, CD71 and ICAM-1 (CD54) also are absent from monocytes. However these antigens are also induced under serum-free culture conditions by means of adherence; therefore they are differentiation- but not serum-dependent antigens (Andreesen et al. 1984).

2.4.2 Characterization of Differentiation by Functional Activity

Morphology and antigen-phenotype are excellent parameters of the maturation process of monocytes into macrophages. In addition, mature macrophages are characterized by their functional activity. A well-known activity of macrophages is the destruction of tumor cells. Three types of cytotoxicity have been described: antibody-dependent cellular cytotoxicity (ADCC), direct contact-dependent cytotoxicity, and cytotoxicity mediated by soluble secreted molecules such as TNF-α. All three types of cytotoxicity depend on the differentiation stage of macrophages. Munn and Cheung (1989) have found that cultivation of monocytes with M-CSF increases ADCC and contact-dependent cytotoxicity against U937 increased during the maturation of monocytes into macrophages (Andreesen et al. 1983b, 1988b). A soluble mediator of cytotoxicity, a newly described tumoricidal activity termed MCT-170, is secreted only by macrophages (Harwix et al. 1992). The capacity to secrete TNF-α is increased several-fold during the in vitro differentiation of human monocytes; in contrast, the secretion of IL-1β and IL-6 is decreased (Wewers and Herzyk 1989; Scheibenbogen and Andreesen 1991).

Table 2. Comparison of functional activity in monocytes and macrophages

Function	Monocyte	Macrophage	Reference
Cytotoxicity			
ADCC	+	+++	Munn and Cheung 1989
Antibody independent	+	+++	Andreesen et al. 1988b
Procoagulant activity	–	+++	Scheibenbogen et al.1992
secretion			
IL-1β	+++	(+)	Wewers and Herzyk 1989
IL-6	+++	++	Scheibenbogen and Andreesen 1991
IL-8	++	++	Scheibenbogen and Andreesen 1991
TNF-α	+	+++	Scheibenbogen and Andreesen 1991
M-CSF	+	+++	Scheibenbogen et al.1990
G-CSF, GM-CSF	+	+++	Krause et al. 1992
Neopterin	+	+++	Andreesen et al. 1990b
1,25 Vitamin D3	+	+++	Kreutz et al. 1993
24,25 Vitamin D3	+++	–	Kreutz et al. 1993
Fibronectin	+	+++	Yamauchi et al. 1987
Phagocytosis	+	+++	Jungi and Hafner 1986
Antigen presentation	++	+	Peters et al. 1987
Enzyme activities			
Nonspecific esterase	++	+++	Musson et al. 1980, Andreesen et al. 1983b
Tartrate-resistant acid phosphatase	–	+++	Andreesen et al. 1983b
Peroxidase	+++	–	Andreesen et al. 1983b
Lysozyme	+	+++	Andreesen et al. 1983b

Furthermore, the production of CSFs (M-CSF, G-CSF, GM-CSF; Scheibenbogen et al. 1990; Krause et al. 1992), neopterin (Andreesen et al. 1990b), fibronectin (Yamauchi et al. 1987), tissue factor (Scheibenbogen et al. 1992), and lysozyme (Musson et al. 1980; Andreesen et al. 1983a) is increased during monocyte differentiation. Vitamin D metabolites are also produced by monocytes/macrophages: 24,25-dihydroxyvitamin D_3 is synthesized only by monocytes whereas macrophages release $1,25(OH)_2D_3$ (Kreutz et al. 1993). Another important macro-

phage function is the processing and presentation of antigen to T-lymphocytes. Schlesier et al. have described monocytes as good antigen-presenting cells; however, this capacity decreases when monocytes mature into macrophages (Peters et al. 1987; Schlesier et al. 1992). In contrast, phagocytosis is improved during monocyte differentiation (Jungi und Hafner 1986). Also, enzyme activities are changed during the differentiation of monocytes into macrophages. Peroxidase activity is lost, whereas tartrate-resistant acid phosphatase is induced during monocyte maturation; Nonspecific esterase activity remains constant (Andreesen et al. 1986). A summary of maturation-associated functions is shown in Table 2.

2.5 Modulation of Serum-Induced Differentiation

The serum-induced differentiation of monocytes into macrophages can be modulated by supplementing the serum with additional factors. Te Velde et al. (1988) have described phenotypical and functional changes in serum containing monocyte cultures supplemented with IL-4. IL-4 induces HLA-DR expression and a decrease in the release of cytostatic and chemotactic factors compared to control cultures with serum alone. As these features are normally regulated in the opposite way during differentiation, IL-4 seems to inhibit differentiation rather than to induce it. IL-13, another factor produced by CD4-positive T-lymphocytes, also increases HLA-DR expression and induces morphological changes of human monocytes (McKenzie et al.1993).

Apart from the regulation of monocyte generation in bone marrow, CSFs also play a role in the further differentiation process of monocytes into macrophages. Addition of M-CSF and GM-CSF to serum results in a better survival rate and stimulates the capacity for antibody-dependent and antibody-independent cytotoxicity (Suzu et al. 1989; Young et al. 1990; Munn and Cheung 1990; Eischen et al. 1991). In addition, both CSFs regulate the expression of the maturation-dependent vitronectin receptor (Nichilo and Burns 1993). GM-CSF has also been shown to induce CD1 expression on monocytes, a marker which is normally found on thymocytes and Langerhans' cells. This indicates a differentiation in the direction of accessory cells (Kasinrerk et al. 1993).

$1,25(OH)_2D_3$, known to induce the differentiation of monocytic cell lines, also supports the serum-induced differentiation of monocytes. Provvedini et al. (1986) have reported an accelerated differentiation, in terms of increased activity of lysosomal enzymes and enhanced adherence, when monocytes were cultured in the presence of $1,25(OH)_2D_3$. In mouse bone marrow macrophages $1,25(OH)_2D_3$ and immunoglobulins increased the expression of the maturation-associated mannose-receptor (Clohisy et al. 1987; Schreiber et al. 1991).

2.6 Differentiation Under Serum-Free Conditions

It is difficult to identify differentiation-inducing signals when serum is used as medium supplement because serum alone is sufficient to induce maturation of human monocytes. Therefore serum-free culture conditions, for example, serum-free media have been developed by several investigators (Helinski et al. 1988; Vogel et al. 1988; Vincent et al. 1992). Most of those serum-free media contain albumin. In contrast, Akiyama et al. (1988) found no positive effect of albumin on monocyte differentiation, but reported that immunoglobulins can induce differentiation in terms of increased 5' nucleotidase activity and decreased peroxidase activity. $1,25(OH)_2D_3$ alone as well as in combination with immunoglobins and albumin is also able to induce monocyte differentiation under serum-free conditions (Kreutz and Andreesen 1990; Kreutz et al. 1992). These cells express differentiation-associated antigens of the MAX series and show functional features of mature macrophages, i.e., high release of TNF-α and neopterin. However, the effect is dependent on the culture substrate; in Teflon culture the addition of immunoglobulin/albumin or M-CSF/albumin is necessary to promote cell survival. Other investigators have also found a positive effect of M-CSF or GM-CSF on cell survival in Teflon cultures (Brugger et al. 1991; Lopez et al. 1993). However, also M-CSF also improve the survival rate on plastic surfaces (Becker et al. 1987). As endogenous M-CSF induction is dependent on cell adherence, monocyte survival in Teflon culture may be diminished because of the weak adherence, for example, an unsufficient induction of endogenous M-CSF.

2.7 Inhibition of Monocyte Differentiation

Differentiation in monocytic cell lines can be induced by cell activators such as interferons and phorbolester. In contrast, the serum-induced differentiation process of blood monocytes is inhibited by activation signals. Interferon-γ, a typical inducer of monocyte/macrophage activation, suppresses monocyte differentiation antigens and increases HLA-DR expression (Firestein and Zvaifler 1987; Andreesen et al. 1990a). Two other T-lymphocyte derived cytokines, IL-4 and IL-13, have been reported to induce monocyte differentiation in terms of increased HLA-DR expression. In addition, cells cultured in the presence of IL-4 are less cytotoxic than control cells, indicating inhibition rather than induction of monocyte differentiation. Therefore, lymphocytes seem to play a crucial role in regulating monocyte differentiation and activation. Another macrophage activator, lipopolysaccharide, is also shown to inhibit monocyte differentiation (Brugger and Andreesen 1991). This indicates that monocyte activation and differentiation seem to be two, noncompatible, mutually exclusive processes.

2.8 Summary

Differentiation of human monocytes into macrophages is the central step in the generation of the heterogenous cell family that constitutes the mononuclear phagocyte system. The in vitro maturation of monocytes is a model only for a complex process which involves (a) signals leading to the migration of the monocyte into tissues and (b) signals which determine the characteristic sunpopulation of macrophage given for a given tissue. Furthermore, cells other than monocytes/macrophages or cytokines released by these cells may contribute to the differentiation process. Certainly not all signals which interfere with monocyte differentiation are known; the knowledge of all differentiation-modulating substances would allow the "design" to generate a special type of macrophage in vitro, for example, for tumor cytotoxicity or antigen presentation, for clinical purposes.

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