PHENOTYPICAL ANALYSIS OF MURINE MACROPHAGE DIFFERENTIATION

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PHENOTYPICAL ANALYSIS OF MURINE MACROPHAGE DIFFERENTIATION

FENOTYPISCHE ANALYSE VAN MACROFAAG-DIFFERENTIATIE IN DE MUIS

PROEFSCHRIFT

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Voor Bernadet, Margriet
en mijn ouders

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

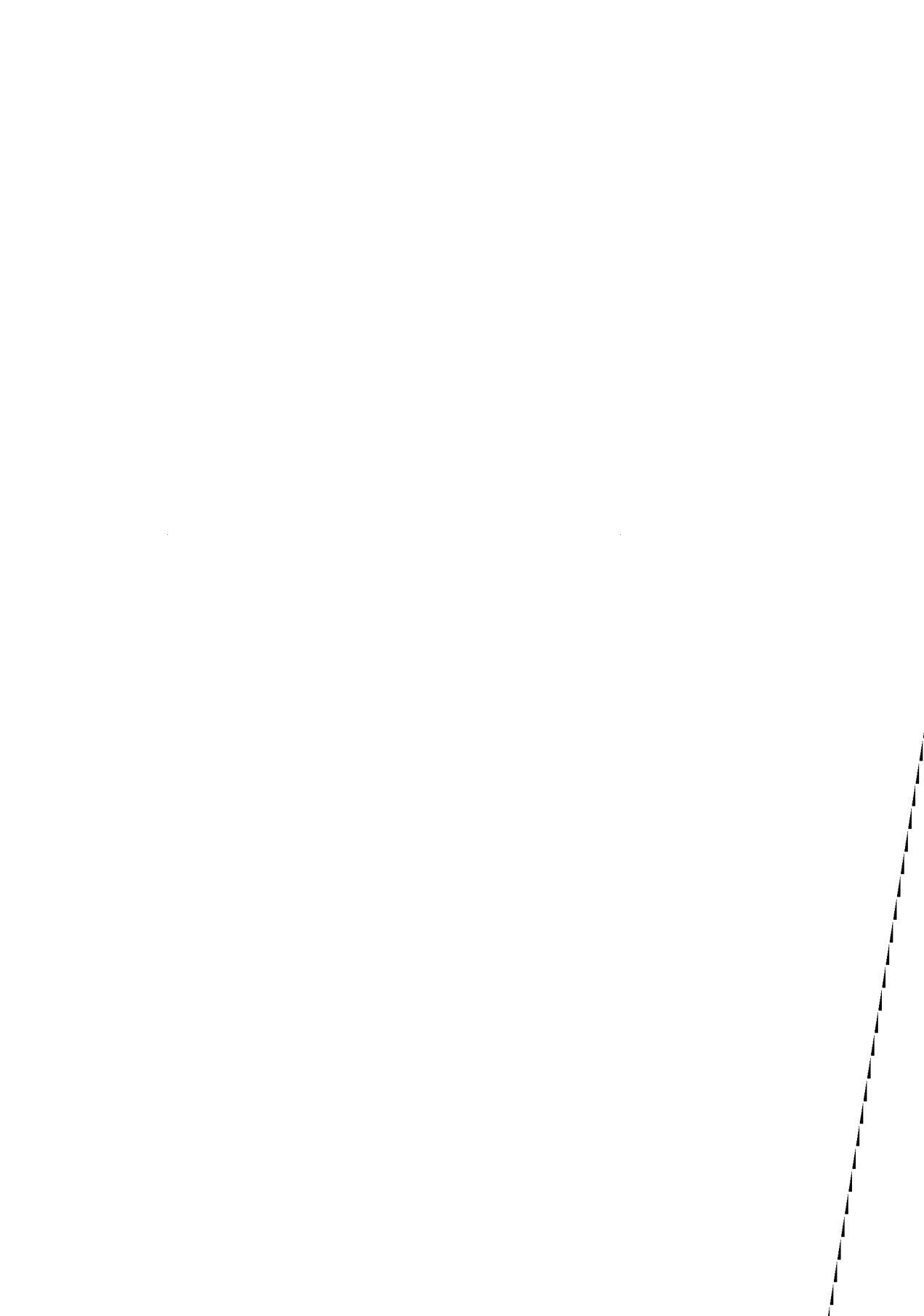
1. GENERAL INTRODUCTION

Hemopoietic cells perform many vital functions in the maintenance of homeostasis and integrity of the body. These functions include, among others, the transport of oxygen by erythrocytes, and the initiation of blood clotting by thrombocytes at sites of injury. Furthermore, hemopoietic cells are the primary effector cells in the defense against micro-organisms and tumor cells. This defense is mainly carried out by two different cell types: **lymphocytes** and **phagocytes**. The distinction between these cell types is, among other things, based upon the different mechanisms that lymphocytes and phagocytes use to recognize foreign material. Lymphocytes recognize invading substances by highly specific antigen receptor molecules which are different and specific for each antigen. Mononuclear and polymorphonuclear phagocytes, on the other hand, discern their foreign targets by receptor molecules with much broader specificity, such as lectin-like receptors recognizing carbohydrate moieties.

From an evolutionary point of view, phagocytes are the most 'ancient' cells in host defense. All multicellular animals contain amoeboid phagocytic cells; in some invertebrates such cells are even the only blood cells present. In general, increasing evolutionary ancientry coincides with increasing phenotypical diversity. The heterogeneity among phagocytes, and especially among the mammalian mononuclear phagocytes, clearly demonstrates the validity of this general rule.

In this thesis, an analysis is presented of the phenotypical differentiation of murine mononuclear phagocytes, i.e. macrophages and their precursors. The term 'differentiation' in this context includes both the process of maturation and of diversification. It should be realized, however, that the term 'differentiation' is also generally used as equivalent to maturation only.

In the next section an introductory overview is given of our current knowledge of macrophage differentiation. The literature cited in this overview primarily deals with studies performed with mice as experimental animals, since the subsequent chapters describe results of experimental studies using murine cells.



CHAPTER 2

INTRODUCTION TO THE DIFFERENTIATION OF MACROPHAGES

(continued)

2. INTRODUCTION TO THE DIFFERENTIATION OF MACROPHAGES

MACROPHAGE DIFFERENTIATION: DEVELOPMENT FROM HEMOPOIETIC STEM CELLS

Hemopoietic precursor cells

In adult mice, bone marrow and spleen are the main sites of hemopoiesis (Metcalf, 1984; 1988). As shown in figure 2.1, blood cell formation appears to be highly hierarchically organized. All hemopoietic cell types are derived from the same so-called 'pluripotent hemopoietic stem cell' (PHSC; Metcalf, 1984; 1988; Lemischka et al., 1986). These PHSC have - within the functional hemopoietic compartment - the highest capacity of both self-renewal and generation of progeny. Derived from PHSC, three main hemopoietic precursor compartments can be distinguished with increasing maturity and differentiation commitment (Ogawa et al., 1983; Metcalf, 1984; 1988):

1. stem cells with multipotential - but not pluripotential - differentiation capacity;
2. committed progenitor cells, which are predetermined to differentiation in only one or two hemopoietic lineages, and
3. immature morphologically distinguishable cells.

The size of each compartment increases strongly with increasing maturity of the cells: stem cells occur at a frequency of approximately $0.2 \times 10^3 / 10^5$ bone marrow cells (BMC), progenitors at $1.5 \times 10^3 / 10^5$ BMC, and proliferating morphologically recognizable cells at $12 \times 10^3 / 10^5$ BMC (Metcalf, 1984). It should be recognized, however, that classification of hemopoietic precursor cells in these three rigid compartments represents an oversimplification. Rather, hemopoietic cells form a continuum of cells with decreasing capacity of self-renewal and increasing commitment to terminal differentiation (Grossman, 1986).

For detection of the various stem- and progenitor cells, both in vivo and in vitro methods are available. Previously, the in vivo CFU-S assay - i.e. the intravenous transfer of hemopoietic cells into lethally irradiated mice which gives rise to splenic colony formation (Till and McCulloch, 1961) - was assumed to detect the most immature, pluripotent stem cells (Metcalf, 1984). Recently, however, it has been shown that the majority of CFU-S are not pluripotential, but already committed to one or a few hemopoietic lineages (Ploemacher and Brons, 1988a). Thus, the CFU-S assay does not detect the ultimate, pluripotential stem cell. The detection of PHSC in vivo - shown by secondary transplantation as so-called pre-CFU-S (Ploemacher and Brons, 1988b; 1989) -

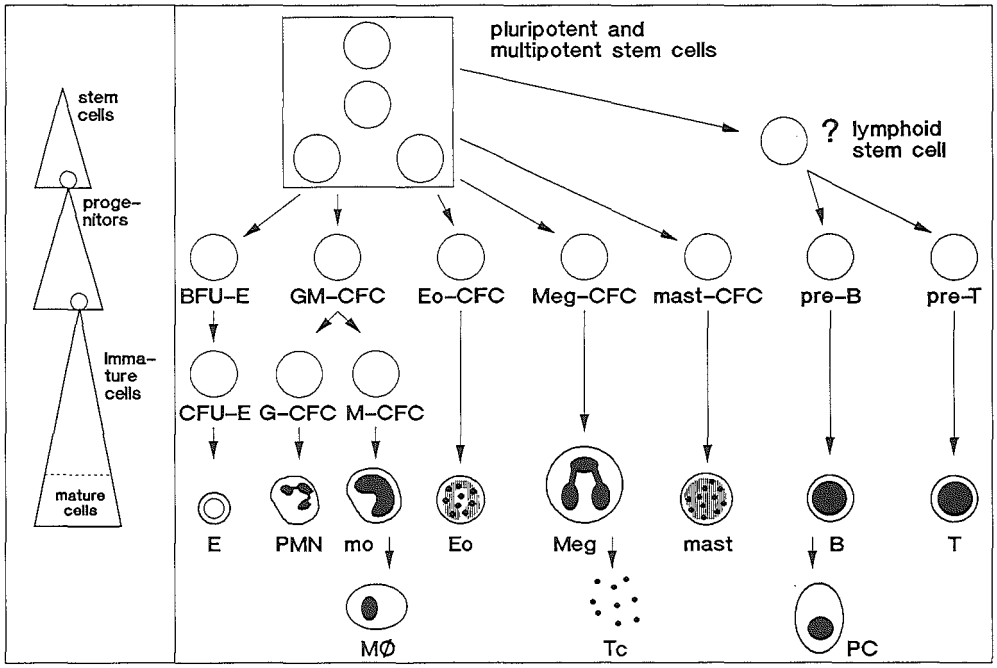


Figure 2.1.

Current view on hemopoietic differentiation in the bone marrow. BFU-E = burst forming unit - erythroid; CFU-E = colony forming unit - erythroid; E = erythrocyte; GM-CFC = granulocyte / macrophage colony forming cell; G-CFC = granulocyte CFC; M-CFC = macrophage CFC; PMN = polymorphonuclear granulocyte; mo = monocyte; Mϕ = macrophage; Eo-(CFC) = eosinophil (CFC); Meg-(CFC) = megakaryocyte (CFC); Tc = thrombocytes; PC = plasma cell. (after Metcalf, 1984; modified).

must therefore be based on their ability to provide long-term reconstitution of lethally irradiated animals in all hemopoietic lineages. In vitro, PHSC can be detected by their ability to maintain hemopoiesis in long-term bone marrow cultures (Dexter and Spooncer, 1987). Furthermore, clonal assays, based on culture of the cells in semi-solid media containing different hemopoietic growth factors, are available for virtually all stem- and progenitor cells in the various hemopoietic lineages (Metcalf, 1984; 1988). Culture systems designed specifically for the study of macrophage precursor differentiation will be mentioned in more detail in a following section.

Bone marrow monocyte formation

Commitment of hemopoietic stem cells towards the mononuclear phagocyte lineage - which comprises macrophages and their morphologically recognizable bone marrow

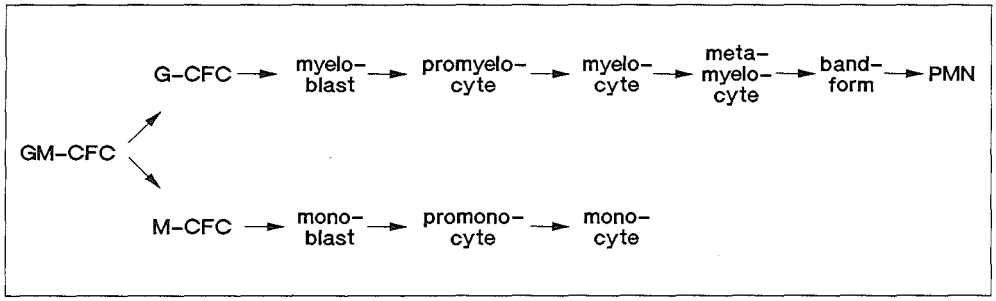


Figure 2.2.
Stages of the granulocytic and monocytic differentiation in the bone marrow. GM-CFC = granulocyte / macrophage colony forming cell; G-CFC = granulocyte CFC; M-CFC = macrophage CFC.

precursors - coincides for a great deal with commitment towards the granulocyte lineage (see fig. 2.1). Thus, a bipotential progenitor cell, the granulocyte-macrophage progenitor, exists. This cell can be identified in vitro as CFU-GM (colony-forming unit granulocyte-macrophage; Metcalf and Burgess, 1982; Metcalf, 1984). In the commonly accepted view (see fig. 2.2), further commitment separates the granulocyte and macrophage lineages, giving rise to the restricted granulocyte colony-forming cells (G-CFC) and macrophage colony-forming cells (M-CFC; Metcalf and Burgess, 1982; MacVittie, 1984).

The most immature cell with unequivocal mononuclear phagocyte characteristics is the monoblast (Goud et al., 1975; van der Meer et al., 1983). The monoblast population may partially overlap with the M-CFC population. In a recent analysis of available kinetic data (Novak et al., 1989), it was noticed that the bone marrow M-CFC population is 5 - 6 times larger than the reported monoblast population (Goud and van Furth, 1975). On the basis of this finding, the authors hypothesized that the majority of M-CFC is in the 'dormant' G_0 stage of the cell cycle and might be activated by an acute inflammatory stimulus (Novak et al., 1989). The bone marrow monoblast is thought to divide only once and thus gives rise to two promonocytes (Goud and van Furth, 1975). The promonocyte, in its turn, also divides once, which leads to the generation of two bone marrow monocytes (van Furth and Diesselhoff-den Dulk, 1970; van Furth et al., 1985a). Finally, monocytes leave the bone marrow compartment and enter the blood stream (van Furth and Cohn, 1968).

Questions about the differentiation commitment of macrophages

Although the conventional scheme of hemopoietic differentiation in the bone marrow, as outlined above, is generally accepted at present, a number of experimental

data question the proposed rigidity of macrophage differentiation commitment. In this respect, two issues deserve attention, namely the separation between the granulocyte and macrophage differentiation pathways, as well as the relationship between mononuclear phagocytes and other hemopoietic lineages.

Numerous studies using murine and human tumor cell lines, characterized as leukemic (pro)myelocytes, revealed that these cells have retained the capacity to differentiate into both macrophages and granulocytes (de Both et al., 1981; Collins, 1987; Zinzar et al., 1989). Moreover, normal human promyelocytes and more mature granulocytic stages could be induced to monocytic differentiation under the influence of interferon-γ (IFN-γ; Perussia et al., 1983). Even cells as mature as metamyelocytes and bandforms were susceptible for induction of monocytic differentiation by IFN-γ (Perussia et al., 1983). Therefore, these data suggest that also granulocytic stages beyond the CFU-GM are capable of development into macrophages.

In the conventional view of hemopoietic differentiation - as depicted in figure 2.1 - macrophages only share a common precursor at the progenitor cell level with granulocytes. Hence, the ultimate precursor cell shared with other hemopoietic lineages must be a multipotent stem cell. However, multiple cell lines with unequivocal B-lymphocytic characteristics show the capacity of macrophage differentiation with no sign of other lineages involved (Boyd and Schrader, 1982; Klinken et al., 1988; Davidson et al., 1988; Kinashi et al., 1989; Hanecak et al., 1989). This suggests the existence of a common progenitor cell with restricted differentiation capacity for the B-lymphocytic and macrophage lineage. Moreover, the human promyelocytic cell line HL-60 - which has the capacity to differentiate into macrophages and granulocytes upon appropriate stimulation - appears to express immunoglobulin light chains (Stavnezer et al., 1986). Together, these findings suggest that the B-lymphocytic and macrophage lineages are more closely related than previously recognized.

Similarly, the macrophage lineage possibly has a closer linkage to the mast cell lineage than previously assumed. This suggestion is based on an immunophenotypical relationship (Valent et al., 1989), as well as on the demonstration of mast cell differentiation in vitro from elicited rat peritoneal macrophages (Sterry and Czarnetzki, 1982).

Therefore, although the bone marrow origin of blood monocytes is beyond doubt, questions remain about the precise relationship of these cells with other hemopoietic lineages at the progenitor level.

Tissue mononuclear phagocytes

Once in the blood, monocytes are distributed over a circulating and a marginating

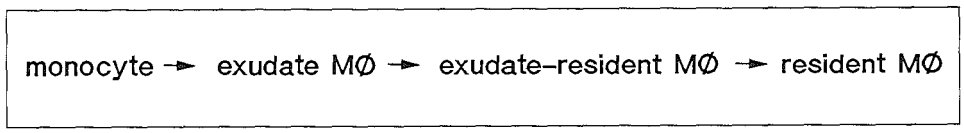


Figure 2.3.
Final stages of the differentiation of monocytes into resident macrophages. Upon entrance of the various tissues, monocytes are thought to traverse multiple developmental stages before resident macrophages (Mϕ) are formed.

pool, the latter most likely being attached to the vascular endothelium (van Furth et al., 1985a; van Furth and Sluiter, 1986). After an average sojourn of 17.4 hr in the circulation, monocytes leave the blood stream randomly by passage of the endothelial lining of blood vessels. Upon entering the various tissues, monocytes undergo their final differentiation steps into tissue macrophages (van Furth and Cohn, 1968; van Furth et al., 1972). On the basis of different patterns of endogenous peroxidase expression, **exudate macrophages** and **exudate-resident macrophages** have been identified as intermediate stages in the differentiation of monocytes into **resident tissue macrophages** (represented in fig. 2.3; van der Meer et al., 1979a; Beelen and Fluitsma, 1982). Actually, all different tissues contain characteristic resident macrophage populations (see table 2.I for a brief survey; cf. van Furth, 1980). In the steady state, resident macrophages occur both freely as well as fixed in the tissue of most organs.

As a response to an inflammatory stimulus, monocytes are recruited from the circulation and develop into distinct inflammatory macrophage types, generally called **exudate macrophages** (van Furth, 1980; van Furth et al., 1985a; Melnicoff et al., 1989). Moreover, in chronic inflammatory lesions (granulomata), macrophages with characteristic morphological features are present, viz. epithelioid cells and multinucleated giant cells (Turk and Narayanan, 1981; Williams and Williams, 1983). As outlined above, exudate macrophages have the capacity to develop into resident macrophages through the intermediate exudate-resident stage (Beelen and Fluitsma, 1982; Hoefsmit et al., 1986).

Dendrocytes

The integration of **Langerhans cells** (LC), **veiled cells** (VC) and **interdigitating cells** (IDC) in the mononuclear phagocyte system - partially based on the results presented in chapter 9 and additionally discussed in chapters 10 and 11 - merits further attention because of the disputed origin of these cells. Most likely, LC, VC and IDC are closely

Table 2.I Peripheral tissue mononuclear phagocyte populations

normal steady state	
connective tissue	histiocytes
bone	osteoclasts
nervous tissue	microglial cells
skin epithelium	Langerhans cells
bone marrow	fixed macrophages, central macrophages
spleen	free and fixed macrophages, interdigitating cells
thymus	fixed macrophages, interdigitating cells
lymph and lymph nodes	free and fixed macrophages, veiled cells, interdigitating cells
liver	Kupffer cells
lung	alveolar and interstitial macrophages
serous cavities	free macrophages
synovial membrane	type A cell
inflammatory response	
	exudate macrophages
	exudate-resident macrophages
	granuloma macrophages
	epithelioid cells
	multinucleated giant cells

related. Austyn (1987) proposed the term "**dendrocytes**" for these cells. In addition to LC, VC and IDC, **dendritic cells** (DC) may be included in the dendrocyte population as DC are the likely in vitro equivalents of the IDC (Breel et al., 1987).

In general, two different origins are proposed for dendrocytes. Firstly, dendrocytes might constitute a separate hemopoietic lineage, distinct from mononuclear phagocytes (Austyn, 1987). Arguments for this view are the lack of phagocytic activity of dendrocytes, their failure to proliferate in vitro, a constitutive expression of MHC class II antigens and their unique functional specialization as stimulators of primary immune responses. Moreover, Janossy et al. (1986) reported that cells with a dendritic cell phenotype and 'classical' macrophages develop as separate lineages in ontogeny. A second, alternative view proposes a monocytic origin of dendrocytes, implicating incorporation of these cells in the mononuclear phagocyte system. The latter view is supported by the observation that interdigitating cells may become actively phagocytosing

under certain experimental conditions (Duijvestijn et al., 1982; Fossum et al., 1984). Moreover, Peters et al. (1987) showed that monocytes differentiate in vitro into cells with morphological and functional dendrocyte characteristics upon culture in the presence of selected serum batches or serum-free media. Similar findings were recently reported by Kabel and coworkers (1989). On the basis of their intermediary characteristics, intra-epithelial Langerhans cells are likely in vivo intermediates between monocytes and dendrocytes (Hoefsmit et al., 1982; Hume et al., 1983; Schuler and Steinman, 1985).

Mature mononuclear phagocyte kinetics

Another point of longstanding debate in macrophage biology regards the proliferative capacity of mature macrophages. This issue relates to the question whether resident macrophage populations are maintained either by local division or by influx of blood monocytes. Previously, several authors have argued in favour of independent maintenance of macrophage populations by local division (Daems and de Bakker, 1982; Sawyer et al., 1982). However, proliferation of mature macrophages was hardly observed and thus regarded unlikely by others who favoured the 'monocyte influx' view (van Furth and Cohn, 1968; van Furth, 1980). More recently, however, it has been recognized that also relatively mature macrophages, such as peritoneal exudate macrophages and resident alveolar macrophages can proliferate in vitro (van der Meer et al., 1985; Lin et al., 1989). Evocation of local stress conditions in vivo, such as partial hepatectomy or transplantation of tumor cells, appeared to induce a strong proliferative response of the Kupffer cells and the macrophages present in the tumor, respectively (Bouwens et al., 1984; Stewart, 1983). Moreover, additional data on steady state in vivo macrophage kinetics revealed that local proliferation does significantly contribute to macrophage population maintenance, though in different degrees dependent on the macrophage population examined (van Furth and Diesselhoff-den Dulk, 1984; van Furth et al., 1985a; Tarling et al., 1987). Hence, both monocyte influx and local macrophage proliferation appear to be involved in resident macrophage population maintenance.

Humoral control of macrophage development

The formation of monocytes in the bone marrow is accurately coordinated by a complex regulatory network (for recent reviews see Bender et al., 1986; Hume et al., 1987; Metcalf, 1988; 1989). It has become clear that macrophage development comprises distinct levels: i.e. proliferation, differentiation commitment as well as maturation of macrophage progenitor cells. Several hemopoietic growth factors appear to be active on multiple levels of this process (see also table 2.II).

Table 2.II Cytokines involved in macrophage development

factor	MW (kDa)	main sources	actions on precursors and mature Mϕ
M-CSF	70-90, 45-50	fibroblasts, Mϕ	proliferation, differentiation, activation
GM-CSF	18-30	T cells, Mϕ, fibroblasts	proliferation, differentiation, activation
IL-1	17	multiple	synergy with other cytokines
IL-2	25	T cells	proliferation, differentiation, activation
IL-3	15-30	T cells	proliferation, differentiation
IL-4	16-20	T cells	differentiation, activation
IL-6	19-21	multiple	synergy with other cytokines
IFN-α/β	17-25	fibroblasts, leukocytes	differentiation
IFN-γ	38-80	T cells	differentiation
FIM	20	Mϕ	proliferation
TNF	17	Mϕ, T cells	proliferation, differentiation
LIF	58	multiple	differentiation

The role of the hemopoietic colony-stimulating factors - **M-CSF, GM-CSF, G-CSF** and **IL-3** (multi-CSF) - in macrophage development has been elaborated to the largest extent. Recent molecular cloning of the genes encoding these glycoproteins has greatly facilitated their characterization (Metcalf, 1988). IL-3 and GM-CSF stimulate in vitro the formation of both macrophage and granulocyte progeny (Metcalf, 1988; 1989). The actions of M-CSF and G-CSF, on the other hand, lead to relatively pure macrophage or granulocyte progeny, respectively (Metcalf and Burgess, 1982; Metcalf, 1988; 1989). Macrophage progenitors, generated in the presence of M-CSF, most likely mature by virtue of endogenously produced **IFN-ß** (Warren and Vogel, 1985; Moore et al., 1985a; 1985b). M-CSF itself is inevitable for survival of mononuclear phagocytes (Tushinski et al., 1982).

In vivo, CSFs tend to act in concert rather than apart. In this respect, M-CSF has been shown to act synergistically with GM-CSF and/or IL-3 in macrophage colony formation in vitro (Williams et al., 1987; Falk and Vogel, 1988; McNiece et al., 1988). Furthermore, **IL-1** and **IL-6** contribute to macrophage generation by synergistic activity with M-CSF, GM-CSF and IL-3 (Bartelmez and Stanley, 1985; Stanley et al., 1986; Hoang et al., 1988; Ikebuchi et al., 1987; Wong et al., 1988; Bot et al., 1989).

In addition to the CSFs and synergistic activities mentioned above, other factors have been demonstrated to exert stimulating activity in macrophage generation. **FIM** - factor increasing monocytopoiesis - is primarily produced in an inflammatory response.

It enhances the production of monocytes by stimulating proliferation of progenitors that are already committed to the macrophage lineage (van Waarde et al., 1977; Sluiter et al., 1987). The T-cell growth factor **IL-2** was convincingly shown to stimulate the proliferation and differentiation of highly purified macrophage precursor cells in vitro (Baccarini et al., 1989). However, application of IL-2 in cultures of unseparated bone marrow cells appeared to inhibit macrophage colony formation significantly (Naldini et al., 1987). The latter effect is presumably caused by IL-2-mediated activation of inhibitory cells or, alternatively, inhibition of accessory cells in macrophage colony formation. **IL-4** has an indirect stimulating effect on hemopoietic precursors (Peschel et al., 1987; Broxmeyer et al., 1988) and appears to induce the maturation of monocytes into macrophages (te Velde et al., 1988). Proliferation of relatively mature bone marrow-derived macrophages is stimulated by **TNF** - tumor necrosis factor -, though only in synergy with M-CSF (Branch et al., 1989). In addition, TNF as well as **IFN-γ**, induce monocytic differentiation in granulocyte / macrophage progenitors (Murphy et al., 1988; Perussia et al., 1983). Another factor, which differentiation-inducing activity has only been demonstrated in leukemic myeloid cells, is **LIF** - leukemia inhibitory factor - (Gearing et al., 1987).

In addition to the cytokines mentioned above - which are glycoproteins of at least 10 kDa - factors of other chemical nature have been shown to stimulate macrophage development as well. **1,25-Dihydroxyvitamin D3**, a steroid which is the biologically active form of vitamin D3, promotes bone marrow macrophage precursor maturation in vitro (Clohisy et al., 1987). Furthermore, inflammatory peptides such as **tuftsin**, **substance P** and **neurotensin**, consisting of only a few amino acids, act as costimulants with M-CSF by activating a distinct macrophage progenitor pool (Babcock et al., 1983; Moore et al., 1988; 1989). Similarly, **LPS** - lipopolysaccharide derived from Gram-negative bacteria - plays a major role in vivo in inflammatory responses, most likely by stimulation of the production of hemopoietic growth factors (Staber and Burgess, 1980; Lotem and Sachs, 1985).

In contrast, inhibition of macrophage progenitor cell proliferation has been reported for **acidic isoferritin** (Broxmeyer et al., 1981; Guimaraes et al., 1988), **corticosteroids** (Shezen et al., 1985), **prostaglandin E_2** (PGE_2; Moore et al., 1984; 1985a; 1985b) **IFN-α/ß** (Moore et al., 1984; 1985a; 1985b; Rigby et al., 1985) and **IFN-γ** (Perussia et al., 1983; Rigby et al., 1985; Coutinho et al., 1986). However, for all of the latter factors it has been shown that reduced proliferation coincides with increased maturation of the cells. Furthermore, inhibition of progenitor cell proliferation in stages prior to the committed granulocyte / macrophage progenitor is caused by **TGF-ß** - transforming growth factor-ß (Keller et al., 1988; Strassmann et al., 1988).

Recently, more knowledge has become available about the intracellular signalling

pathways of macrophage growth and differentiation factors. Expression of protein kinases as coded by the oncogenes c-fms - which is identical to the receptor for M-CSF - and c-mos, appears to play an important role in this respect (Sherr et al., 1988; Farrar et al., 1988; Kurata et al., 1989). Detailed elaboration of this issue lies, however, beyond the subject of this thesis.

Only recently the effect of various cytokines has been studied in vivo, either by administration of purified (recombinant) factors or by construction of transgenic animals (Kindler et al., 1986; Metcalf et al., 1987; Broxmeyer et al., 1987; Lang et al., 1987; Hume et al., 1988). In general, these in vivo studies confirm the results previously observed in vitro. However, these results are obtained with individual cytokines and do not yet reveal the - major or minor - significance of each of the factors in macrophage differentiation in the steady state in vivo. Overall, from the multitude of factors listed above, it becomes clear that macrophage differentiation is a complex process, carefully regulated at multiple levels.

MACROPHAGE DIFFERENTIATION: GENERATION OF DIVERSITY

Macrophage functions

Macrophages perform a large variety of functions contributing to different aspects of host defense (summarized in table 2.III).

Endocytosis

The macrophage's best known function is endocytosis, i.e. phago- and pinocytosis, of micro-organisms, effete cells, debris and other waste products (Cohn and Steinman, 1982; van Oss, 1986). Macrophages express different receptor types to discriminate 'non-self' or 'altered-self': lectin-like receptors bind to carbohydrates on target cells and foreign molecules; other receptors which recognize the Fc part of immunoglobulins or complement components bind to immune complexes and opsonized targets (Gordon et al., 1988a; Kinet, 1989). One of the complement receptors, type 3 (CR3), is extremely versatile. In addition to binding the complement component C3bi, bacteria, yeasts and intracellular parasites are internalized via CR3, without prior opsonization (Wright and Jong, 1986; Bullock and Wright, 1987; Russell and Wright, 1988). Furthermore, CR3 is involved in macrophage adhesion to vascular endothelium (Detmers and Wright, 1988).

Cytotoxicity

Macrophages can display a strong cytotoxic activity towards potentially harmful

Table 2.III Summary of mononuclear phagocyte functions

*	endocytosis of micro-organisms, effete cells and debris
*	cytotoxic activity against micro-organisms and tumor cells
*	regulation of the inflammatory response
*	regulation of the immune response
*	regulation of hemopoiesis

micro-organisms, virally infected cells or tumor cells. To this purpose, macrophages may synthesize an array of cytotoxic products such as oxygen metabolites, nitrogen oxides, bactericidal and tumoricidal proteins such as lysozyme and TNF (Nathan, 1987; Decker et al., 1987; Gabay, 1988; Rappolee and Werb, 1988; Stuehr and Nathan, 1989).

Regulation of the inflammatory response

In addition to an effector function in host defense, macrophages are involved in the regulation of virtually all stages of the inflammatory response. In response to injury and infection, macrophages produce mediators like interleukin 1 (IL-1), TNF, prostaglandin E_2 (PGE_2) and macrophage inflammatory proteins (MIP), which have pro-inflammatory activity (Dinarello, 1987; Larrick and Kunkel, 1988; Dinarello and Savage, 1989; Davatelis et al., 1989). Furthermore, molecules which attract other leukocytes, like leukotriene B_4 and complement components, are secreted (Nathan, 1987; Rappolee and Werb, 1988). In chronic inflammation, macrophages form granulomas together with T lymphocytes. Here, local TNF production by macrophages appears to play a pivotal role in the induction and maintenance of these granulomas (Kindler et al., 1989). In the recovery phase of the inflammatory response, macrophages are directly involved in wound repair by production of TGF-ß, which stimulates fibroblast collagen synthesis, and of TNF, which now induces blood vessel growth (Leibovich et al., 1987; Danon et al., 1989; Pierce et al., 1989).

Regulation of the immune response

Similarly, in the antigen-specific limb of the inflammatory response, mononuclear phagocytes play an important immunoregulatory role (Unanue and Allen, 1987; Geppert and Lipsky, 1988). Dendrocytes (dendritic, interdigitating, veiled and Langerhans cells) are uniquely equipped for the antigen-specific activation of resting T cells in primary immune responses (Austyn, 1987). However, uptake and processing of antigens is more

efficiently performed by classical macrophages by virtue of their endocytic capacity and extensive lysosomal compartment. Expression of class II MHC molecules by macrophages and dendrocytes is essential for antigen presentation to T cells (Unanue and Allen, 1987). In addition, classical macrophages require cell surface expression of IL-1 in order to activate T cells. Dendrocytes apparently do not produce IL-1, although exogenous IL-1 amplifies their antigen presentation capacity several fold (Steinman, 1988). Likely, dendrocytes produce an as yet unknown cytokine with similar auxiliary activity as IL-1.

Macrophages also play a direct role in the humoral immune response by the production of IL-1 and IL-6. These factors synergistically stimulate B cell growth and differentiation (Vink et al., 1988; Van Damme et al., 1988).

In contrast to the foregoing, macrophages may also suppress immune responses, either by inhibiting T or B cell proliferation directly (Allison, 1978; Piguet et al., 1981; Stout and Fisher, 1983) or by inhibiting dendritic cell antigen presentation function (Holt et al., 1988). Immunosuppression by macrophages can be mediated by production of PGE_2 (Piguet et al., 1981; Shibata and Volkman, 1985a) or of TGF-ß (Wahl et al., 1988).

Regulation of hemopoiesis

Macrophages also play an important role in the regulation of hemopoiesis. A considerable part of both bone marrow and splenic red pulp hemopoietic stroma consists of macrophages (Hume et al., 1983; Weiss and Sakai, 1984). Characteristic are the stellate, central macrophages in erythropoietic islands (Crocker et al., 1988), but central macrophages have been shown in granulopoietic clusters as well (Crocker and Gordon, 1985). Except from providing a mechanical framework together with other stromal cells, macrophages produce a wealth of hemopoietic growth factors, including IL-1, IL-6, GM-CSF, G-CSF, and erythropoietin (Rich, 1986; Bagby, 1987; Metcalf, 1988).

In erythropoiesis, macrophages play a dual, vital role. In addition to the production of erythropoietin, macrophages constitute an important link in the metabolism of iron (Finch and Huebers, 1982; Deiss, 1983). Effete erythrocytes are phagocytosed and the iron contents is either stored intracellularly in the form of ferritin, or released in the plasma after being bound to apotransferrin.

Furthermore, macrophages stimulate B lymphopoiesis in the bone marrow (Pietrangeli and Osmond, 1985; King et al., 1988) as well as T lymphopoiesis in the thymus (Zepp et al., 1984; Papiernik et al., 1987a).

Macrophages may also inhibit hemopoiesis by the production of mediators like IFN-α/ß, PGE_2 or erythroid inhibitory activity (EIA) (Moore et al., 1984; 1985a; 1985b; Rigby et al., 1985; Sassa et al., 1987).

Clearly, macrophages are versatile cells, performing essential functions in multiple processes. Complete absence of mononuclear phagocytes in a pathologic situation has never been reported, indicating that lack of cells of the mononuclear phagocyte lineage is incompatible with life (Johnston, 1988).

Macrophage heterogeneity

Inter- and intrapopulation heterogeneity

Obviously, not all mononuclear phagocytes perform the multitude of functions mentioned above. A marked heterogeneity with respect to functions as well as phenotypes characterizes this cell type (Johnston, 1988; Gordon et al., 1988b). In general, two types of heterogeneity can be distinguished, namely inter-population and intra-population heterogeneity (Walker, 1982; Walker and Hester, 1983; Dougherty and McBride, 1984).

Inter-population heterogeneity refers to differences among populations of macrophages located at, or obtained from different anatomical sites. In this respect, especially free macrophage populations such as peritoneal and alveolar macrophages have been extensively compared. One striking difference between these two populations, already observed at the morphological level, is represented in figure 2.4. Alveolar and peritoneal macrophages differ in many functional aspects as well (Morahan, 1980; Walker, 1982; Walker and Hester, 1983; Gordon et al., 1988b). Other examples of inter-population heterogeneity are the differential production of arachidonic acid metabolites by splenic and peritoneal macrophages (Nusrat et al., 1988) or the differential binding of lectins by resident macrophages in different organs (Tsukada and Spicer, 1988).

Intra-population heterogeneity refers, evidently, to differences within the macrophage population from one particular morphological location, thus revealing subpopulations. For instance, human blood monocytes can be separated into distinct subpopulations with different activities in tumor cell killing or accessory function for B- or T-cell proliferation (Whisler et al., 1982; Figdor et al., 1982). Similarly, rat and murine peritoneal exudate macrophages elicited with fetal calf serum appear to consist of four subpopulations, clearly differing in endogenous peroxidase localization and 5'-nucleotidase activity (Beelen and Walker, 1983; Ginsel et al., 1983).

Origins of macrophage heterogeneity

Macrophage heterogeneity may be explained according to different views (Lee, 1980; Walker, 1982; Walker and Hester, 1983; Bursuker and Goldman, 1983; Dougherty and McBride, 1984; Treves, 1984; Shibata and Volkman, 1985b; Gordon, 1986; Gordon et al., 1988b). In figure 2.5, the distinct, simplified views dealing with macrophage heteroge-

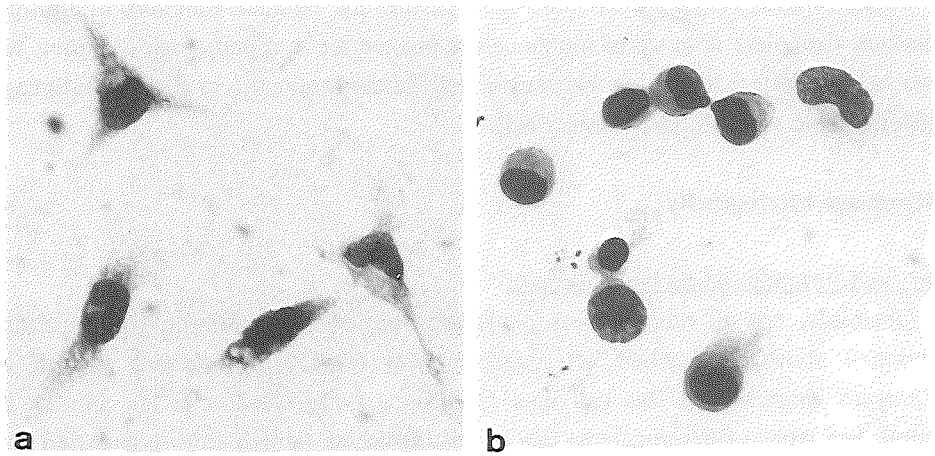


Figure 2.4.

Morphological appearance of peritoneal macrophages (a) and alveolar macrophages (b). Cells were isolated, cultured overnight on cover slips and stained with May Grünwald - Giemsa. Note that the cells stretch differently on the glass surface, despite a similar treatment (isolation, culture medium etc.). Original magnification x 920.

neity are summarized. Briefly, according to a first model, macrophages may pass through different functional stages upon maturation. Secondly, macrophages may be activated by local stimuli to perform different functions. Thirdly, different lineages may develop in the bone marrow, leading to mature macrophage populations with different functions and phenotypes. Finally, different - independently regulated - mononuclear phagocyte compartments may exist: peripheral resident macrophage populations which are maintained by local proliferation within the peripheral tissues, and inflammatory exudate macrophages which are derived from the circulating monocyte pool. Examples of the experimental evidence supporting each of the distinct views are given hereafter.

1. Different maturation stages. Functional and phenotypical changes with maturation of macrophages have been shown by many investigators. For example, maturing macrophages in bone marrow cultures lose the capacity to bind the lectin wheat-germ agglutinin, but show increasing secretion of apoprotein E (de Water et al., 1985; Werb and Chin, 1983). On the other hand, production of plasminogen activator and IFN were found to be temporary phenomena, correlated with intermediate stages of differentiation (Neumann and Sorg, 1980; Werb and Chin, 1983). Furthermore, natural cytotoxic activity against particular tumor cells and micro-organisms has been demonstrated at the level of macrophage precursor cells (Lohmann-Matthes et al., 1979; Decker et al., 1986; Baccarini et al., 1988). Immaturity of these cells is indicated by the

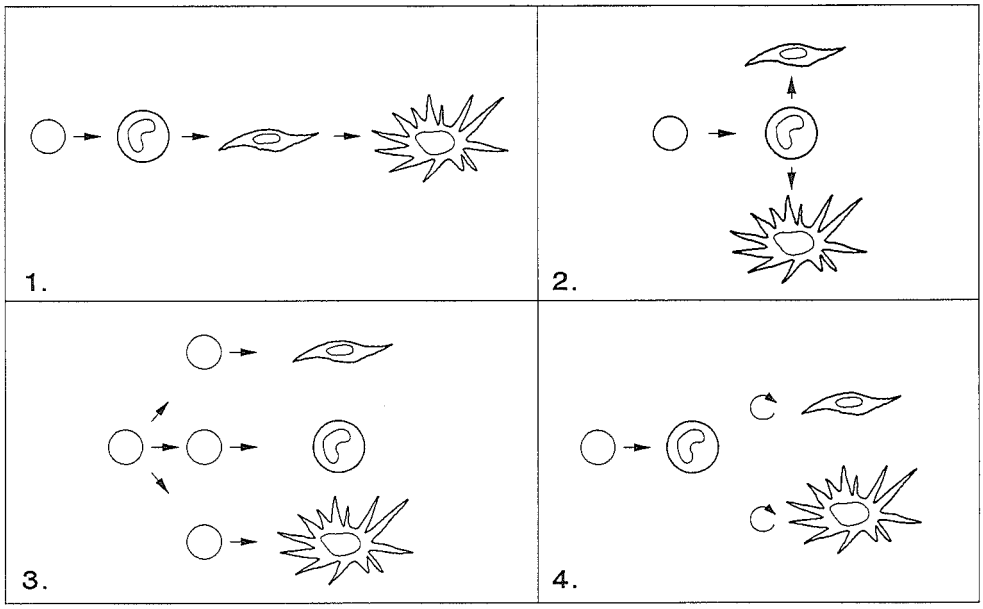


Figure 2.5.

Different views explaining the heterogeneity of the mononuclear phagocyte system. 1. different functional and phenotypical stages are passed upon maturation of macrophages; 2. different stages are the result of differential activation by local influences in peripheral tissues; 3. different mature cells originate from distinct precursors, revealing various differentiation lineages; 4. mature populations are independently maintained by local proliferation, whereas bone marrow-derived blood monocytes differentiate to exudate macrophages under inflammatory conditions.

lack of phagocytic activity and adherence. However, these natural killer cells unequivocally belong to the mononuclear phagocyte lineage as they mature into macrophages in response to M-CSF. Concomitantly with maturation, the cells lose their cytotoxic property.

2. *Different activation stages.* Activation of macrophages appears to be necessary in order to potentiate these cells for particular functions or to enhance the performance of existing functions. For instance, the tetrapeptide tuftsin (Thr-Lys-Pro-Arg) is a stimulator of many macrophage functions including phagocytosis, oxygen metabolite production and antigen presentation (Fridkin and Gottlieb, 1981; Najjar, 1983). Similarly, IL-4 and GM-CSF appear to be potent activators of mature macrophage antigen presentation and cytotoxicity (Zlotnik et al., 1987; Wirth et al., 1989; Morrissey et al., 1989). However, it should be realized that 'macrophage activation' as such does not exist: macrophages may be activated to perform particular functions. Simultaneously,

however, other functional capabilities will be diminished (Morahan, 1980; Adams and Hamilton, 1986). This notion was nicely demonstrated by Campbell et al. (1984; 1988), who showed that a given macrophage population preferentially expresses either tumoricidal or bactericidal activity, but not both activities at the same time. Moreover, a bactericidal population may lose this activity and gain tumoricidal potential by overnight culture in the presence of IFN-γ.

The sequential steps in activation of relatively mature macrophages to tumoricidal activity have been elaborated extensively (Johnson et al., 1983a; Adams and Hamilton, 1984). A so-called responsive macrophage is sensitized by cytokines like IFN-γ to become a primed macrophage. A second signal, such as LPS, is needed for the development of cytolytically competent cells. Similar sequential activating events are likely to exist for other functions as well. For instance, antigen presentation requires the expression of class II MHC molecules. Such expression can be induced by IFN-γ. However, class II expression by itself is not sufficient for adequate antigen presentation, and must be accompanied by the expression of IL-1 or similar accessory molecules (Unanue and Allen, 1987; Steinman, 1988).

3. Different lineages of differentiation. The existence of multiple macrophage differentiation lineages in the bone marrow is a controversial subject. Bursuker and Goldman (1982; 1983) originally proposed that different lineages of bone marrow macrophage precursor cells are differentially expanded under steady state or inflammatory conditions. These authors distinguished two precursor populations, one leading to mature macrophages with a high 5'-nucleotidase (NT) activity, and the other giving rise to cells with a low NT activity. Regarding the NT expression as a clonal marker, the authors postulated that resident peritoneal macrophages are derived from the macrophage precursor population leading to cells with high NT activity (Bursuker and Goldman 1982; 1983). In contrast, exudate macrophages elicited by various agents are in this view derived from the other precursor population giving rise to macrophages with low NT activity. Under inflammatory conditions, the latter population is preferentially expanded. The validity of this interpretation may be questioned, as the NT level is possibly not a reliable clonal marker (de Water et al., 1985). Nevertheless, a number of recent studies support the view that macrophage precursors with varying differentiation potential exist. Macrophage precursors, derived either from spleen or from bone marrow, as well as the progeny obtained from these cells, appear to differ markedly with regard to their cytotoxic activity and target selectivity (Baccarini et al., 1986). Furthermore, different colonies of spleen macrophages obtained in vitro showed a differential ability for antigen presentation, partially correlating to the number of macrophages expressing class II molecules in the particular colony (Walker, 1987). In addition, repopulation of the spleen after selective macrophage elimination using

liposomes containing DMDP (dichloromethylene diphosphonate), revealed a striking difference in kinetics of reappearance of the different subpopulations (van Rooijen et al., 1989). In these studies, it was found that red pulp macrophage numbers returned to a normal level at one week after elimination. In contrast, complete repopulation of marginal zone macrophages took more than one month. The different repopulation kinetics strongly suggests that splenic macrophage subpopulations have different precursors.

4. Different, independently maintained populations. The existence of independently maintained mononuclear phagocyte compartments as a source of macrophage heterogeneity is also disputed. The essence of this concept is that resident macrophage populations are self-maintaining, whereas blood monocytes are precursors of exudate macrophages involved in inflammatory responses (Sawyer et al., 1982; Daems and de Bakker, 1982; Volkman et al., 1983; Shibata and Volkman, 1985a; 1985b). In this respect, Shibata and Volkman (1985a; 1985b) demonstrated the independent regulation of three mononuclear phagocyte populations, namely splenic suppressor macrophages, circulating monocytes and splenic macrophage colony-forming cells. As pointed out in an earlier section, the ability of full self-maintenance of resident macrophage populations is, however, questioned. Recent support for the existence of macrophage compartments, that are maintained independently from the bone marrow, comes from elegant studies using in vivo labeling of resident peritoneal macrophages with the extremely stable fluorescent dye PKH-1 (Melnicoff et al., 1988). These authors demonstrated that the resident peritoneal macrophage population was maintained for at least 49 days without significant replacement by - unlabeled - recruited monocytes. They concluded that the resident peritoneal macrophage population was maintained by proliferation of either the resident macrophages themselves or of locally present macrophage precursors, as 49 days is far beyond the half life of resident peritoneal macrophages. Potential precursors of peritoneal macrophages have indeed been demonstrated in the omental milky spots (Beelen et al., 1980). Most likely, these local precursors are also labeled by intraperitoneal application of the dye.

In conclusion, a variety of mechanisms exists that may account for the observed heterogeneity of macrophages. Of these mechanisms, differences in maturation and activation stages caused by environmental conditions are most convincingly demonstrated. Thus, mechanisms 1 and 2 (fig. 2.5) undoubtedly contribute to the generation of macrophage heterogeneity. In addition, increasing experimental evidence supports the existence of separate differentiation lineages and the independent maintenance of various macrophage populations as additional sources of macrophage heterogeneity (mechanisms 3 and 4).

MACROPHAGE DIFFERENTIATION: EXPERIMENTAL TOOLS

With regard to the scope of this thesis and the methods used in the next chapters, an introduction will follow here on two tools which were frequently applied in the study of macrophage differentiation, i.e. in vitro growth of mononuclear phagocytes and monoclonal antibodies directed against molecules on or in these cells.

Mononuclear phagocytes in culture

In general, mononuclear phagocytes can relatively easily be manipulated and maintained in vitro. This enabled the development of a variety of culture methods for different types of mononuclear phagocytes which will be briefly commented hereafter.

Isolated macrophage precursors

Macrophage progenitors - either derived from bone marrow or from other sources - may be cultured, provided that a source of growth factor (CSF) is present. This source of CSF can either be 'crude', such as conditioned medium (CM) from L929 fibroblasts or lung, or an extract from pregnant mouse uterus (Metcalf, 1984). Nowadays, purified growth factors, primarily M-CSF and GM-CSF, produced using recombinant DNA technology form an attractive alternative. Depending on the source of CSF, macrophage progenitors will proliferate as well as differentiate in vitro, revealing colonies within 4-14 days. For the enumeration of macrophage precursors in vitro, clonal cultures are routinely performed in agar or methyl cellulose (Metcalf, 1984). Liquid cultures are more convenient when cells are to be harvested for experimental manipulation. Liquid cultures on cover slips will reveal round, immature cells - mostly monoblasts - as well as more mature cells - promonocytes and macrophages - that have spread over the surface (fig. 2.6). Large numbers of bone marrow-derived mononuclear phagocytes can be retrieved from liquid cultures in Teflon bags, as adherent mature cells are easily detached (van der Meer et al., 1979b; 1983). Depending on the time of harvest, such culture systems contain a variable proportion of monoblasts, promonocytes and macrophages.

So-called high-proliferative-potential colony-forming cells (HPP-CFC) will form large macrophage colonies in vitro when combinations of M-CSF with other growth factors are applied (Bradley and Hodgson, 1979). Two different HPP-CFC populations are stimulated by either M-CSF plus IL-1 on the one hand, or by M-CSF plus IL-3 or GM-CSF on the other hand (McNiece et al., 1986; 1988). HPP-CFC, however, are not yet committed to the macrophage lineage and have been shown to possess multipotentiality (McNiece et al., 1987).

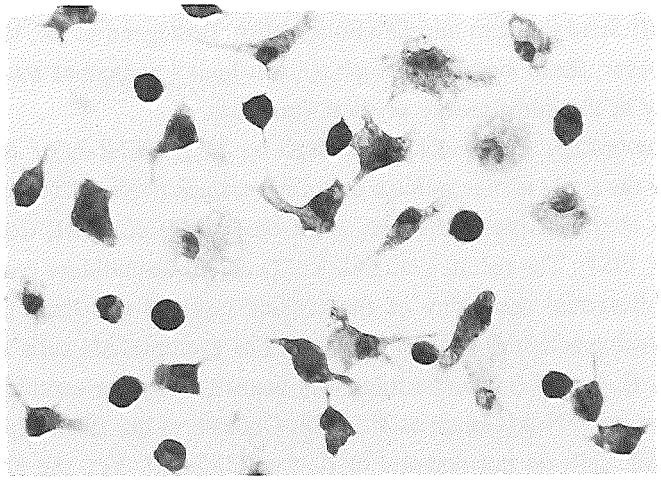


Figure 2.6.

Morphological appearance of mononuclear phagocytes obtained from liquid bone marrow culture. Bone marrow cells were harvested and cultured for 7 days on a cover slip placed in medium containing M-CSF. The resulting progeny was fixed with glutaraldehyde and stained with May Grünwald-Giemsa. Immature monoblasts appear as round cells, whereas more mature promonocytes and macrophages are increasingly stretched. Original magnification x 650.

Growth factor-dependent macrophage cell lines

Growth factor-dependent macrophage cell lines may be isolated, originating from cytokine-stimulated bone marrow cultures, (Cinatl et al., 1982; Johnson et al., 1983b; Ohki and Nagayama, 1988). The main advantage of such cell lines is that a large, clonal population of macrophages is available for experimentation. Application of different growth factors, or different sources of macrophages (bone marrow, spleen, peritoneal cavity, lung) results in phenotypically and functionally different cell lines (Ohki and Nagayama, 1988; Lombard et al., 1988). In this respect, cell lines maintained by IL-3-containing CM appear to express immature characteristics compared to M-CSF dependent lines (Ohki and Nagayama, 1988).

Macrophage tumor cell lines

Growth factor-independent macrophage tumor cell lines have been extensively used for the study of macrophage phenotypes and functions (Ralph, 1980; 1984). These lines appear to be clonally derived and are thought to be arrested in a particular stage of differentiation (Greaves, 1979; 1982). A discrimination can be made between myeloid and myelomonocytic cell lines with immature characteristics, and lines with features of mature macrophages. The latter have been shown to exert, for instance, phagocytosis,

tumor cytotoxicity, production of cytokines, antigen presentation, and chemotactic activity (Ralph, 1980; 1984; Schwarzbaum and Diamond, 1983; Terheggen et al., 1985; Klasen et al., 1988). However, these mature macrophage cell lines - as mature macrophages - differ considerably in the performance of these functions.

Immature macrophage tumor cell lines, such as M1, WEHI-3, or RMB-1, are attractive tools for the study of macrophage maturation, since these cells can be induced to express mature characteristics (Ichikawa, 1969; de Both et al., 1981; Cooper et al., 1982; Ralph, 1980; 1984). The recent availability of purified recombinant growth factors has revealed a differential sensitivity of the various myeloid cell lines towards the differentiation inducing activities of such cytokines. For example, M1 cells are induced to differentiate by IL-6, LIF (leukemia inhibitory factor), or by the combinations IL-1 plus TNF, or IL-1 plus IFN-ß (Gough et al., 1988; Onozaki et al., 1988; Chiu and Lee, 1989). However, M1 cells do not respond to IL-3 or GM-CSF. Yet, the latter factors induce differentiation in the myeloid clone 7-M12 cells (Lotem et al., 1988). Most likely, differentiation inducing factors trigger a cascade of genes to be expressed, including genes coding for autocrine differentiation factors. In this respect, IFN-ß has been shown to be such an autocrine intermediate in the macrophage differentiation pathway (Onozaki et al., 1988).

The induction of *differentiation* by cytokines like IL-1, IL-3, IL-6, or GM-CSF on myeloid cell lines seemingly contrasts with the stimulation of *proliferation* in normal hemopoietic progenitors. Thus, it may be questioned whether differentiation induction of myeloid cell lines provides a valid model of macrophage differentiation. To explain the apparent contradiction in cytokine effect on myeloid cell lines and normal progenitors, Metcalf (1989) postulated a single genetic control element that dictates a particular cell either to self-renew or to generate committed progeny. Obviously, for leukemic cells this 'switch' dictates for self-renewal, whereas normal hemopoietic precursors tend to generate committed progeny in the absence of inducing cytokines. The action of cytokines on either leukemic cells or normal precursors would thus change the 'switch' towards generation of committed progeny and self-renewal, respectively.

Immortalization of mononuclear phagocytes

Immortalization of mononuclear phagocytes is of increasing importance to elucidate macrophage heterogeneity. To this purpose, two methods may be applied, i.e. infection of cells with retroviruses carrying oncogenes, and somatic cell hybridization with a suitable tumor cell line.

Retroviral infection. Blasi et al. (1985) showed that in vitro infection of bone marrow cells with the recombinant J2 retrovirus, which carries the raf/mil and myc oncogenes, preferentially immortalizes monocytic cells. The resulting cell lines exhibit

the same biological properties as normal tissue macrophages (Blasi et al., 1987). Using the same recombinant retrovirus, however, Roberson and Walker (1988) were unable to immortalize freshly in vitro cloned splenic macrophages. Yet, after 4 months of additional culture about one third of all clones could be rendered growth factor-independent by viral infection. Most likely, a 'pre-leukemic event' had to take place before the macrophage clones were apt to be immortalized by the retroviral oncogenes. Notably, the heterogeneity of the original clones with respect to antigen presenting capacity was fully maintained in the resulting tumor cell lines.

Somatic cell hybridization. An alternative method for macrophage immortalization is somatic hybridization with tumor cells. Several groups have established mature murine macrophage hybrids using this method (Tzehoval et al., 1984; Uchida et al., 1985; Takeda et al., 1985; Zuckerman et al., 1986; Chou et al., 1987). As a tumor cell fusion partner, mostly the P388D1 macrophage or NS-1 myeloma cells were applied. The initiating macrophage populations were either splenic adherent cells or elicited peritoneal exudate macrophages. In addition, functional hybrids between isolated dendritic cells and SP2/0 myeloma cells were recently reported by Breel et al. (1988). An important observation for all hybrids is that parental cell heterogeneity is reflected in the resulting hybrids. Differential functional capacities were noted among different macrophage hybrids in antigen presentation to T helper cells, induction of T suppressor activity, inhibition of lymphocyte mitogenesis, as well as tumor cytotoxicity (Ju and Dorf, 1985; Liu et al., 1985a; 1985b; Kawasaki et al., 1986; Tzehoval et al., 1984; 1989). Furthermore, different hybrids appeared to be heterogeneous with regard to the expression and inducibility of class II MHC molecules and other cell surface antigens (Chou et al., 1987; Martin et al., 1988; Tzehoval et al., 1989). The distinct phenotypes of the particular hybrids appeared to be stable over a long time of in vitro maintenance. Altogether, these data suggest that somatic cell hybridization is a suitable method for the immortalization of particular macrophage phenotypes.

Monoclonal antibodies against mononuclear phagocyte antigens

Monoclonal antibodies (mAbs), recognizing molecules on or in cells, are powerful tools for the discrimination of different cell types. Furthermore, the phenotypical make-up of a cell reflects its competence in the performance of distinct functions as many of these functions are carried out or assisted by molecules at the cell surface. As a logical consequence, many investigators of macrophage biology have produced and described mAbs directed against macrophage antigens in order to unravel the complexity of the mononuclear phagocyte system. It is virtually impossible - within the limits of this introductory section - to give a complete survey and description of all anti-macrophage

mAbs that have been described in the literature until now. Therefore, a selection has been made, including those mAbs that have been characterized with regard to the differential recognition of macrophage differentiation stages. This selection is represented in tables 2.IV - 2.VII. Brief descriptions of the various reactivities focus on the binding of the mAbs to mononuclear phagocytes, thereby largely omitting crossreactivity with other hemopoietic and non-hemopoietic cells. Frequently, multiple mAbs have been developed against a single mononuclear phagocyte antigen. Therefore, the characteristics of the various antigens are discussed, rather than the individual mAbs.

Immature mononuclear phagocyte antigens

Only few mAbs have been described that are directed against antigens on precursor stages of macrophage development (the recognized antigens have been listed alpha-

Table 2.IV mAbs against macrophage precursor antigens

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
Lgp100a Ly-9	100	30-C7	-	-	(203)
L3T4 MT4 Ly-4	52	GK1.5 H129.19	CD4	interaction with class II MHC molecules	(97) (287)
MB1 Ag	-	MB1	-	-	(128)
MIV 52 Ag MIV 113 Ag MIV 116 Ag	-	MIV 52 MIV 113 MIV 116	-	-	(207) (111)
Nk-1.1	-	PK136	-	-	(189)
Qa-m7	39-45/12	5035-50.1	-	class I MHC-like (?)	(311)
Thy-1	25-30	a.o. 59-AD2.2	-	signal transduction (?)	(203)

- : data are presently unknown

betically in table 2.IV). The **Thy-1** antigen was found to be expressed by HPP-CFC as well as by M-CFC, using complement-mediated lysis of labeled bone marrow cells (Boswell et al., 1984; Berman and Basch, 1985). Thy-1 expression appears to decrease upon maturation of the cells. However, the level of Thy-1 expression by macrophage progenitors is probably low, since Müller-Sieburg and coworkers reported that in flowcytometric analysis, granulocyte-macrophage precursors were predominantly found in a Thy-1 negative bone marrow fraction (Müller-Sieburg et al., 1988). Recently, tingible body macrophages in lymph node follicles were reported to express Thy-1 antigens (Smith et al., 1988). The detection of Thy-1 on these cells, however, is most likely due to the application of sensitive, non-routine procedures, since such a reactivity was not found using regular immunohistochemical techniques (van Ewijk et al., 1981). Using FACS, Fredrickson and Basch (1989) recently demonstrated that the majority of murine stem- and progenitor cells, including M-CFC, express **L3T4** antigens (CD4). Similarly, both GM-CFC and M-CFC express **Qa-m7** and **Lgp100a** (Harris et al., 1985; Bertoncello et al., 1986; Miller et al., 1985). A proportion of isolated splenic macrophage precursors as well as non-adherent precursors obtained from bone marrow cultures appear to express **Nk-1.1**, indicative of the natural killer activity of these cells (Baccarini et al., 1988; Li et al., 1989). In addition, also the mAbs **MB1**, and **MIV 52, MIV 113**, and **MIV 116** exhibit preferential binding to antigens of non-adherent macrophage precursors from M-CSF stimulated bone marrow cultures (Gordon and Hirsch, 1982; Leenen et al., 1986a; Falkenberg et al., 1989).

Antigens expressed by multiple stages of mononuclear phagocyte differentiation

Of the mAbs reactive with immature as well as mature stages in macrophage differentiation - the recognized antigens have been listed alphabetically in table 2.V -, those directed against the common leukocyte antigen as well as those against the transferrin receptor deserve special attention in the context of this thesis. MAbs against the lymphocyte function-associated antigen LFA-1 will be discussed in the next section because of the structural similarity between LFA-1 and Mac-1 antigens.

The **common leukocyte antigen** (CLA, T-200, Ly-5, CD45) is expressed in all stages of leukocyte differentiation. In the mononuclear phagocyte lineage, CLA is found on GM-CFC and M-CFC, as well as on monocytes and peritoneal macrophages (Watt et al., 1983; Springer, 1980). In bone marrow cultures, CLA expression increases from the monoblast to the macrophage stage (Nibbering et al., 1987). The function of this universal leukocyte antigen remained unclear, until Tonks et al. (1988) showed that CLA has intracellular protein tyrosine phosphatase activity. Thus, CLA is likely involved in the regulation of cellular activation and de-activation processes as it may counteract protein kinase activities (Clark and Ledbetter, 1989).

Table 2.V **mAbs against antigens expressed by multiple stages of mononuclear phagocyte differentiation**

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
CLA	170-220	a.o.	CD45	protein tyrosine phosphatase	
T-200		30-G12			(203)
Ly-5		YBM/42			(421)
DNL3.7 Ag	-	DNL3.7	-	-	(226)
DNL4.4 Ag	-	DNL4.4	-	-	(226)
HSA	-	M1/69	-	-	(332)
		B2A2			(319)
		J11d			(47)
H11 Ag	-	30-H11	-	-	(203)
H-2K/D	46 / 12	a.o. M1/42	-	cellular recognition	(332)
IL-2-R	55	3C7	CD25	low affinity	(277)
		AMT-13		interleukin-2 receptor	(278)
LFA-1	180 (α)	a.o. M7/14	CD11a		(80)
Ly-15		H35-89.9		cell adhesion molecule	(286)
	95 (β)	M18/2	CD18		(310)
Ly-6C	14-17	a.o. Monts-1	-	signal transduction (?)	(171)
		6C3			(106)
Pgp-1	95	IM7.8.1	CD44	-	(362)
Ly-24		AMF-8			(68)
Tf-R	2 x 100	a.o. H129.121	CD71	transferrin receptor	(373)
7/4 Ag	-	7/4	-	-	(146)

- : data are presently unknown

abbreviations: CLA - common leukocyte antigen; HSA - heat stable antigen; Tf-R - transferrin receptor

As pointed out in a previous section, macrophages play an important role in the iron metabolism by scavenging senescent erythrocytes and making the iron content reusable. In addition, **transferrin receptors** also appear to be involved in iron internalization by mature macrophages. Andreesen et al. (1984) showed that terminal differentiation of blood monocytes into macrophages coincides with induction of transferrin receptor expression as measured by anti-transferrin receptor binding. Concomitantly, the differentiated macrophages appeared to store the transferrin-transported iron in the form of ferritin. In immature mononuclear phagocytes, as in other cell types, expression of transferrin receptors is required for cell growth (Trowbridge and Shackelford, 1986). The observed fraction of GM-CFC that does not bind anti-transferrin receptor mAbs reflects the known rest-stage of a proportion of these progenitors (Lesley et al., 1984). Many anti-transferrin receptor antibodies have been reported that inhibit proliferation of cells by hindering iron uptake (Trowbridge and Shackelford, 1986). MAbs of the IgM class appear to inhibit receptor recycling completely, whereas IgG anti-transferrin receptor mAbs induce increased internalization and degradation of transferrin receptors (Lesley and Schulte, 1985; Lesley et al., 1989). Thus, both IgM and IgG anti-transferrin receptor mAbs decrease cellular iron uptake, although entirely different mechanisms are involved. An as yet unresolved enigma, however, is how a single IgG anti-transferrin receptor mAb may inhibit the proliferation of a particular cell type completely, whereas the proliferation of another cell type remains unaffected by the same antibody (Trowbridge et al., 1982b; Kemp et al., 1987).

Mature mononuclear phagocyte antigens

MAbs that bind to mature stages of mononuclear phagocyte differentiation can be divided into two classes: mAbs recognizing mature macrophages (table 2.VI), and mAbs directed specifically against dendrocytes (table 2.VII). The reactivities of especially those mAbs that have been used in the studies described in this thesis will be discussed in the next paragraphs.

To my knowledge, no mAbs have been described with selective pan-macrophage reactivity. As such, this finding emphasizes again the extensive heterogeneity of the mononuclear phagocyte system.

Perhaps the most universal macrophage marker is **F4/80**, which is expressed by most mature macrophage populations (Austyn and Gordon, 1981; Hume et al., 1983; Lee et al., 1985). F4/80 Ag expression increases with cell maturity after the bone marrow M-CFC stage (Hirsch et al., 1981). However, F4/80 Ag is not expressed at regularly detectable levels by macrophages in lymphoid microenvironments (Witmer and Steinman, 1984; Kraal et al., 1987). The reason for lack of F4/80 Ag expression by these cells is possibly the high local concentration of lymphocyte-derived cytokines, some of which down-regulate F4/80 Ag expression (Ezekowitz and Gordon, 1982). Of the

Table 2.VI **mAbs against mature macrophage antigens**

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
AcM.1 Ag	-	AcM.1	-	-	(354)
asialo-GM1	-	SH34	-	-	(328)
BMA-1 Ag	# x 38	BMA-1	-	-	(281)
BM8 Ag	125	BM8	-	-	(234)
B23.1 Ag	-	B23.1	-	-	(201)
ER-HR3 Ag	55-67 69-78	ER-HR3	-	-	(88)
ER-TR6 Ag	-	ER-TR6	-	-	(403)
ER-TR9 Ag	-	ER-TR9	-	involved in neutral polysaccharide uptake	(404)
FcRI	2 x 50	3A2	CD64	IgG2a Fc receptor; protein kinase	(173)
FcRII	47-70	2.4G2	CDw32	IgG1 / IgG2b Fc receptor	(371)
Forssmann glycolipid	-	M1/22.25 33B12	-	-	(332) (329)
F4/80 Ag	160	F4/80	-	-	(10)
H-2 Ia	2 x 25-34	a.o. M5/114, ER-TR1,-2,-3	-	Ag-peptide binding for presentation	(27) (403)
H-2 I-J	-	JK10-23	-	involved in T_{suppr} induction	(233)
MAA-1 Ag	-	MAA-1	-	-	(118)
Mac-1 Ly-40	170 (α) 95 (ß)	a.o. M1/70 M18/2	CD11b CD18	C3bi receptor cell adhesion molecule	(334) (310)
Mac-2	32	M3/31 M3/38	-	-	(148)

Table 2.VI **mAbs against mature macrophage antigens (continued)**

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
Mac-3 LAMP-2	110	M3/84	-	-	(149)
Mac-4 54-2 Ag	180	M3/37 54-2	-	-	(333) (202)
MA158.2	-	158.2	-	-	(187)
MBR-1 MBR-2 MBR-3 30-E2 Ag	-	MIV 55 MIV 38 14G8 30-E2	-	-	(111) (207) (197) (203); (236)
MM9 Ag	-	MM9	-	-	(353)
MOMA-1 Ag	-	MOMA-1	-	-	(191)
MOMA-2 Ag	-	MOMA-2	-	-	(193)
M43 Ag	-	M43	-	-	(348)
M57 Ag	-	M57	-	-	(348)
M102 Ag	-	M102	-	-	(348)
M143 Ag	-	M143	-	-	(348)
SER	170-185	SER-4	-	R of sialylated glycoconjugates	(348)
TR-1N Ag TR-3N Ag	57 / 46	TR-1N TR-3N	-	-	(280)
WE15 Ag	-	WE15	-	-	(353)
3AE8 Ag	-	3AE8	-	signal transduction (?)	(56)

- : data are presently unknown
abbreviations: FcR - Fc receptor; SER - sheep erythrocyte receptor

dendrocytes, Langerhans cells express readily detectable levels of F4/80 Ag, whereas isolated dendritic cells appear to express this antigen only marginally (Hume et al., 1983; 1984; Crowley et al., 1989).

Another frequently used macrophage marker, **Mac-1**, is equivalent to the type 3 complement receptor (Beller et al., 1982). Immunohistochemical analysis showed that anti-Mac-1 mAb preferentially detects 'free' macrophages above tissue-fixed cells (Flotte et al., 1983). Mac-1 expression increases with maturation of the cells (Walker et al., 1985), although the stage at which this marker is first detectable is unclear. Miller et al. (1985) found no Mac-1 expression by GM-CFC or M-CFC, whereas Gordon et al. (1985) reported Mac-1 expression by 50% of GM-CFC and 70% of M-CFC. In order to unravel the multi-functionality of the type 3 complement receptor, many studies have made advantage of the fact that anti-Mac-1 mAbs tend to be directed against functional sites of the receptor (Wright and Jong, 1986; Bullock and Wright, 1987; Russell and Wright, 1988). Additionally, anti-Mac-1 mAb has been shown to activate macrophages in a manner closely resembling IFN-γ mediated activation (Ding et al., 1987).

Mac-1 and **LFA-1** molecules share the same ß-chain and belong to the integrin superfamily of adhesion molecules (Hemler, 1988). As such, LFA-1 - but not Mac-1 - is structurally involved in the binding of tumor targets to activated, cytotoxic macrophages and T cells (Strassmann et al., 1986, Pierres et al., 1982). LFA-1 expression is first induced in bone marrow precursors, as part of the GM-CFC and M-CFC are LFA-1 positive (Miller et al., 1985). Upon maturation, LFA-1 expression increases: all peripheral blood monocytes are LFA-1 positive (Strassmann et al., 1985). Mature macrophages show a more selective expression of LFA-1 compared to Mac-1: only activated cells are LFA-1 positive, whereas resident or thioglycollate-elicited macrophages are LFA-1 negative (Strassmann et al., 1985).

Mac-2 antigens are preferentially expressed by macrophages in response to specific differentiative signals, such as thioglycollate elicitation (Ho and Springer, 1982). In bone marrow cultures, Mac-2 is hardly expressed by non-adherent precursors, but clearly detectable at more mature cells (Walker et al., 1985). Therefore, Mac-2 may be regarded as a maturation marker. In situ, Mac-2 was found to be expressed by both tissue macrophages and dendrocytes (Flotte et al., 1983).

Mac-3 antigens closely follow the Mac-2 distribution pattern: tissue macrophages as well as dendrocytes are Mac-3 positive (Flotte et al., 1983). The observed granular cytoplasmic staining by anti-Mac-3 (Flotte et al., 1983) is most likely due to staining of lysosomes, since Mac-3 was found to be identical to the lysosomal membrane protein LAMP-2 (Chen et al., 1985a; 1985b). Increase of cellular volume and lysosome content also explains the observed increase in Mac-3 expression with maturation of macrophages (Ralph et al., 1983; Walker et al., 1985).

The macrophage-specific antigen **BM8** is expressed relatively late in the differentiation of macrophages, since it is not found on bone marrow cells and blood monocytes (Malorny et al., 1986). In bone marrow cultures, BM8 Ag is only expressed by the mature macrophage stages. Like F4/80 Ag, BM8 Ag is hardly expressed in the lymphoid areas of the spleen. BM8 Ag is also found on Langerhans cells, thus reinforcing the close phenotypical linkage between dendrocytes and macrophages.

Another antigen, characteristic for a major macrophage population, is detected by **MOMA-2** (Kraal et al., 1987). In contrast to BM8 Ag, MOMA-2 Ag is already expressed by bone marrow precursors and blood monocytes. The tissue distribution of MOMA-2 Ag correlates closely with acid phosphatase expression. Hence, macrophages in lymphoid areas are easily detected by MOMA-2. Isolated dendrocytes express this antigen weakly.

A distinctive, post-monocytic population is detected by **MOMA-1** (Kraal and Janse, 1986). In the spleen, MOMA-1 detects a characteristic subpopulation of white pulp macrophages, i.e. the highly non-specific esterase positive marginal metallophilic macrophages. In lymph nodes, MOMA-1 stains the various sinusoidal macrophage subpopulations. Elimination of the MOMA-1 positive population in neonates by antibody injections seriously disturbs the immune responses against thymus-dependent (TD) and thymus independent type 2 (TI-2) antigens (Kraal et al., 1988). However, the response against thymus independent type 1 antigens (TI-1) was not altered. These data suggest therefore that MOMA-1 positive macrophages are important cells for uptake and presentation of TD and TI-2 antigens in neonates.

ER-TR9 recognizes an antigen that is specifically expressed by another macrophage subpopulation, situated in the splenic marginal zone adjacent to the rim of marginal metallophils (van Vliet et al., 1985; Dijkstra et al., 1985). In the lymph node medulla, however, ER-TR9 and MOMA-1 most likely detect the same sinusoidal macrophage population. ER-TR9 antibody injection in vivo completely blocked the characteristic uptake of neutral polysaccharides by marginal zone macrophages (Kraal et al., 1989). Puzzling, however, is the observation that complete elimination of the population did not influence the immune response against such TI-2 antigens.

Another macrophage subset-specific marker is **ER-HR3** Ag (de Jong et al., 1987). High numbers of ER-HR3 positive cells are found in the bone marrow stroma and the splenic red pulp; both are hemopoietic sites. In the course of macrophage differentiation, ER-HR3 Ag is increasingly expressed and occurs on a subpopulation of blood monocytes. Furthermore, distinctive macrophage subpopulations are recognized, including macrophages in the connective tissue of the intestine and in the intertubular areas of the renal medulla. Neonatal injection of ER-HR3 antibody led to a transient decrease of bone marrow hemopoietic progenitor cells, but not of CFU-S. This decrease appeared to be partially compensated by an increase of splenic CFU-S and CFU-GM.

These experiments suggest therefore that ER-HR3 antibody injection selectively depresses bone marrow hemopoiesis.

Dendrocyte antigens

MAbs that bind specifically to dendrocytes are scarce (table 2.VII). Of these, **MIDC-8** appears to be best suited for the immunohistochemical detection of dendrocytes. MIDC-8 lacks the cross-reactivity with epithelia, exhibited by **NLDC-145** (Breel et al., 1987; Kraal et al., 1986). MAb **33D1**, on the other hand, showed only poor reactivity in immunohistochemical analysis (Witmer and Steinman, 1984). MIDC-8 binds to a cytoplasmic component of dendrocytes, which might be a disadvantage in some experimental settings. NLDC-145 Ag is expressed by all dendrocyte types and is also found on a subpopulation of alveolar macrophages and some bone marrow-derived macrophage colonies (Breel et al., 1987; 1988b). Expression of this antigen appears to be inducible by cytokines (Breel et al., 1988a). Both MIDC-8 and NLDC-145 recognize Langerhans cells, thus reinforcing the suggestion that Langerhans cells constitute an intermediate developmental stage between dendritic cells on the one hand, and classic macrophages on the other (Hoefsmit et al., 1982; Hume et al., 1983).

In conclusion, a large variety of mAbs against different stages of macrophage development has been produced and described in the literature. In contrast to earlier, optimistic expectations, mAbs have not yet unraveled the origins of macrophage heterogeneity. It was hoped that clearly distinctive mAb reactivity patterns would be obtained, enabling the unequivocal identification of macrophage maturation and activation stages, and - if existing - distinct differentiation lineages or independently

Table 2.VII mAbs against dendrocyte antigens

Ag	mol.mass (kDa)	mAb	CD-designation	Ag-function	references
MIDC-8 Ag	-	MIDC-8	-	-	(38)
NLDC-145 Ag	145	NLDC-145	-	-	(192)
33D1 Ag	-	33D1	-	-	(272)

- : data are presently unknown

maintained macrophage populations. However, the complexity of the reactivity patterns of the presently available mAbs makes clear that the mononuclear phagocyte system is even more heterogeneous than previously thought.

CHAPTER 3

PURPOSE OF THE STUDY AND

INTRODUCTION TO EXPERIMENTAL WORK

3. PURPOSE OF THE STUDY AND INTRODUCTION TO EXPERIMENTAL WORK

As outlined in the previous chapter, many questions concerning the process of differentiation - i.e. maturation and diversification - of macrophages remain unanswered. In this thesis, a few of these questions have been approached:

- Do distinct lineages of macrophage differentiation exist ?
- If so, can such lineages be distinguished already at the macrophage precursor level ?
- Additionally, do such differentiation lineages express distinctive antigens ?
- Can macrophage precursors be distinguished on the basis of their phenotype - i.e. the diversity of antigens expressed on their cell surface ?
- Are macrophage precursors heterogeneous with respect to their phenotype ?
- Can maturation of macrophages in vitro be modulated by mAbs against cell surface antigens ?
- Can mature macrophage subpopulations be distinguished in situ on the basis of their phenotype ?
- Do dendrocytes belong to the mononuclear phagocyte system ?

As may be deduced from the questions above, we feel that thorough analysis of the phenotypes of mononuclear phagocytes may significantly aid to unravel the complexity of the mononuclear phagocyte system. In general, the purpose of this study is therefore the establishment of a correlation between mononuclear phagocyte phenotype and differentiation stage. To that end, novel mAbs were raised and specifically screened for the recognition of different stages in macrophage differentiation.

Vital elements in the development of mAbs are both the methods of screening and the targets used. Therefore, screening methods have first been worked out, as far as these were not yet available. Parts of these methodical studies have been reported elsewhere (Leenen et al., 1985; 1987). As targets for screening, multiple mature macrophage populations were isolated or investigated in situ using immunohistochemistry. However, detection of antigens that are selectively expressed by early stages in macrophage development is problematic, since macrophage precursors are relatively scarce and hidden in the complex bone marrow population. Hence, a model of macrophage differentiation - described in **chapter 4** - was developed using a panel of macrophage tumor cell lines, arrested in distinct stages of macrophage differentiation.

We decided to focus initially on macrophage precursors as, in particular, the phenotypical changes in the first stages of macrophage differentiation are unexplored. An additional argument for studying the early differentiation stages is the unresolved

question whether multiple macrophage lineages exist or not. An answer to this question may be found in the macrophage precursor population, as multiple lineages - if they exist - are initiated early in macrophage development. An approach to this issue is described in **chapter 5**, where we characterize macrophage precursor hybrid cell lines obtained by immortalization of cells from bone marrow cultures.

As clonal cell populations, the macrophage precursor hybrids are unique immunogens for the development of a panel of anti-macrophage precursor antibodies. In **chapter 6** four different mAbs, generated against macrophage precursor hybrids, are presented that selectively recognize precursor stages in macrophage differentiation, both in the differentiation models used for screening as well as in normal bone marrow.

Another mAb obtained from these fusions, ER-MP21, appeared to be directed against the murine transferrin receptor. In cultures of mononuclear phagocytes, ER-MP21 caused a strong inhibition of macrophage precursor proliferation, whereas proliferation of more mature stages was hardly affected. In **chapter 7**, we substantiate the indicated correlation between macrophage differentiation stage and proliferation inhibition by ER-MP21. Furthermore, we approach the mechanism underlying this differential inhibition of proliferation by anti-transferrin receptor mAb.

In **chapter 8** the reactivity of ER-MP23, a mAb also obtained from the mentioned fusions, is characterized. The recognized antigen is only marginally expressed by macrophage precursors, but readily detectable on a characteristic mature macrophage population associated with connective tissue. Most likely, ER-MP23 is a unique marker for a separate macrophage differentiation lineage or an independently maintained population.

A mAb, ER-BMDM1, raised against more mature stages of macrophage differentiation, is described in **chapter 9**. This mAb appeared to recognize a cell surface aminopeptidase occurring on a subpopulation of both macrophages and dendrocytes. The latter finding strengthens the mononuclear phagocyte origin of dendrocytes. Analysis of ER-BMDM1 aminopeptidase expression in various differentiation models revealed a strong correlation between macrophage maturation stage and ER-BMDM1 Ag expression.

Taking advantage of newly available mAbs against mature macrophage subpopulations, we decided to investigate the macrophage content of the spleen with respect to the occurrence of phenotypically distinct subpopulations (**chapter 10**). This study led to the discovery of hitherto unknown macrophage subpopulations, especially in the splenic red pulp compartment. In general, this study shows that the phenotypical approach contributes significantly to our knowledge of mononuclear phagocyte heterogeneity.

CHAPTER 4

MURINE MACROPHAGE CELL LINES CAN BE ORDERED IN A LINEAR DIFFERENTIATION SEQUENCE

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ABSTRACT

The present study investigated whether transformed macrophage cell lines represent certain stages in macrophage differentiation. Cell surface markers linked to macrophage differentiation were characterized in 11 murine macrophage cell lines and compared with markers on isolated resident and exudate peritoneal macrophages. Furthermore, the capacity of the cell lines to phagocytose latex microspheres was analyzed. This analysis indicated that cell lines arrested at an early differentiation stage were characterized by the expression of the 'immature' markers Thy-1 and MIV 113, and the lack of expression of 'mature' macrophage markers such as Mac-1, Mac-2 and F4/80. More mature cell lines, which do not express 'immature' markers, show an increase in the expression of 'mature' macrophage markers. Furthermore, the expression of the 'mature' markers was found to be correlated with the phagocytic capacity of the cells. We have ordered the cell lines in a linear differentiation sequence based on these data. We propose that this sequence represents various stages in the differentiation of macrophages. This panel of cell lines provides a new model of early macrophage differentiation.

INTRODUCTION

Macrophages originate in the bone marrow from pluripotent hemopoietic stem cells (Van Furth and Cohn, 1968). The differentiation of macrophages is accompanied by the acquisition of common macrophage characteristics, including the expression of the differentiation antigens Mac-1 and F4/80, and the capacity for immune phagocytosis (Springer et al., 1979; Van Furth et al., 1980; Hirsch et al., 1981). The expression of 'immature' markers such as Thy-1 or Lgp-100 is lost during the course of macrophage differentiation (Boswell et al., 1984; Berman and Basch, 1985; Miller et al., 1985). The identification and characterization of various stages in macrophage differentiation has been severely hampered by the fact that the precursor cells in the bone marrow occur only at a very low frequency (Van der Meer et al., 1983). Culture systems which select for the differentiation of macrophages are useful for the study of macrophage development (Stuart et al., 1977; Van der Meer et al., 1979b; Walker et al., 1985). However, cell populations that are derived from these cultures are heterogeneous with respect to the cellular differentiation stages represented. Furthermore, the frequency of occurrence of precursor cells is low in these systems.

We therefore considered whether transformed cell lines could be used to identify the various stages of macrophage development. According to recent insights, tumor cells appear to be arrested at defined stages of differentiation. The phenotype expressed by

these cells usually reflects the cell lineage and stage of differentiation (Greaves, 1979; 1982).

In the present study we investigated the expression of common 'mature' and 'immature' macrophage markers by a panel of 11 macrophage cell lines. We quantitatively determined the expression of the 'mature' markers Mac-1, Mac-2 and F4/80 and of the 'immature' markers Thy-1 and MIV 113. As a functional indicator of macrophage differentiation, we assessed the capacity of the cell lines to phagocytose latex beads. On the basis of the results of these experiments, we proposed a linear differentiation sequence into which the macrophage cell lines could be ordered. This sequence provides a useful model for the study of macrophage differentiation.

METHODS

Mice. Male and female BALB/c mice, 15-20 weeks of age, were obtained from Bomholtgard Ltd. (Ry, Denmark) and kept under routine laboratory conditions.

Macrophage populations and cell lines. Resident peritoneal cells were obtained by peritoneal lavage with 5 ml RPMI-1640 (Flow Laboratories, Irvine, Scotland). Thioglycollate elicited peritoneal exudate cells were isolated from mice that had been injected intraperitoneally with 1 ml Brewer's thioglycollate (Difco Laboratories, Detroit, USA) 4 days previously. The main characteristics of, and original references pertaining to the cell lines used in this study are summarized in table 4.I. The cells were cultured under 7% CO_2 in RPMI-1640 which had been supplemented with 5% fetal calf serum (FCS), 5% newborn calf serum (NCS), 5% horse serum (HS) and 5 x 10^{-5} M 2-mercaptoethanol. Penicillin, streptomycin and fungizone were added as antibiotics.

Preparation of cells. For flowcytometric analysis, cells were isolated and washed twice with RPMI-1640. The cells were then fixed with 0.5% or 1% paraformaldehyde (Merck, Darmstadt, FRG) in DPBS for 30 min at room temperature at a concentration of 10^6 cells/ml. The fixation was terminated by centrifugation at 250 x g for 10 min through a cushion of FCS. The cells were subsequently washed with DPBS and with DPBS supplemented with 0.02% (w/v) gelatin (DPBS-gel) and 50 μl aliquots pipetted into microtitre plates. A coating of cells was applied to Terasaki trays for use in the ELISA procedure (Leenen et al., 1985). Briefly, the cells were collected, washed with RPMI-1640 and aliquots placed in Terasaki trays. The cells were allowed to settle to the bottoms of the wells and were then fixed to the plates using 0.05% (v/v) glutaraldehyde (Polysciences, Warrington, USA). The plates were stored at 4°C in DPBS-gel sup-

Table 4.I Cell lines

Cell line	Cell type	Reference
M1	myeloblast	Ichikawa (1969)
RMB-1	myelocyte	De Both et al. (1981)
RMB-3	myelocyte	De Both et al. (1981); to be published
WEHI-3B	immature macrophage	Warner et al. (1969)
WEHI-3	immature macrophage	Warner et al. (1969)
Pu5-1.8	macrophage	Ralph et al. (1975)
J774	macrophage	Ralph et al. (1983)
P388D1	macrophage	Koren et al. (1975)
RAW264.7	macrophage	Raschke et al. (1978)
RAW309Cr.1	macrophage	Raschke et al. (1978)
WR19M.1	macrophage	Raschke et al. (1978)
P815	mast cell	Ralph and Nakoinz (1981)
RLD-1	immature T cell	De Both et al. (1983)
P3-X63-Ag8.653	B cell	Kearney et al. (1979)

plemented with 20 mM sodium azide. After being treated in this way, the plates could be stored for several months without substantial loss of membrane antigenic integrity (Van Ewijk et al., 1984).

Antisera and conjugates. The specifications of and references for the antisera used are summarized in table 4.II. Optimally diluted culture supernatants of hybridoma cell lines grown in RPMI-1640 with 10% FCS, were used. As second-stage antibodies, we applied affinity-purified sheep anti-rat immunoglobulin coupled to E. coli ß-galactosidase (Radiochemical Centre, Amersham, UK), or fluorescein-conjugated rabbit anti-rat immunoglobulin (Nordic Immunological Laboratories, Tilburg, The Netherlands).

Immunofluorescence and flow-cytometric analysis. Fluorescence labeling and subsequent analysis were performed as described by van Ewijk et al. (1981, 1984). At least 10^4 cells from each individual sample were analyzed with a FACS II (Becton and Dickinson, Sunnyvale, CA, USA). Before each analysis, the FACS was calibrated using

Table 4.II Antisera

Antiserum	Antigen	Specificity	References
M1/70	Mac-1, complement receptor type 3	not-fixed macrophages, granulocytes, natural killer cells	Springer et al. (1979); Beller et al. (1982); Flotte et al, (1983)
M3/38	Mac-2	macrophages, dendritic cells, epithelium	Ho and Springer (1982); Flotte et al. (1983)
M3/84	Mac-3	macrophages, dendritic cells, epithelium, endothelium	Flotte et al. (1983); Ho & Springer (1983)
2.4G2	Fc receptor type II	macrophages, granulocytes, B cells	Unkeless (1979)
F4/80	F4/80	macrophages	Austyn & Gordon (1981)
59-AD2.2	Thy-1	T cells, stem cells, myeloid progenitors	Ledbetter & Herzenberg (1979); Boswell et al. (1984); Berman & Basch (1985)
MIV 113	MIV 113	leukocyte- and erythroid progenitors	Willmer et al. (1984); Leenen et al. (1986a)
30G12	T-200	all leukocytes	Ledbetter & Herzenberg (1979)

fluorescent latex particles (Polysciences). The fluorescence signals were amplified logarithmically. The mean peak fluorescence values obtained, expressed as logarithmic channel numbers, were converted to a linear scale according to the method of Jongkind et al. (1986). As a control, negative values were obtained from cells stained only with FITC-conjugated second-step antiserum. These negative control values, expressed as mean linear values ($\overline{F_{lin}}$), were subtracted from the $\overline{F_{lin}}$ obtained for cells stained with monoclonal antibody and second-step reagent, thus yielding in a net mean linear fluorescence value, represented as $\Delta\overline{F_{lin}}$. Resident peritoneal macrophages and peritoneal exudate macrophages were identified in the lavage mixtures on the basis of their forward-light-scatter characteristics.

ELISA. The ELISA procedure was essentially performed as described by van Soest et al. (1984) with minor modifications (Leenen et al., 1985). In individual experiments, antibody binding was expressed as a percentage of the positive control (anti-T-200)

antibody binding, since T-200 was expressed by all macrophage cell lines at a constant, high level. This standardization was employed in order to compare experiments with different sets of test plates. However, the FACS analysis of cells stained with anti-T-200 and rabbit anti-rat Ig-FITC revealed significant differences between the individual cell lines with respect to the expression of T-200 (table 4.III). In order to compare the marker expression of different cell lines, it was necessary to correct for these differences in T-200 expression. Therefore, ELISA data were standardized to an arbitrarily chosen T-200 expression of 800 channels, using the data given in table 4.III. Since P3 cells do not express the T-200 antigen, these cells were excluded from the ELISA experiments.

Phagocytosis. The phagocytic capacities of the various cell lines were assessed, using fluorescent polystyrene latex beads (Polysciences; diameter 1.8 μm). Cells with adherent properties were cultured at a concentration of 5 x 10^5 cells/ml for 2 h on coverslips. These cover slips were then washed twice in RPMI-1640 and covered with a layer of

Table 4.III. Expression of T-200 antigen as determined with FACS analysis

	Mean fluorescence channel (linear scale) ± SEM
M1	424±46
RMB-1	484±50
RMB-3	480±58
WEHI-3B	723±96
WEHI-3	1107±40
Pu5-1.8	1179±65
J774-1.6	699±99
P388D1	1106±89
RAW264.7	746±106
RAW309Cr.1	1209±153
P815	1419±289
RLD-1	574±91
P3	18±3

Data result from at least 5 determinations per cell line.

latex beads suspended in RPMI-1640, with or without 10% NCS as an opsonin (cf. Diesselhoff-Den Dulk and Van Furth, 1981). The ratio of cells to latex beads was 1:100. Phagocytosis was allowed to occur for 1 h at 37°C. After phagocytosis, the coverslips were gently washed with RPMI-1640. By treating the coverslips with acetone during 1 min, the cells were fixed and, at the same time, the fluorescence of the extracellular, non-phagocytosed, beads was quenched. The coverslips were mounted on microscopic slides with 10x DPBS/glycerol (1:9) and examined using a fluorescence microscope. Cells from non-adherent cell lines were isolated from culture and washed twice with RPMI-1640. They were resuspended in RPMI-1640, with or without 10% NCS. Latex beads were added to the cells in a 100:1 ratio. After 1 h of phagocytosis, cytocentrifuge preparations were made. These were then treated with acetone, coverslipped, and examined as indicated above.

RESULTS

Surface antigen expression

In the course of macrophage differentiation, the cellular phenotype changes. 'Immature' markers such as Thy-1 and MIV 113 are lost, whereas 'mature' markers such as Mac-1, Mac-2 and F4/80 are increasingly expressed. In the first part of this study, we determined the markers expressed by the various cell lines in order to investigate the differentiation stage at which the cells were arrested. The cell lines examined have been listed in table 4.I. The monoclonal antibodies used to detect the various differentiation markers have been mentioned in table 4.II.

Expression of 'mature' macrophage markers (Mac-1, Mac-2, F4/80)

Figure 4.1 shows the expression of Mac-1, Mac-2 and F4/80 antigens by the cell lines, as determined by FACS analysis. In table 4.IV the corresponding ELISA data are given. In figure 4.1 and in the following figures, the cell lines have been ordered according to the most likely differentiation sequence, as will be discussed. The data obtained indicated that M1 and RMB-1 did not express Mac-1 (figure 4.1, table 4.IV). RMB-3, WEHI-3B, WEHI- 3, Pu5-1.8 and J774-1.6 showed a low level of Mac-1 expression, whereas P388D1, RAW264.7, RAW309Cr.1 and WR19M.1 expressed Mac-1 at an intermediate to high level.

Mac-2 was not expressed by M1, RMB-1, RMB-3 and WEHI-3B. WEHI-3 expressed Mac-2, but at a low level, whereas the other macrophage cell lines demonstrated an intermediate to high level of Mac-2 antigen expression.

Significant expression of the F4/80 antigen was only found in the cell lines with

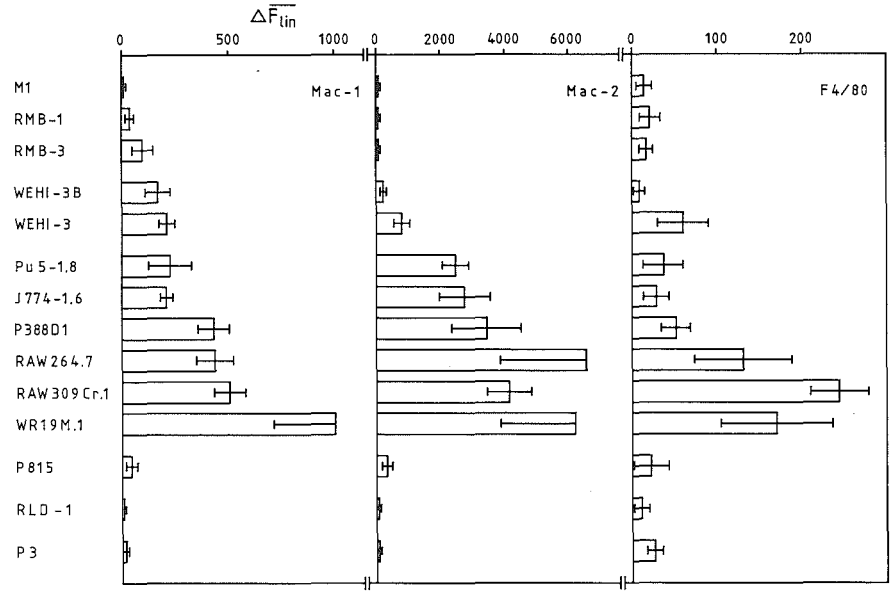

Figure 4.1.

Expression of Mac-1, Mac-2 and F4/80 antigens by macrophage and control cell lines, as determined by flow-cytometry. $\Delta\overline{F_{lin}}$ represents the net linear fluorescence channel, i.e. the mean linear fluorescence channel of cells stained with monoclonal antibody and rabbit anti-rat Ig-FITC minus the mean linear fluorescence channel of cells stained only with anti-rat Ig-FITC (negative control values). The bar represents the standard error of the data obtained in at least three individual experiments.

the highest levels of Mac-1 and Mac-2 expression, i.e. RAW264.7, RAW309Cr.1 and WR19M.1. The non-macrophage cell lines (P815, RLD-1, P3) demonstrated no detectable expression of these three differentiation antigens.

Expression of 'immature' macrophage markers (Thy-1, MIV 113)

Figure 4.2 shows the expression of the immature macrophage antigen Thy-1, as determined by FACS analysis. The expression of MIV 113 was determined only by the ELISA procedure, and is represented in table 4.IV. Thy-1 was highly expressed by RMB-1, RMB-3 and WEHI-3 (fig. 4.2). MIV 113 was expressed only by M1, RMB-1 and RMB-3 (table 4.IV). These 'immature' macrophage markers were not expressed by any of the other macrophage cell lines. The immature T cell line RLD-1 expresses both Thy-1 and MIV 113, whereas the other control cell lines did not express these markers.

Phagocytosis

In a second series of experiments, the various macrophage cell lines were tested for

Table 4.IV. Phenotypes of macrophage- and control cell lines as determined by ELISA

	Mac-1	Mac-2	F4/80	Thy-1	MIV 113
M1	1± 1	2± 1	1± 1	1± 1	31± 8
	(0-2)	(1-4)	(0-2)	(0-2)	(18-43)
RMB-1	0± 1	2± 1	1± 1	51± 7	10± 2
	(0-1)	(0-3)	(0-2)	(41-59)	(6-13)
RBM-3	6±1	6± 2	7± 2	39± 6	21± 5
	(2-8)	(3-11)	(2-10)	(32-45)	(11-32)
WEHI-3B	5± 1	6± 2	2± 2	2± 2	0± 1
	(3-8)	(5-7)	(1-5)	(0-5)	(0-1)
WEHI-3	11± 3	32±15	10± 3	173±14	0± 1
	(4-15)	(3-66)	(7-12)	(132-193)	(0-1)
PU5-1.8	19± 5	92±10	3± 3	7± 3	0± 3
	(7-32)	(72-11)	(1-7)	(3-8)	(0-6)
J774-1.6	15± 4	30± 6	14± 4	3± 2	1± 2
	(10-21)	(25-37)	(6-18)	(0-8)	(0-5)
P338D1	31± 5	96±16	7± 4	2± 3	0± 1
	(23-40)	(57-116)	(1-14)	(0-8)	(0-1)
RAW264.7	35± 7	118±19	30± 7	3± 2	1± 2
	(20-50)	(89-144)	(20-37)	(0-6)	(0-3)
RAW309CR.1	62±11	76±18	60±12	5± 3	0± 2
	(41-68)	(35-143)	(47-77)	(0-9)	(0-3)
WR19M.1	63±15	138±27	54±10	6± 3	1± 2
	(42-82)	(111-156)	(50-57)	(4-10)	(0-6)
P815	1± 4	8± 4	2± 2	2± 2	2± 2
	(0-4)	(4-11)	(0-4)	(0-4)	(0-5)
RLD-1	0± 1	2± 2	1± 1	84±15	17± 8
	(0-1)	(0-4)	(0-2)	(0-2)	(6-37)

Data represent relative expression as a percentage of standardized positive control (see Methods) ± standard error of at least 3 determinations. The range is expressed in parentheses.

the capacity to phagocytose latex beads, both in the presence and in the absence of NCS. In the presence of NCS, the microspheres were opsonized, and were therefore ingested by Fc-receptor-mediated phagocytosis.

Figure 4.3 shows the results of these experiments. A marked difference in phagocytic capacity was observed between the various cell lines. Both in the absence and in the presence of NCS, the phagocytosis by M1, RMB-1, RMB-3, WEHI-3B and WEHI-3 was at the same level as in the non-phagocytic control cell lines. In the absence of NCS, the

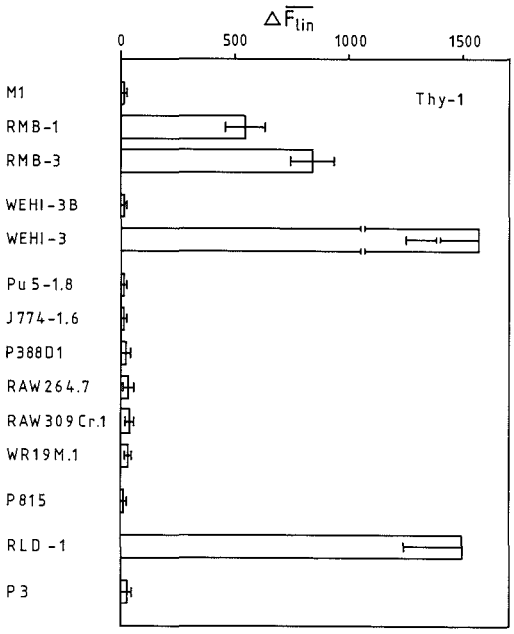

Figure 4.2.
Expression of Thy-1 antigen by macrophage and control cell lines, as determined by flow-cytometry. The bar represents the standard error of the data obtained in at least three experiments.

other macrophage cell lines (Pu5-1.8, J774-1.6, P388D1, RAW264.7, RAW309Cr.1 and WR19M.1) ingested the latex beads at about the same level. In the presence of NCS, however, a difference in phagocytic capacity was observed; the level of phagocytosis was well correlated with the expression of 'mature' macrophage markers (cf. to fig. 4.1).

Phenotype stability

It may be argued that the expression of cell surface markers changes during prolonged passaging of cells. To investigate the stability of the expression of cell surface markers by a particular macrophage cell line, we analyzed the phenotypes of different sublines of J774 as well as those of different passages of the same subline. In Table 4.V the results of this analysis are summarized. The expression by cells from the earlier passage of J774-1.6, termed J774-1.6-1, was identical to that of J774-1.6 cells tested in previous assays. The cells from a later passage (J774-1.6-2) were obtained after approximately 6 months of cell culture. The earlier passage (J774-1.6-1) demonstrated, compared to J774-1.6-2, a lower level of Mac-1 expression, whereas it expressed Mac-2 at a higher level. Comparison of J774-1.6-2 with the other J774 sublines reveals that of all markers tested, only the expression of Mac-2 showed any significant differences.

Phenotypes of peritoneal macrophages

Figure 4.4 illustrates the marker phenotypes of resident and exudate peritoneal

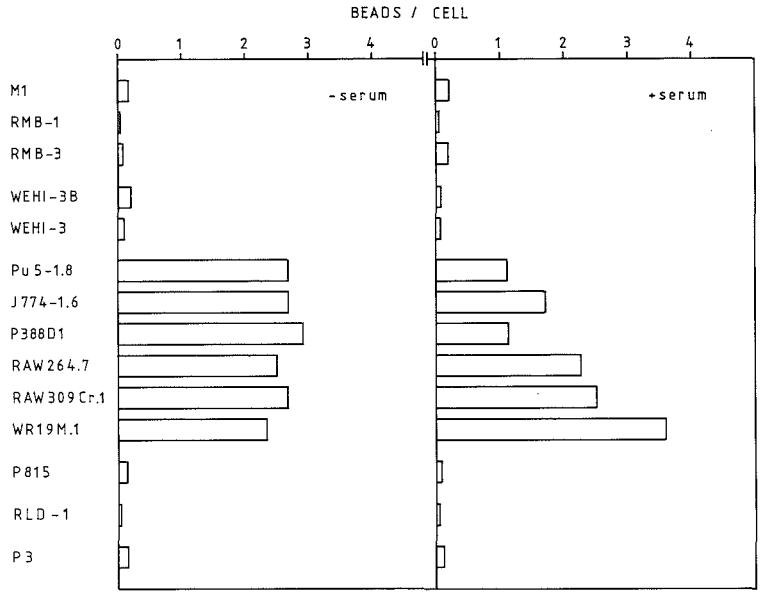

Figure 4.3.

Phagocytosis of non-opsonized (- serum) and opsonized (+ serum) latex beads by macrophage and control cell lines, expressed as the mean number of beads per cell as determined in two independent experiments. At least 200 cells were observed for each determination.

Table 4.V. Phenotypes of J774 sublines as determined by ELISA

	Mac-1	Mac-2	Mac-3	2.4G2	F4/80	Thy-1	MIV 113
J774-1.6-1	15±4	30±6	27±8	14±4	14±4	3±2	1±2
	(10-21)	(25-37)	(13-35)	(6-18)	(4-19)	(0-8)	(0-5)
J774-1.6-2	35±6	2±1	20±6	12±6	19±5	0±1	N.D.
	(25-42)	(0-6)	(6-30)	(7-27)	(8-27)	(0-1)	
J774.2	47±8	14±7	27±8	17±4	15±5	0±1	2±3
	(40-52)	(2-26)	(16-40)	(11-22)	(9-23)	(0-1)	(0-5)
J774.C3C	41±6	3±1	38±8	15±3	16±6	1±1	1±1
	(36-45)	(1-5)	(26-48)	(13-17)	(8-26)	(0-1)	(0-2)
J774-A.1	36±5	11±6	23±6	11±3	21±4	1±1	4±1
	(35-37)	(3-22)	(15-28)	(8-13)	(16-26)	(0-1)	(3-6)

Data represent relative expression as percentage of standardized positive control ± standard error of at least 3 determinations. The range is expressed in parentheses.

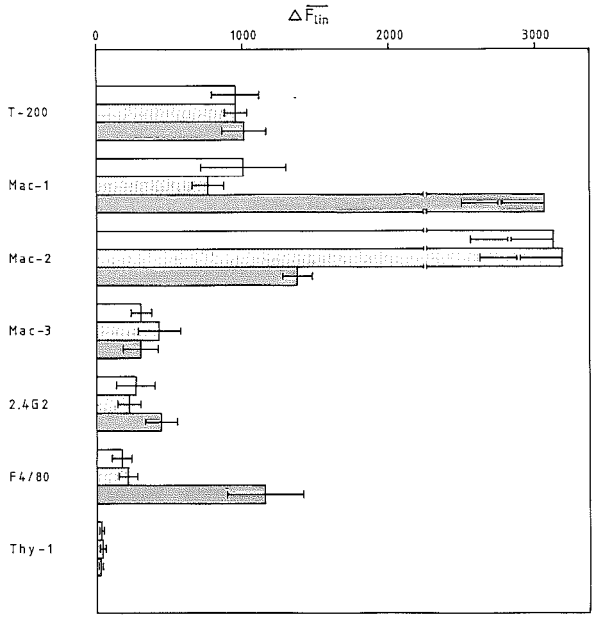

Figure 4.4.
Comparative phenotypical analysis of WR19M.1 (▭), peritoneal exudate macrophages elicited with thioglycollate (▨) and resident peritoneal macrophages (▨) as determined by flowcytometry.

macrophages and of the cell line WR19M.1. A striking similarity of antigenic composition was observed between WR19M.1 and peritoneal exudate macrophages that had been elicited with thioglycollate. Resident peritoneal macrophages, on the other hand, were characterized by a significantly higher level of Mac-1 and F4/80 expression and a lower level of Mac-2 expression.

DISCUSSION

In the present study we determined the phenotypes of a panel of 11 macrophage cell lines. The results imply that, among these cell lines:

1. The expression of the 'immature' macrophage marker Thy-1 occurs only in RMB-1, RMB-3 and WEHI-3 cells. The 'immature' marker MIV 113 is only expressed by M1, RMB-1 and RMB-3 cells.
2. The 'mature' macrophage markers Mac-1, Mac-2 and F4/80 are not expressed, or are only expressed at low levels, by cell lines that express 'immature' markers.
3. The 'mature' markers are expressed by the various cell lines in varying degrees. However, the expression of Mac-1, Mac-2 and F4/80 shows significant correlation.
4. Phagocytosis of latex beads is only observed in Pu5-1.8 J774-1.6, P388D1, RAW264.7, RAW309Cr.1 and WR19M.1 cells. The phagocytic capacity varies between these lines when latex beads are opsonized with serum.

5. A correlation exists between the capacity to phagocytose opsonized latex beads and the expression of 'mature' markers.

Accordingly, the macrophage cell lines investigated can be ordered as a linear differentiation sequence. We propose that the most likely sequence in order of increasing maturity, is the following: M1, RMB-1, RMB-3, WEHI-3B, WEHI-3, Pu5-1.8, J774-1.6, P388D1, RAW264.7, RAW309Cr.1, WR19M.1. This sequence is also reflected in Table 4.I and figures 4.1 - 4.3.

The aligned cell lines can be divided into three groups: a precursor group (M1, RMB-1, RMB-3), a group of immature macrophages (WEHI-3B, WEHI-3) and a third group comprising more mature cells (Pu5-1.8, J774-1.6, P388D1, RAW264.7, RAW309-Cr.1, WR19M.1). The precursor cell lines can be induced to differentiate into either granulocytic or macrophage cells, depending on the nature of the inducing agent (Sachs, 1978; De Both et al., 1981). The immature macrophage cell lines WEHI-3B and WEHI-3 show some characteristics of mature macrophages but lack others (Ralph and Nakoinz, 1981; Van Loveren et al., 1983; Ralph, 1984). WEHI-3B can be induced to differentiate further (Cooper et al., 1982). The mature lines exhibit many functional and phenotypic characteristics of mature macrophages (Ralph, 1980; 1984; Ralph et al., 1983).

The characteristics we studied in order to determine macrophage maturity show a clear correlation with the normal macrophage differentiation sequence. In the bone marrow, Mac-1 is not expressed by hemopoietic progenitors (Miller et al., 1985) and found on immature macrophages only at a low level (Springer et al., 1979; Springer, 1980). Furthermore, differentiation of blood monocytes into peritoneal exudate macrophages is accompanied by an increase in the level of Mac-1 expression (Springer et al., 1979; Springer, 1980). Thus, it appears that the level of expression of Mac-1 generally correlates with macrophage differentiation. For Mac-2, a similar increase of antigen expression during differentiation has been described. In analyses of bone marrow cultures stimulated with CSF-1, Walker et al. (1985) demonstrated the presence of Mac-2 on only a small minority of non-adherent macrophage precursors, whereas virtually all mature macrophages expressed Mac-2 at a high level. Springer et al. (1979) showed a strong increase in the level of Mac-2 expression during the differentiation of blood monocytes to peritoneal exudate macrophages. The expression of F4/80 by differentiating macrophages in bone marrow culture was studied by Hirsch et al. (1981) and appeared to increase steadily during macrophage differentiation. In addition, the capacity to phagocytose latex beads has been shown to be correlated with macrophage differentiation (Van Furth et al., 1980; Van der Meer et al., 1983).

The expression of the 'immature' markers used in this study is inversely correlated to the degree of macrophage differentiation. Thy-1 is present on pluripotent hemopoietic

stem cells, early macrophage precursors, and on other progenitor stem cells, but not on mature macrophages (Berman and Basch, 1985; Boswell et al., 1984; Schrader et al., 1982). The marker recognized by MIV 113 antibodies is present on undifferentiated M1 cells but is lost after induction of macrophage differentiation (Willmer et al., 1984; Leenen et al., 1986). In bone marrow cultures we demonstrated the presence of this marker on macrophage progenitors but not on differentiated macrophages (Leenen et al., 1986). Together, these results indicate that the markers used in the present study are, either positively or negatively, correlated to macrophage differentiation.

How stable is the phenotype of macrophage cell lines? The phenotypic comparison of different J774 sublines (table 4.V) indicates that the 'mature' macrophage markers are expressed at comparable levels on these sublines. The expression of Mac-2, however, shows significant differences. The lack of Thy-1 expression by M1 and WEH1-3B questions whether these lines have a phenotype, that reflects to their apparent differentiation stages. We have found that the expression of Thy-1 by precursor and immature macrophage cell lines may vary. During prolonged culture of the RMB and WEH1-3B cell lines, variant clones were derived that had gained or lost the ability to express of Thy-1 (unpublished results). Therefore, we regard it as possible that M1 and WEH1-3B may also have lost the ability to express Thy-1 during years of cell culture.

It may be asked whether macrophage cell lines are arrested absolutely at definite stages of differentiation. Macrophage precursor cell lines exhibit spontaneous differentiation to some extent (De Both et al., 1981; Leenen et al., 1986). Furthermore, precursor and immature cell lines can be induced to undergo differentiation into macrophages by a number of stimuli, such as dexamethasone, lipopolysaccharide or various conditioned media (Sachs, 1978; Ralph et al., 1983; Ralph, 1984). The 'immature' marker Thy-1 is not expressed by any of the J774 sublines investigated in this study. However, Van Furth et al. (1985c) reported the expression of Thy-1 by the J774.1 subline. This phenomenon suggests a phenotypic shift in this particular subline towards a less mature stage of differentiation. Morphometric data and the low level of phagocytosis observed by Van Furth et al. (1985c) support the suggestion that a phenotypic shift occurred in the J774.1 subline. Together, these results indicate that the arrest of tumor cell line maturation is not absolute (cf. Greaves, 1982). Therefore, reliable use of the cell line model of macrophage differentiation presented requires frequent control of marker expression stability.

How does this cell line model relate to normal macrophage differentiation? The precursor cell lines M1, RMB-1 and RMB-3 appear to be arrested around the CFU-GM differentiation stage, since cells from these lines can be induced to undergo both granulocytic and macrophage differentiation. A more detailed phenotypic study of RMB-1 published elsewhere reinforces this conclusion (Delwel et al., 1987). The striking phenotypic resemblance between WR19M.1 and thioglycollate elicited peritoneal exudate

macrophages (fig. 4.4) suggests that WR19M.1 cells are arrested at a stage comparable to that of the exudate macrophage.

One could argue that the cell lines investigated in this study might be representatives of different macrophage lineages rather than representatives of certain differentiation stages. However, the 'mature' differentiation markers investigated in our study are general macrophage markers, expressed by virtually all mature macrophage populations. They do not distinguish between different subpopulations. The question of whether the macrophage cell lines investigated represent different populations of macrophages is beyond the scope of this paper.

In conclusion, the macrophage cell lines investigated in this study can be ordered into a linear differentiation sequence on the basis of a combined phenotypic and functional analysis. The panel of cell lines studied provides a model of macrophage differentiation, in which stages are represented from the level of the committed stem cell for the granulocytic and macrophage lineages on the one hand, to the level of exudate macrophages at the other hand. This model is of particular value for studying the precursors of macrophages, since cells at differentiation stages between CFU-GM and the monoblast stage are not abundant in normal bone marrow or in CSF-1 induced bone marrow cultures.

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CHAPTER 5

MURINE MACROPHAGE PRECURSOR CHARACTERIZATION. I. PRODUCTION, PHENOTYPE AND DIFFERENTIATION OF MACROPHAGE PRECURSOR HYBRIDS

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SUMMARY

This study reports on the earliest stages of mononuclear phagocyte differentiation. A crucial question in this developmental process is whether mature macrophage heterogeneity is already appointed at the precursor cell level. To this purpose, we produced clonal populations of mononuclear phagocytes from bone marrow culture by somatic cell hybridization with two HAT-sensitive myeloid cell lines. A panel of 22 stable hybrids was obtained from these fusions. Differentiation stage analysis of the hybrids indicated that all cell lines had immature mononuclear phagocyte characteristics. The hybrids exhibited typical myeloid morphology and mainly non-adherent growth. Mature macrophage features, such as expression of the cell surface antigens Mac-1, Mac-2 and F4/80, phagocytosis of latex beads, and expression of non-specific esterase and acid phosphatase activity, were virtually absent. The immature macrophage markers Thy-1, MIV 25 and MIV 52, on the other hand, were readily expressed, although heterogeneity was observed among different hybrid cell lines.

We then analyzed the differentiation potential of 7 hybrids by culture of the cells in the presence of post-endotoxin serum supplemented with IFN-γ, and found that the expression of mature macrophage characteristics was induced. However, the various hybrids showed divergent patterns of mature macrophage marker induction. R0C2 cells, for instance, showed extensive morphological and phenotypical differentiation without concomitant induction of phagocytosis. In contrast, W1C4 cells showed significant induction of phagocytosis without simultaneous increase of phosphatase and esterase activity. R1C1 cells were unique in the strong induction of Ia antigen expression.

Together, our data indicate that (i) early macrophage differentiation stages can be rescued by somatic cell hybridization, and that (ii) the obtained hybrid cell lines are able to mature according to divergent differentiation programs.

INTRODUCTION

Mononuclear phagocytes originate from the bone marrow, where precursors develop from pluripotent hemopoietic stem cells (van Furth and Cohn, 1968). Monocytes are transported via the peripheral blood and mature to macrophages upon entrance of the various tissues. Mature macrophages constitute a heterogeneous population with regard to functional, morphological and phenotypical aspects (for reviews see Morahan, 1980; Walker and Hester, 1983; Gordon et al., 1988b). However, consensus about arranging mononuclear phagocytes in discrete subsets - as can be done for lymphoid cells - has not been reached. It remains to be conclusively established whether specialized subsets exist,

or, alternatively, the mononuclear phagocyte lineage as a whole forms a continuum in which different activation- and maturation stages - induced by extrinsic factors - perform distinct immunological functions. Arguments have been given for both views (Walker and Hester, 1983; Gordon et al., 1988b; Bursuker and Goldman, 1983; Treves, 1984; Gordon, 1986). Furthermore, the question may be raised whether mononuclear phagocyte heterogeneity is already appointed at the precursor level, as has been suggested previously (Bursuker and Goldman, 1983; Baccarini et al., 1986; Walker, 1987).

To address these questions, macrophages and their precursors have to be analyzed at the clonal level. An attractive approach is immortalization of single cells through hybridization with a suitable tumor cell line. In this way, representatives of different mature macrophage populations have been immortalized by using a mature macrophage cell line as fusion partner (Uchida et al., 1985; Tzehoval et al., 1984; Zuckerman et al., 1986; Chou et al., 1987). The resulting mature macrophage hybrids showed extensive heterogeneity with respect to phenotypes and functional capacities, such as phagocytosis, antigen presentation, IL-1 and prostaglandin secretion. Even during prolonged culture of the various mature macrophage hybrids, the distinct phenotypes are stably maintained. Likely, the distinct mature macrophage hybrids represent naturally occurring macrophage populations. Furthermore, the phenotypical diversity of these hybrids suggests that the different mature macrophage phenotypes are predetermined, rather than induced by extrinsic factors, as the hybrids are kept under identical culture conditions.

In the present study, we investigate whether macrophage heterogeneity is already appointed at the precursor level. To enable clonal analysis of macrophage precursor stages, we used somatic cell hybridization of bone marrow derived mononuclear phagocytes with myeloid tumor cell lines. By choosing tumor cell lines with an immature phenotype as fusion partners, we aimed at specific immortalization of macrophage precursors.

We analyzed the obtained hybrids with regard to several aspects indicative of macrophage maturity, viz. morphology, expression of mature and immature markers recognized by monoclonal antibodies, expression of non-specific esterase and acid phosphatase as well as phagocytosis of latex beads. In addition, the potential of the hybrids to undergo terminal differentiation upon induction with several agents was investigated.

The present data indicate that: (a) macrophage precursors can be immortalized by somatic cell hybridization; (b) the resulting macrophage precursor hybrids retain the capacity to differentiate; (c) different hybrids follow divergent differentiation pathways upon induction with an identical stimulus

MATERIALS AND METHODS

Mice. Male and female BALB/c mice were purchased from Olac (Bicester, Oxon, UK). Animals were kept under routine conditions and sacrificed at 15 - 20 weeks of age. C57Bl/6J mice were used for the production of post-LPS serum (PES). These mice were obtained from the Netherlands Energy Research Foundation (ECN, Petten, The Netherlands).

Antisera and conjugates. The specifications of the monoclonal antisera, used in the present study, are described in table 5.I. Culture supernatants from hybridoma cell lines grown in RPMI-1640 or DMEM (Flow Laboratories, Irvine, Scotland) with 10% FCS or SERUM-PLUS™ (KC Biological, Lenexa, KS, USA) were used.

As second-stage antibodies, we applied affinity purified sheep-anti-(rat Ig)-ß-galactosidase conjugate (Radiochemical Centre, Amersham, UK) or rabbit-anti-(rat IgG + IgM)-ß-galactosidase (Zymed Laboratories, San Francisco, CA).

M-CSF stimulated bone marrow culture. Bone marrow-derived mononuclear phagocytes (BMDM) were obtained by culturing bone marrow cells at 1.10^5 cells/ml under 10% CO_2 in DMEM supplemented with 20% LCM as a source of M-CSF, 10% FCS or 10% SERUM-PLUS™, penicillin and streptomycin. LCM was harvested after culturing a confluent layer of L cells for 5 days in DMEM with antibiotics.

As a source of immature BMDM, non-adherent cells were isolated after 4 days of culture in plastic flasks (Costar, Cambridge, MA)(Walker et al., 1985). This population consisted of about 55% mononuclear cells and 45% mature and immature polymorphonuclear cells. Lymphoid and erythroid contamination of this population was less than 5%.

Relatively mature BMDM were isolated from Teflon culture bags (van der Meer et al., 1979b) after 7 days of culture. This population consisted of approximately 65% mature and 35% immature mononuclear phagocytes (monocytes/macrophages and monoblasts + promonocytes, respectively; van der Meer et al., 1983).

Cell lines. The myeloid cell lines RMB-TG and WEHI-TG, used as fusion partners for the generation of hybrids, are the HGPRT-negative variants of RMB-1 and WEHI-3B, respectively (de Both et al., 1981; Warner et al., 1969; Geurts van Kessel et al., 1983). These cell lines were kindly provided by Dr. Ad Geurts van Kessel and cultured under 10% CO_2 in DMEM supplemented with 10% FCS or 10% SERUM-PLUS™ and antibiotics. The mature macrophage cell line RAW309Cr.1 (Raschke et al., 1978) and the pre-T cell line RLD-1 (de Both et al., 1983) were used as control cell lines in

Table 5.I. Monoclonal antibodies

antibody	antigen	specificity	reference
2.4G2	Fc-receptor II	Mϕ, granulocytes, B cells	(371)
30-G12	T-200	leukocytes	(203)
53-7.3.13	Lyt-1	T cells, B cell subset	(203)
53-6.72	Lyt-2	suppressor-/cytotoxic T-cells	(203)
59-AD2.2	Thy-1	T-cells, hemopoietic stem cells, myeloid cells	(203)
B2A2	heat-stable Ag	most hemopoietic cells	(319)
ER-TR6	ER-TR6 Ag	Mϕ, interdigitating cells, T cells, epithelium	(403)
F4/80	F4/80 Ag	Mϕ	(10)
H129.19	MT4	helper T cells, some monocytes/Mϕ	(287)
H129.37	LFA-1-α	leukocytes, hemopoietic precursors	(286, 287)
H129.121	transferrin-receptor	cells in cycle	(373)
IM7.8.1	Pgp-1	hemopoietic cells, esp. phagocytes	(362)
M1/42	class I MHC	all tissues	(332)
M1/70	Mac-1, complement receptor-3	non-fixed Mϕ, granulocytes, natural killer cells	(334)
M1/75	heat-stable Ag	erythrocytes, erythroblasts	(332)
M3/38	Mac-2	Mϕ, dendritic cells, epithelium	(148)
M3/84	Mac-3	Mϕ, dendritic cells, epi-, endothelium	(149)
M5/114	class II MHC	immunologically reactive cells	(27)
MIV 25	MIV 25 Ag	Mϕ precursors, granulocytes, B and T cells	(111, 207)
MIV 43	MIV 43 Ag	hemopoietic cells	(111)
MIV 38	MBR-2	mature Mϕ, endothelium, B cells, erythrocytes	(111, 207, 236)
MIV 52	MIV 52 Ag	Mϕ precursors, hemopoietic cells	(111, 207)
MIV 55	MBR-1	mature Mϕ, endothelium, B cells, erythrocytes	(111, 207, 236)
MECA-20	MECA-20 Ag	endothelium	(105a)
MEL-14	homing-receptor	recirculating lymphocytes	(122a)
RA3 6B2	B-220	B cells, myeloid cells	(65a)
RB6 8C5	Gr-1	granulocytes	(152a)

phagocytosis experiments. These cells were cultured as described above.

Generation of macrophage precursor hybrids. BALB/c BMDM obtained after 7 days of culture (see above) and RMB-TG and WEHI-TG cells were separately washed twice in serum-free RPMI. Then, 10^7 BMDM were mixed with either 10^7 RMB-TG or 2.8×10^6 WEHI-TG cells and washed another time. Hybridization occurred by gently applying 1 ml pre-warmed (37°C) 71 % polyethylene glycol (PEG-4000; Merck, Darmstadt, FRG)

in serum-free RPMI-1640 supplemented with 5% DMSO to the cell mixture. After 2 min at 37°C, the PEG was gradually diluted with 10 ml pre-warmed serum-free RPMI. Next, the cells were centrifuged (5 min, 50xg, room temperature) and resuspended in DMEM (α-modification) supplemented with 15% FCS, 40 U HGF/ml as present in HECS (Aarden et al., 1985), hypoxanthine (1.10^{-4} M), aminopterin (4.10^{-7} M), thymidine (1.6 x 10^{-5} M), 2-ME (5.10^{-5} M) and antibiotics. Cells were aliquotted in 24-well tissue culture plates (Costar). Selection of hybrids occurred for 3 weeks in the described HAT-containing medium, followed by culture for 1-2 weeks in HT-containing medium. After that period hybrid cell lines were cultured in α-DMEM or in DMEM, as described for RMB-TG and WEHI-TG.

Karyotype analysis. Metaphase preparations of the parental cell lines and the hybrids R0C2, R1B1, R1C1, R2C4, W1C3, W1C4 and W6A2 were kindly prepared by Dr. An Hagemeijer and co-workers from our department using R-banding with Acridine Orange after heat denaturation (Hagemeijer et al., 1979).

Induction of macrophage differentiation. For maximal induction of macrophage differentiation, we used post-endotoxin serum (PES) as differentiation inducing agent (Fibach and Sachs, 1974) supplemented with recombinant IFN-γ (Perussia et al., 1983). For the production of PES, C57Bl/6J mice were injected i.v. with 400 μg Salmonella typhosa LPS-W (Difco Laboratories, Detroit, MI, USA) in PBS and blood was collected 6 hr later by cardiac puncture of CO_2-anaesthetized animals. Supernatant from transfected CHO-cells was used as a source of recombinant IFN-γ (Dijkmans et al., 1985). This was kindly provided by Dr. Jan Trapman, Dept. Pathology, Erasmus University, Rotterdam. Other differentiation inducing agents tested, were Con A-stimulated spleen cell conditioned medium (20%), dexamethasone (5.10^{-5} M), E.coli-LPS (0.1 μg/ml), DMSO (1-2%), and LCM (20%).

Hybrid and parental cell lines were set into culture for 5 days at initial density of $1\text{-}3.10^5$ cells/ml in DMEM supplemented with 10% SERUM-PLUS™, antibiotics and 1% PES + 0.5 U, 1 U or 10 U rIFN-γ/ml as differentiation inducing agents. Control cultures were performed similarly, without PES + IFN. Adherent cells were isolated from plastic flasks either by repeated pipetting or by incubating the cultures with 0.02% Na-EDTA in PBS for 10 min at room temperature. With respect to the characteristics studied here, no differences were observed between the populations isolated by either method.

Phagocytosis. The phagocytic capacities of parental cell lines and hybrids before and after induction of differentiation were determined as described previously (Leenen et

al., 1986b).

Non-specific esterase and acid phosphatase activity. Non-specific esterase activity was determined according to Li et al. (1973) using naphthylacetate as substrate. Acid phosphatase activity was demonstrated according to Loyda et al. (1967).

ELISA. The expression of surface antigens by hybrids and parental cell lines was quantitatively assessed by means of a sensitive fluorescence micro-ELISA, using target cells coated to Terasaki trays with 0.05% glutaraldehyde (Leenen et al., 1985). As shown previously (Leenen et al., 1985), this low glutaraldehyde concentration does not influence the detectability of the investigated antigens. Briefly, wells were incubated for 1 hr with monoclonal antibodies, rinsed thoroughly with PBS-Tw (0.05% v/v), incubated for 1 hr with optimally diluted anti-(rat Ig)-ß-galactosidase conjugate, rinsed again and finally incubated for 1 hr at 37°C with the fluorogenic substrate 4-methylumbelliferyl galactopyranoside. The amount of generated fluorescent product was then determined with a scanning microfluorometer.

In order to enable comparison of antigen expression by different cell lines as well as comparison of data obtained in different experiments, the obtained fluorescence values were standardized as follows. Day-to-day variables in the ELISA-procedure are:

1. Cell density on the test plates.
2. Specific activity of the enzyme conjugate used as second stage reagent. This variability is caused by decreasing enzyme and antibody activity with time as well as by variations in daily made dilutions.
3. Amplification of the fluorescence signal by the scanning equipment.

In order to exclude variations in cell density, Terasaki trays were prepared by coating equal amounts of cells, measured as protein content, to the wells. The variation in specific enzyme activity and amplification of the fluorescence signal was corrected for by expressing the obtained experimental fluorescence values as percentages of an internal positive control. The latter value was determined in the same assay by measuring the fluorescent product generated on a separate Terasaki tray which was saturated with purified rat Ig and subsequently incubated with the anti-(rat Ig)-conjugate and fluorogenic substrate. Thus, antigen expression is given in arbitrary units (AU), implying that for 100 AU a fluorescence signal was obtained that equalled the internal positive control. Antigen expression, determined in this way, appeared to be constant on different Terasaki trays of a single cell line.

RESULTS

In the first part of this section we describe the generation and karyotype analysis of the macrophage hybrids presently under study. In the second part, the immunophenotype of these hybrids is analyzed in order to obtain information about the differentiation stage at which the hybrids are arrested. The differentiation potential of a selected panel of hybrids is presented in the last part of this study.

I. Generation and karyotype of macrophage precursor hybrids

Mononuclear phagocytes, obtained by culturing bone marrow for 7 days in the presence of M-CSF (BMDM-7), were hybridized with HGPRT-negative variants of the myeloid cell lines RMB-1 and WEHI-3B (RMB-TG and WEHI-TG, respectively). Culture of the hybridized cells in HAT-medium resulted in the appearance of clonal growth after 1 - 2 weeks. The fusion of BMDM-7 with RMB-TG resulted in 16 stable hybrids from 60 wells plated, whereas 6 stable hybrids were obtained from 60 wells plated with the fusion mixture of BMDM-7 with WEHI-TG. The hybrids resulting from fusion with RMB-TG (hereafter called R-hybrids) appeared to exhibit non-adherent growth and a typical immature myeloid morphology (fig. 5.2a). Similarly, the hybrids gained after fusion with WEHI-TG (W-hybrids) showed a myeloid morphology, although most of the W-hybrids appeared to grow slightly adherent.

The results of the karyotype analysis of 7 representative hybrids and the parental cell lines are given in table 5.II. Initial analysis of 3 hybrids, 3 months after fusion, showed a doubled chromosome number compared to the parental cell lines. After 26 months, only the R1B1 hybrid showed significant loss of chromosomes, whereas the other investigated hybrids appeared to be stable with respect to their chromosome content. Remarkably, hybrid W6A2 cells contained 93 chromosomes on an average, which is more than the double set.

II. Immunophenotype of macrophage precursor hybrids

The immunophenotype of the hybrids was determined in a semi-quantitative micro-ELISA using the panel of monoclonal antibodies listed in table 5.I. Their phenotype was compared with the phenotype of the parental cell lines RMB-TG and WEHI-TG, and of bone marrow derived mononuclear phagocytes obtained after 7 days of culture (BMDM-7). The latter population was similar to the one used for fusion. Furthermore, a comparison was made with non-adherent cells, obtained after 4 days of bone marrow culture (BMDM-4). This population consisted of mononuclear cells (≈ 55%) and poly-

Table 5.II. Karyotype analysis of macrophage precursor hybrids and parental cell lines

cell line	number of chromosomes	
	3 months after fusion	26 months after fusion
RMB-TG	41 ± 1	41 ± 1
R-hybrids		
R0C2	nd	80 ± 3
R1B1	80 ± 1	75 ± 3
R1C1	81 ± 3	82 ± 4
R2C4	nd	81 ± 1
WEHI-TG	nd	41 ± 1
W-hybrids		
W1C3	80 ± 1	81 ± 3
W1C4	nd	78 ± 3
W6A2	nd	93 ± 2

Mean number of chromosomes ± SD was calculated from 3-10 metaphases of each cell line

morphonuclear cells (≈ 45%).

The expression of class I and class II MHC antigens as well as the expression of mature and immature macrophage markers is given in figure 5.1. The expression of other lympho-hemopoietic markers is given in table 5.III.

MHC antigens

Class I MHC antigens were present on most R-hybrids as well as on 2 (of 6) W-hybrids. It should be noted, however, that in later experiments (see table 5.IV) class I antigen expression was found on W-hybrids that were previously scored negative.

Class II MHC antigens were expressed neither by R- nor by W-hybrids. A low level of class II antigen expression was observed on BMDM-4, whereas BMDM-7 showed the highest level of class II expression.

Mature macrophage antigens

As indicators for macrophage maturity we investigated the expression of Mac-1, Mac-2 and F4/80 Ag (fig. 5.1). Comparison of the expression of these antigens by the immature BMDM-4 and the more mature BMDM-7 cells clearly showed an increase in expression of these markers during the course of macrophage differentiation. The

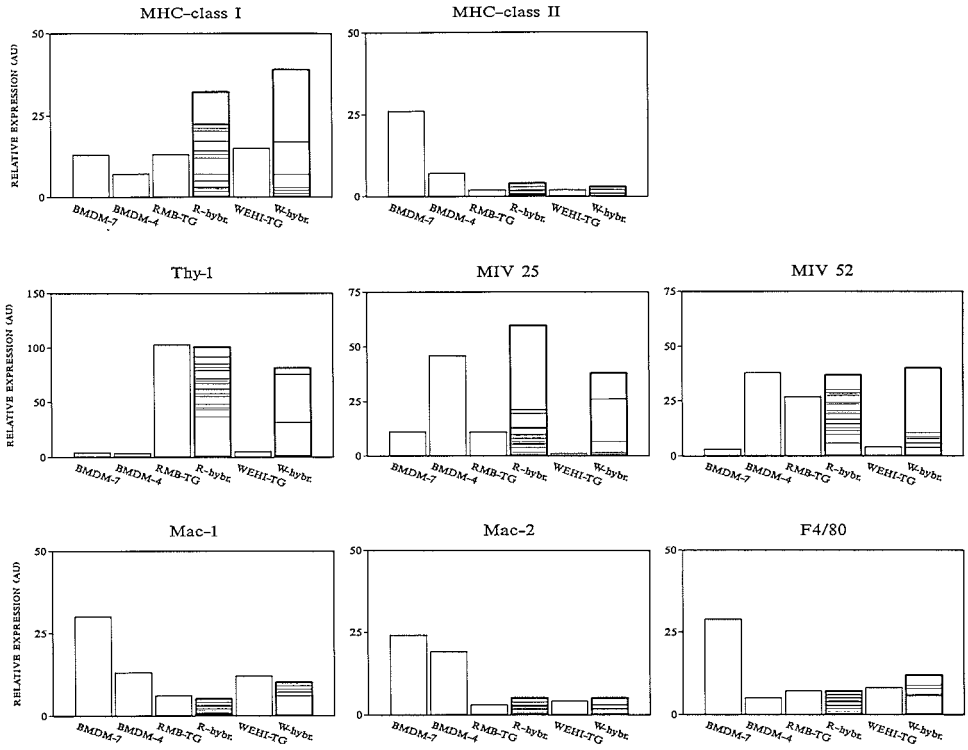

Figure 5.1.

Immunophenotype of BMDM-7, BMDM-4, R- and W-hybrids and their parental cell lines as determined with ELISA. Each dash in the complex bars represents the mean antigen expression for a single hybrid, determined in at least three assays. The level of antigen expression by the investigated populations is given relative to the internal positive control in arbitrary units (AU) as described in Materials and Methods.

BMDM-7 population always showed the highest expression of Mac-1, Mac-2 and F4/80 Ag. None of the mature markers was expressed beyond background by any of the R-hybrids. The W-hybrids, on the other hand, showed a low, but detectable, level of Mac-1 and F4/80 Ag expression, whereas Mac-2 was clearly absent.

Immature macrophage antigens

As examples of immature macrophage antigens, we examined the expression of Thy-1, MIV 25 Ag and MIV 52 Ag on hybrid cells (fig. 5.1). The latter two markers were clearly expressed by immature BMDM, present in 4-days' bone marrow cultures, but hardly or not by the more mature BMDM obtained from 7 days' bone marrow cultures.

Thy-1 was expressed at high levels by all R-hybrids as well as by the RMB-TG parental line. The W-hybrids showed qualitative heterogeneity with respect to the

expression of Thy-1. Three W-hybrids showed Thy-1 expression at a level comparable to the R-hybrids, whereas the other 3 W-hybrids showed no expression of Thy-1, just as the parental WEHI-TG cells.

MIV 25 Ag was differentially expressed by the R-hybrids. Most R-hybrids showed a low, but significant level of expression just as RMB-TG, whereas some R-hybrids expressed MIV 25 Ag at a high level comparable to BMDM-4.
The W-hybrids appeared to express MIV 25 Ag with similar heterogeneity as Thy-1, some W-hybrids exposing MIV 25 Ag in fairly high amounts and others being negative for this marker, just as WEHI-TG.

MIV 52 Ag was expressed by virtually all R-hybrids though at varying levels. All W-hybrids but one expressed MIV 52 Ag at only low levels.

Taken together, it can be concluded that both R- and W-hybrids lack a high expression of mature macrophage markers. On the other hand, immature macrophage markers are expressed by most hybrids, though at varying levels.

Other lympho-hemopoietic antigens

Table 5.III shows the immunophenotype of hybrids, parental cell lines and BMDM-populations with regard to other markers, informative for the cell types under study. These additional phenotyping experiments were undertaken to exclude the possibility that the hybrids represent cells of other hemopoietic lineages, for instance lymphocytes. Antigens expressed by all R- and W-hybrids as well as by the reference populations are HSA (recognized by B2A2), Pgp-1, MIV 43 Ag, T-200 and the transferrin receptor. These are general markers found on most hemopoietic cell types. On some hybrids a generally low expression of Gr-1, Lyt-1, B-220, ER-TR6 Ag and Mac-3 was found. Variability was observed in different assays with regard to the expression of LFA-1 and MEL-14 Ag. Most likely, this was due to minor variations in culture conditions. Mature markers such as the Fc-receptor type II, MBR-1 and -2, and MECA-20 Ag were not found on the hybrids. These were, on the other hand, readily expressed by most cells of the BMDM-7 population used for fusion. Lyt-2, MT4 and the heat-stable antigen recognized by M1/75 were neither expressed by any of the hybrids, nor by any of the control populations.

Thus, it can be concluded that both R- and W-hybrids express general hemopoietic markers, but virtually no markers characteristic for non-macrophage lineages.

III. Induction of macrophage differentiation

In the previous part we showed that the hybrid cells are arrested at a precursor stage. The question is now whether these cells indeed can differentiate into a more

Table 5.III. **Immunophenotypical analysis of macrophage precursor hybrids, parental cell lines and bone marrow derived macrophages**

antibody	antigen	BMDM-7	BMDM-4	RMB-TG	R-hybrids	WEHI-TG	W-hybrids
B2A2	HSA	nd	nd	+	+	+	+
IM7.8.1	Pgp-1	+	+	+	+	+	+
MIV 43	MIV 43 Ag	+	+	+	+	+	+
30-G12	T-200	+	+	+	+	+	+
H129.121	transferrin-R	+	±	+	+	+	+
RB6 8C5	Gr-1	±	+	±	± H	-	-
53-7.3.13	Lyt-1	-	-	±	± H	-	± H
RA3 6B2	B-220	-	+	±	H	±	H
ER-TR6	ER-TR6 Ag	-	-	+	± H	-	± H
M3/84	Mac-3	+	±	+	+ H	±	H
H129.37	LFA-1-α	±	V	V	V	V	V
MEL-14	MEL-14 Ag	V	-	-	V	-	V
2.4G2	FcR-II	±	-	-	-	-	-
MIV 38	MBR-2	+	-	-	-	-	-
MIV 55	MBR-1	+	-	-	-	-	-
MECA-20	MECA-20 Ag	+	-	-	-	-	-
53-6.72	Lyt-2	-	-	-	-	-	-
H129.19	MT4	-	-	-	-	-	-
M1/75	HSA	-	-	-	-	-	-

- = relative expression < 5 arbitrary units (AU); ± = relative expression 5 - 15 AU;
+ = relative expression > 15 AU; H = heterogeneity among different hybrids;
V = interexperimental variations observed

mature phenotype. Therefore, we attempted to induce terminal differentiation of a panel of hybrids. This panel was selected on the basis of a divergent expression of macrophage precursor markers (see table 5.IV).

Induction of macrophage differentiation was most evident when post-endotoxin serum (PES) was used in combination with IFN-γ (see below). Application of DMSO, LCM, LPS or dexamethasone, well-known differentiation inducing agents for myeloid tumor cell lines, caused no morphological or phenotypical changes, whereas application of Con A-stimulated spleen cell conditioned medium induced similar, though less pronounced effects as PES (data not shown).

As markers for macrophage differentiation we investigated the morphology, im-

Table 5.IV. **Class I and immature macrophage antigen expression by macrophage precursor hybrids and parental cell lines selected for induction of differentiation**

cell line	relative expression (AU)*			
	class I Ag	Thy-1	MIV 25 Ag	MIV 52 Ag
RMB-TG	21	74	32	33
R0C2	27	72	24	36
R1B1	25	55	20	11
R1C1	24	48	3	59
R2C4	21	64	14	25
WEHI-TG	17	5	2	13
W1C3	14	61	17	26
W1C4	11	4	2	14
W6A2	26	59	4	12

* data were determined by ELISA and represent the mean of 3 individual experiments

munophenotype, phagocytic capacity, and non-specific esterase and acid phosphatase activity of induced and control hybrid cells.

Morphology

Figure 5.2 shows the morphology of R0C2 hybrid cells before (fig. 5.2a) and after

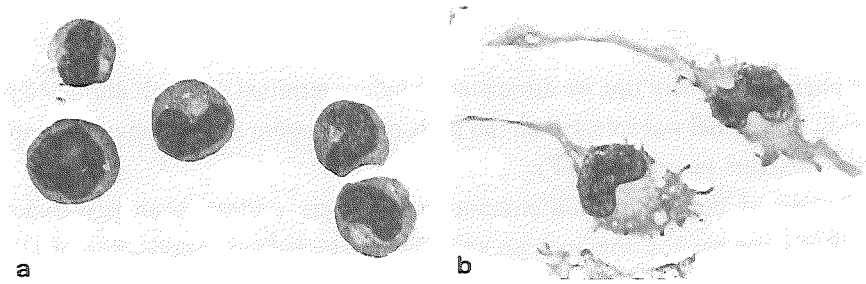

Figure 5.2.
Morphology of R0C2 hybrid cells before (a) and after (b) induction of differentiation using 1% PES and 1 U/ml IFN-γ. May-Grünwald Giemsa staining was performed on a cytocentrifuge preparation (a) or on adherent cells cultured on a cover slip (b). Original magnification x 640.

induction of differentiation (fig. 5.2b). Non-induced cells grew in suspension and had a typical myeloid appearance with a fairly basophilic cytoplasm. Induced cells, on the other hand, showed a more mature macrophage morphology with firm adherence to the culture substrate and pronounced pseudopodia. This morphologic differentiation was most striking for R0C2 cells. In general, virtually all hybrids showed an increase in the number of adherent cells in culture upon induction of differentiation.

Immunophenotype

The phenotypical changes, observed upon induction of differentiation, are shown in figure 5.3. In three independent experiments the immunophenotypes of control cells and cells induced for 5 days with 1% PES supplemented with 0.5, 1 and 10 U/ml IFN-γ, respectively, were determined. The data are compared to simultaneously obtained values of non-induced control cells.

The induction of mature macrophage markers as well as the decrease of expression of immature macrophage markers was most obvious for the R0C2 hybrid cell line. Especially Mac-1 and Mac-2 expression was highly induced, whereas the expression of Thy-1 and MIV 52 Ag decreased strongly. The expression of the immature marker MIV 25 Ag decreased or increased, depending on the concentration of IFN-γ applied together with PES. MIV 25 Ag expression was also induced by IFN-γ only (data not shown). Similarly, induction of class I Ag depended on the concentration of IFN-γ. Class II Ag was not induced on R0C2 cells. In contrast, both class I and class II antigens were highly induced on R1C1 cells. R1B1 and R2C4 cells, on the other hand, were neither inducible for class I nor for class II antigen expression by PES + IFN-γ. The slight induction of Mac-1 and F4/80 Ag expression was a more common characteristic, observed for most hybrids.

Similar differential changes, though less pronounced, were observed for the W-hybrids. On W1C3 cells, for instance, MIV 25 Ag was induced concomitantly with the induction of mature macrophage markers. W1C4 cells, on the other hand, showed a much higher induction of class I Ag, while no changes in MIV 25 Ag expression were observed.

It should be noted that the phenotypical changes described here were determined on relatively large numbers of cells coated to wells of Terasaki trays. Investigation of antigen expression at the single cell level, using the immuno-ß-galactosidase assay (Leenen et al., 1987), learned that marker changes occurred on the vast majority of the cells in the populations (data not shown).

Phagocytosis

The phagocytic capacities of induced and control cell lines are shown in figure 5.4.

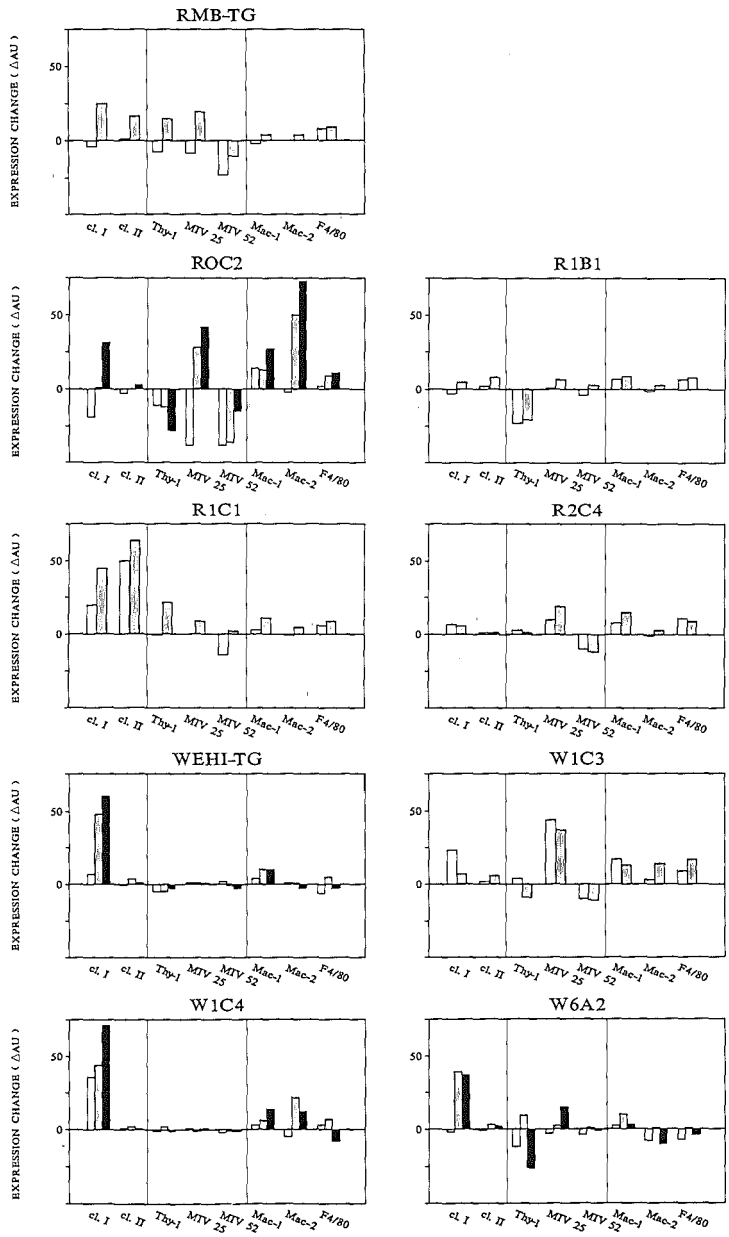

Figure 5.3.

Immunophenotypical changes upon induction of differentiation. In three independent experiments differentiation was induced using 1% PES + 0.5 U (open bars), 1 U (hatched bars) or 10 U IFN-γ/ml (black bars). Change in expression is given compared to the phenotype of control cells in arbitrary units (AU) as described before. Immunophenotypes of induced and control cells were determined simultaneously. Some hybrids, which showed significant cell death in the experiment using 10 U IFN-γ/ml, were not further evaluated in that particular experiment.

Mature macrophage (RAW309Cr.1) and pre-T (RLD-1) cells were included as positive and negative controls, respectively.

Non-induced R-hybrids showed a low level of phagocytosis, whereas control W-hybrids - in general - phagocytosed to a slightly higher extent. Upon induction of differentiation, the ability to ingest latex beads increased only marginally for the R-hybrids. In contrast, the phagocytic capacity of induced W1C4 and W6A2 cells increased significantly to a level comparable with the mature RAW309Cr.1 cells.

Non-specific esterase and acid phosphatase activity

Finally, we investigated whether - as a sign of differentiation - non-specific esterase and acid phosphatase activity was induced in the differentiated hybrid cells. In tables 5.V and 5.VI the results of these experiments are given. Control hybrids and parental cell lines expressed these mature macrophage enzymes to a limited extent (termed 'dull' in tables 5.V and 5.VI). Only the control W6A2 hybrid showed a significant percentage of cells with a high esterase and phosphatase activity ('bright'). After induction, however, R0C2, R2C4, W1C3 and W6A2 hybrids demonstrated a considerable increase of esterase and phosphatase levels. R1B1, R1C1 and W1C4 hybrids, on the other hand, did not show an increase of enzyme expression.

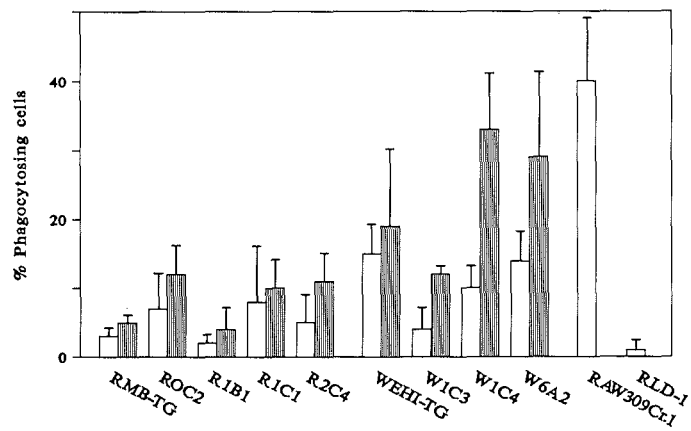

Figure 5.4.

Phagocytic capacity of control (open bars) and differentiated (hatched bars) macrophage precursor hybrids. For induction of differentiation, cells were cultured for 5 days with 1% PES + 1 U/ml IFN-γ. RAW309Cr.1 mature macrophages and RLD-1 pre-T cells served as positive and negative controls, resp. Data represent the mean of three separate experiments ± SD. At least 300 cells were observed for each determination.

Table 5.V. **Non-specific esterase activity of control and differentiated macrophage precursor hybrids and parental cell lines**

cell line		% positive cells exp. 1 dull	exp. 1 bright	exp. 2 dull	exp. 2 bright
RMB-TG	co	nd	nd	98	2
	PI	nd	nd	98	2
R0C2	co	100	0	98	2
	PI	49	51	52	48
R1B1	co	nd	nd	99	1
	PI	nd	nd	99	1
R1C1	co	100	0	100	0
	PI	97	3	95	5
R2C4	co	100	0	100	0
	PI	83	17	70	30
WEHI-TG	co	100	0	nd	nd
	PI	100	0	nd	nd
W1C3	co	100	0	99	1
	PI	62	38	63	37
W1C4	co	100	0	100	0
	PI	100	0	99	1
W6A2	co	74	26	86	14
	PI	48	52	72	28

Table 5.VI. **Acid phosphatase activity of control and differentiated macrophage precursor hybrids and parental cell lines**

cell line		% positive cells exp. 1 dull	exp. 1 bright	exp. 2 dull	exp. 2 bright
RMB-TG	co	nd	nd	99	1
	PI	nd	nd	98	2
R0C2	co	98	2	98	2
	PI	90	10	65	35
R1B1	co	nd	nd	98	2
	PI	nd	nd	95	5
R1C1	co	99	1	99	1
	PI	98	2	96	4
R2C4	co	100	0	98	2
	PI	75	25	87	13
WEHI-TG	co	100	0	nd	nd
	PI	100	0	nd	nd
W1C3	co	100	0	98	2
	PI	96	4	85	15
W1C4	co	100	0	100	0
	PI	99	1	100	0
W6A2	co	89	11	80	20
	PI	70	30	60	40

co = control cells; PI = induced for 5 days with 1% PES + 1U/ml IFN-γ; nd = not determined

DISCUSSION

In this paper we demonstrate the possibility to rescue the differentiation stage of macrophage precursors by somatic cell hybridization of bone marrow derived mononuclear phagocytes with myeloid tumor cell lines. This approach enables the investigation of the complexity of the macrophage precursor pool, as the present hybrids represent clonal populations of macrophage precursor cells, which occur in the bone marrow only at low frequency.

We define the present panel of hybrids as precursor cell lines since they show:

- the presence of immature macrophage cell surface markers;
- a low level of expression, or absence of mature macrophage cell surface markers;
- weak expression of non-specific esterase and acid phosphatase activity;
- a low level of phagocytic capacity;
- myeloid morphology and primarily non-adherent growth.

The macrophage precursor hybrids appear to be arrested around the CFU-GM stage of differentiation, since they resemble their parental cell lines with respect to the expression of markers indicative of macrophage differentiation stage. In this context, we have previously shown that RMB cells most likely correspond to the CFU-GM stage (Leenen et al.,1986b; Delwel et al., 1987).

Our data suggest that W-hybrids, in general, represent slightly more mature stages of differentiation compared to R-hybrids, since:

- all W-hybrids express low, but detectable levels of the mature markers Mac-1 and F4/80;
- several W-hybrids lack the expression of one or more of the investigated precursor markers;
- most W-hybrids show a higher level of phagocytosis of latex beads;
- some W-hybrids are slightly adherent.

The characteristics of both R- and W-hybrid cell lines differ markedly from the "mature" BMDM-7 population that was used for fusion. A more close phenotypic correlation was observed with the "immature" BMDM-4 population. The observed difference with the BMDM-7 cells is most likely explained by a selective immortalization of macrophage precursors, present in the BMDM-7 population, since somatic cell hybrids generally express the differentiation-associated features of both fusion partners (Erikson et al., 1981; van Dongen et al., 1986; Breel et al., 1988a). However, one cannot exclude the possibility that the tumor cell phenotype 'dominates' the hybrid phenotype (Davis and Adelberg, 1973).

We selected a panel of 7 hybrids for the investigation of their differentiation potential using post-endotoxin serum together with interferon-γ as inducing agent. Taken

together, these hybrids show many morphological, phenotypical and functional changes upon induction of differentiation (summarized in table 5.VII). Marker changes - when they occur - comprise an increase in the expression of mature macrophage characteristics and a decrease of immature macrophage characteristics. Noteworthy, the myeloid fusion partners, RMB-TG and WEHI-TG, do hardly respond to the inducing agents used.

Not all hybrids obtain, upon induction of differentiation, the common macrophage feature of phagocytosis (fig. 5.4, table 5.VII). A possible explanation for this apparent discrepancy is the relative immaturity of the hybrid cells, even after induction of differentiation. In this context, it should be realized that phagocytic activity is acquired by mononuclear phagocytes at the monoblastic stage (van der Meer et al., 1983). Monoblasts, however, already resemble mature BMDM with regard to the expression of the mature macrophage markers studied here (Nibbering et al., 1987). It is evident from the changes in marker expression (see fig. 5.3), that the differentiated hybrid cells do not yet have a fully mature macrophage phenotype and thus have not yet reached a differentiation stage comparable to the monoblast.

Why do the various macrophage precursor hybrids respond in different ways to identical differentiation inducing agents? (see also table 5.VII). R1C1 cells, for instance, were induced to express Ia antigens at high levels, whereas R0C2 cells changed drastically with regard to morphology and phenotype without concomitant induction of Ia antigen expression. R0C2 cells were also induced to express acid phosphatase and non-specific esterase activities, however without accompanying induction of phagocytosis. In contrast, W1C4 cells were induced to phagocytose latex beads without significant increase in their phosphatase and esterase content. It may be argued that these divergent patterns of marker changes are a consequence of the artificial immortalization of the macrophage precursor cells. We have, however, at least two reasons to assume that this is not the case. As shown in table 5.II, the chromosome content of all investigated hybrids but one is stable for more than 2 years of culture. Therefore, variations in chromosomal composition is no valid explanation of the observed heterogeneous differentiation pathways. In addition, extensive analysis of mature macrophage hybrids by other investigators led to the conclusion that these cell lines are consistent representatives of normal macrophage populations, notwithstanding their artificial origin (Uchida et al., 1985; Tzehoval et al., 1984; Zuckerman et al., 1986; Chou et al., 1987). Although non-physiological effects, obviously, cannot be ruled out, we feel that the observed phenotypical changes in the macrophage precursor hybrids most likely reflect regular events occurring upon differentiation of normal macrophage precursors. Hence, the divergent differentiation pathways suggest that the immortalized macrophage precursors constitute a heterogeneous population with respect to their differentiation potential. Different differentiation programs appear to determine the sequence and

Table 5.VII. Summary of induced differentiation characteristics in macrophage precursor hybrids

cell line	increase of adherence and stretching	increase of class I antigen expression	increase of class II antigen expression	decrease of immature macrophage antigen expression	increase of mature macrophage antigen expression	increase of phagocytosis	increase of NSE expression	increase of AP expression
R0C2	++	+	-	++	++	-	++	+
R1B1	+	-	-	+	±	-	-	-
R1C1	-	++	++	-	±	-	-	-
R2C4	+	-	-	-	+	-	+	+
W1C3	+	+	-	-	+	-	+	±
W1C4	-	++	-	-	+	+	-	-
W6A2	-	++	-	+	-	+	+	+
RMB-TG	-	+	+	-	-	-	-	-
WEHI-TG	-	++	-	-	±	-	-	-

changes are represented arbitrarily as ± (weak), + (moderate), and ++ (strong).
NSE = non-specific esterase; AP = acid phosphatase

level of expression of mature macrophage characteristics. Therefore, the present data argue in favour of the existence of distinct macrophage precursor subsets, which give rise to different mature macrophage subsets. In this context, we speculate that R1C1 cells - which highly express Ia antigens upon induction of differentiation - represent the precursors of the mature macrophage subset with a high level of Ia expression, as observed by Walker (1987). Previously, also other investigators suggested the existence of distinct macrophage precursor subsets by using either clonal phenotypical analysis of M-CSF stimulated cultures (Bursuker and Goldman, 1983; Walker, 1987), or functional comparison of macrophage precursors from spleen and bone marrow (Baccarini et al., 1986).

Can the different macrophage precursor subsets, as represented in the macrophage precursor hybrid panel, be phenotypically distinguished at the level of the precursor itself? The macrophage precursor hybrids, altogether, show only slight mutual differences with respect to the phenotypical and functional characteristics studied here. Significant heterogeneity is only observed in the expression of some immature macrophage antigens (table 5.IV) as well as some other lympho-hemopoietic antigens (table 5.III). The differential expression of these antigens, however, shows no obvious correlation with the observed pathways of differentiation. For instance, the presence or absence of MIV 25 Ag from a particular hybrid does not correlate to the ability or disability to express, upon induction of differentiation, any of the mature macrophage features listed in table 5.VII. Hence, the markers used in the present study do not decisively discriminate distinct macrophage precursor subsets. However, the level of expression of immature macrophage antigens appears to be a characteristic feature of different macrophage precursor subsets. This is suggested by the similarity between R2C4 and W1C3 cells. Both hybrids display similar differentiation programs (table 5.VII), which suggests that R2C4 and W1C3 represent macrophage precursors belonging to the same subset. This relationship is reflected in the striking phenotypical resemblance between R2C4 and W1C3 (table 5.IV).

In summary, our report describes the generation of hybrids with characteristics of macrophage precursors as determined from morphological, phenotypical and functional analysis. These macrophage precursor hybrids show only minor mutual differences. Upon induction of differentiation, however, populations arise with divergent features of mature macrophages. Therefore, these results suggest that representatives of distinct macrophage precursor subsets have been immortalized. For a further phenotypical characterization of the early stages of macrophage differentiation, we prepared a panel of monoclonal antibodies, using the present macrophage precursor hybrids as immunogens. In the accompanying paper, we show that the selected antibodies can be successfully used for the isolation of macrophage precursor cells from murine bone marrow.

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CHAPTER 6

MURINE MACROPHAGE PRECURSOR CHARACTERIZATION. II. MONOCLONAL ANTIBODIES AGAINST MACROPHAGE PRECURSOR ANTIGENS

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SUMMARY

The aim of the present study was the phenotypical analysis of early stages in macrophage differentiation. To this purpose, we produced a panel of syngeneic rat hybridomas, which secreted antibodies (mAbs) against macrophage precursor antigens. As immunogens we used immortalized macrophage precursors (see preceding paper). We screened the obtained mAbs in the following in vitro models of macrophage differentiation:

(i) a panel of macrophage cell lines ordered in a linear differentiation sequence;
(ii) immature and mature mononuclear phagocytes obtained from bone marrow culture;
(iii) a panel of macrophage precursor hybrids, and
(iv) differentiated and control macrophage precursor hybrid cells.

Four mAbs, ER-MP12, ER-MP20, ER-MP54 and ER-MP58, were selected. These mAbs recognize antigens which disappear in the course of macrophage differentiation.

Next, we investigated whether these mAbs also recognized macrophage precursors in normal bone marrow. To that end, ER-MP positive and -negative bone marrow fractions were isolated using a fluorescence activated cell sorter. Fractions were cultured in M-CSF containing conditioned medium, and the resulting mature macrophage progeny was quantified using the MTT-assay. The present experiments indicate that ER-MP12 and ER-MP20 detect a subpopulation of bone marrow macrophage precursors, whereas ER-MP58 stains virtually all macrophage precursors.

Biochemical analysis of radio-iodinated antigens revealed that these mAbs recognize different molecules. ER-MP12 and ER-MP20 bound to single-chain (glyco)proteins of 140 kDa and 14 kDa, respectively. ER-MP54 precipitated multiple polypeptides, of which the major chains have an apparent molecular weight of 90, 80-85 and 70-75 kDa.

Based on the molecular weight of the recognized antigens as well as on the mAb specificities, we conclude that ER-MP12, ER-MP54 and ER-MP58 recognize hitherto unknown antigens of murine macrophage precursor cells. The antigen detected by ER-MP20 is most likely identical to Ly-6C.

INTRODUCTION

Macrophages play a key role in the host defense against infections (recently reviewed by Gordon, 1986; Unanue and Allen, 1987; Johnston, 1988). During the differentiation of macrophages from hemopoietic stem cells, many phenotypical and functional changes occur (reviewed by Volkman, 1984; van Furth, 1985; Schook and Tew, 1988). The earliest events in macrophage differentiation are, however, largely

unknown, since the phenotypical characteristics of bone marrow macrophage precursors are only poorly defined. Presently available monoclonal antibodies (mAbs), are mainly directed against antigens expressed by mature macrophages (Springer, 1980; Austyn and Gordon, 1981; Malorny et al., 1986; Kraal et al., 1987).

The aim of the present study was the phenotypical analysis of early macrophage differentiation stages. Therefore, we produced a panel of monoclonal antibodies against murine macrophage precursor antigens. We selected the mAbs for recognition of antigens which disappear upon macrophage differentiation. This criterion is based on the assumption that functionally important antigens, which play decisive roles in the early steps of macrophage differentiation, will be exclusively present at precursor stages. Once - at more mature stages - the precursor-specific function of such an antigen is no longer needed, the expression of the respective antigen will decrease.

Since macrophage precursors occur in the bone marrow only at low frequency, we used immortalized macrophage precursors (Leenen et al., 1989d) as immunogens. For screening of the reactivity of the obtained mAbs, we made use of several in vitro models of macrophage differentiation:

- a panel of macrophage cell lines, aligned in a linear differentiation sequence (Leenen et al., 1986b)
- a population of immature bone marrow-derived mononuclear phagocytes (BMDM), isolated after 4 days of culture, compared to a population of relatively mature BMDM, isolated after 7 days of culture (Walker et al., 1985)
- a panel of macrophage precursor hybrids, representing various macrophage precursor subsets as well as macrophage precursors in different stages of differentiation (Leenen et al., 1989d)
- induction of differentiation in the R0C2 macrophage precursor hybrid cell line (Leenen et al., 1989d).

On the basis of the reactivity in these macrophage differentiation models we identified 4 mAbs - ER-MP12, -20, -54 and - 58 -, which specifically bind to antigens on macrophage precursor stages. Next we applied these mAbs to investigate the phenotype of macrophage precursors from bone marrow.

MATERIALS AND METHODS

Animals. Male and female BALB/c mice were purchased from Olac (Bicester, Oxon, UK) and sacrificed at 15 - 20 weeks of age. Female Lewis rats, used for immunization, were obtained from Radiobiological Institute TNO, Rijswijk, The Netherlands. Male and female (Louvain x Lewis) F1 rats, used for ascites production, were raised in the

laboratory animals centre of our faculty. Animals were kept under routine laboratory conditions with free access to food and water.

Antisera and conjugates. In this study, we used culture supernatants from the hybridomas M1/70, M3/38 and F4/80, which contained mAbs directed against the mature macrophage antigens Mac-1, Mac-2 and F4/80 Ag, respectively. As sources of ER-MP mAbs, described below, we used either hybridoma culture supernatants or optimally diluted, partially purified antibody preparations. The latter were obtained by precipitation of immunoglobulins from ascitic fluid (1:1 diluted in PBS) using 18% Na_2SO_4 (final concentration). Next, the precipitates were dissolved in PBS and desalted using Sephadex G-25M columns (Pharmacia, Uppsala, Sweden).

As second stage conjugate in ELISA experiments, we applied affinity purified sheep-anti-(rat Ig) coupled to E.coli ß-galactosidase (Radiochemical Centre, Amersham, UK). In flowcytometric experiments, we used FITC-coupled $F(ab)_2$ fragments of rabbit-anti-(rat IgG) (Cappel, Malvern, PA).

Macrophage cell lines. Macrophage cell lines, used in the present study, were cultured in DMEM (Flow Laboratories, Irvine, Scotland) with 10% FCS or SERUM-PLUS™ (KC Biological, Lenexa, KS) and antibiotics, unless otherwise stated. Macrophage precursor hybrids R0C2, R0C4, R0D6, R1B1, R1B4, R1C1, R1C3, R1D6, R2A5, R2A6, R2C1, R2C4, R2D4, R2D5, R6A1, W1A3, W1C3, W1C4, W1D1, W1D3 and W6A2 as well as their parental cell lines RMB-TG and WEHI-TG were targets for hybridoma screening (Leenen et al., 1989d). The R0C2 macrophage precursor hybrid was induced to differentiate along the monocytic/macrophage pathway by culturing the cells for 5 days in the presence of 1% post-endotoxin serum + 0.5, 1 or 10 U/ml rIFN-γ, as described previously (Leenen et al., 1989d).

A panel of macrophage cell lines, ordered in a differentiation sequence, was used as a model of macrophage differentiation (Leenen et al., 1986b). These cell lines can be divided into three groups, based on the differentiation stage at which the cells are arrested:

- macrophage precursor cell lines: M1 (Ichikawa, 1969), RMB-1 and RMB-3 (de Both et al.,1981; Leenen et al., 1986b)
- immature macrophage cell lines: WEHI-3B and WEHI-3 (Warner et al., 1969)
- mature macrophage cell lines: Pu5-1.8 (Ralph et al., 1974), J774-1.6 (Ralph et al., 1975), P388D1 (Koren et al., 1975), RAW264.7, RAW309Cr.1 and WR19M.1 (Raschke et al., 1978).

M-CSF stimulated bone marrow culture. Bone marrow-derived mononuclear

phagocytes (BMDM) were cultured according to the methods described before (Leenen et al., 1989d). As a source of immature BMDM, non-adherent cells were isolated after 4 days of culture in plastic flasks (Costar, Cambridge, MA) (Walker et al., 1985). This population (designated BMDM-4) consisted of about 55% mononuclear cells and 45% mature and immature polymorphonuclear cells. Lymphoid and erythroid contamination of this population was less than 5%. BMDM with mature macrophage phenotype were isolated from Teflon culture bags after 7 days of culture (BMDM-7; van der Meer et al., 1979b).

Immunization and generation of rat x rat hybridomas. We used macrophage precursor hybrid cells, W1C3 and pooled R1B1 + R1C1, as immunogens. A priming immunization was given i.p. with 3.10^7 cells, emulsified in complete Freund's adjuvant (Difco, Detroit, MI). After 5 weeks, the rats were boosted i.p. with 2.10^7 cells, suspended in incomplete Freund's adjuvant. Four days after the booster immunization, the rats were sacrificed, the spleens were taken out aseptically and cell suspensions were made in RPMI-1640 supplemented with 10% FCS.
The HAT-sensitive rat myeloma cell line Y3-Ag1.2.3 (abbr. Y3), used as fusion partner for B-cell hybridoma production (Galfre et al., 1979) was kindly donated by Dr. G. Galfre, MRC, Cambridge, UK. For fusion, Y3 cells were mixed with immune spleen cells at a ratio of 1:10. This mixture was washed three times with serum-free RPMI-1640. Cell fusion was induced with PEG-4000 as described previously (Leenen et al., 1989d). Next, the cells were spun down (5 min, 50 x g, room temperature), gently resuspended in DMEM (α-modification) supplemented with 10% FCS, 40 U HGF/ml as present in HECS (Aarden et al., 1985), hypoxanthine (10^{-4}M), azaserine (1 μg/ml), 2-mercaptoethanol (5.10^{-5}M) and antibiotics, and plated in 24-well culture plates (Costar) at a density of 3.10^5 cells per well. In our hands, the use of both α-modified DMEM and azaserine, instead of aminopterin, were essential in order to obtain satisfying numbers of syngeneic rat hybridomas. After initial screening, selected hybridomas were subcloned by limiting dilution. The subclasses of immunoglobulins, secreted by the hybridomas, were determined by standard Ouchterlony immunodiffusion using anti-(rat Ig subclass) antisera from Nordic (Tilburg, The Netherlands) and Miles (Kankakee, IL). The monoclonal antibodies, presented here, have been coded ER-MP (Erasmus University Rotterdam - macrophage precursor).

ELISA. The expression of antigens, recognized by the ER-MP monoclonal antibodies, was semi-quantitatively determined using a sensitive micro-ELISA, extensively described earlier (Leenen et al., 1989d; Leenen et al., 1985). Resulting experimental values were expressed in arbitrary units (AU) relative to an internal positive control in order to

enable inter-experimental comparison as well as comparison between different cell types (Leenen et al., 1989d).

Immunofluorescence labeling, flowcytometric analysis and cell sorting. Fluorescence labeling was performed on an aseptically prepared bone marrow cell suspension, that had been washed at least twice with serum-free DMEM. All further labeling steps were performed at 4°C. 1-2.10^7 cells were incubated with monoclonal antibodies in 0.5 ml for 1 hr. Next, the cells were washed at least twice with DMEM supplemented with 0.02% gelatin (w/v; DMEM/gel) and incubated for 1 hr in 0.5 ml optimally diluted FITC-coupled anti-(rat Ig), supplemented with 2% normal mouse serum to avoid non-specific antibody binding. The cells were washed again twice and resuspended in DMEM/gel. Cells were analyzed and sorted using a FACS II (Becton and Dickinson, Sunnyvale, CA). The tubing of the FACS was extensively flushed with 70% ethanol prior to sterile cell sorting. The resulting fluorescence signals were amplified logarithmically. Percentages of positive cells were calculated according to Huiskamp and Van Ewijk (1985). In two experiments, 4,000 and 10,000 cells from gated populations were sorted, respectively, into 96-well culture plates (Costar), previously filled with 100 μl BMDM-culture medium per well.

Macrophage precursor quantification. We determined the presence of macrophage precursors in the sorted bone marrow fractions by quantifying the progeny of these cells after 7 days of culture in BMDM-culture medium. This quantification was performed using the colorimetric MTT assay, as originally described by Mosmann (1983). This method has recently been adapted for quantification of macrophage precursor progeny (Kriegler et al., 1987). Briefly, 10 μl MTT-solution (5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) in PBS) was added to each well. Wells, filled with BMDM-culture medium, in which no bone marrow cells had been seeded, served as background controls. The plates were incubated for 4 hr at 37°C under 7% CO_2 in order to allow the conversion of the tetrazolium salt into the dark blue formazan product. This reaction has been shown to be linearly related to cell number as far as similar cells are compared (Mosmann, 1983). Finally, 100 μl acidic iso-propanol (0.04 M HCl in isopropanol) was added to each well. The formazan product was dissolved by thorough mixing. The plates were monitored on a Titertek Multiskan MCC (Flow Laboratories) 96-well ELISA plate reader at a wavelength of 570 nm. The obtained background values were subtracted from the experimental data.

Antigen characterization. The molecular weight of the antigens, recognized by the ER-MP mAbs, was determined using immunoprecipitation, essentially as described by

Pont et al. (1985) and Borst et al. (1987). Briefly, 10^7 cells were labeled with 1 mCi $Na^{125}I$ using the glucose oxidase - lactoperoxidase iodination method (Hubbard and Cohn, 1975). Cells were lysed at 4°C in 1% Triton X-100 in 10 mM Tris.HCl, pH 8.0, 0.15 M NaCl, supplemented with 0.5 % BSA, 10 mM EDTA, 1 mM phenylmethyl-sulfonyl fluoride, 5 mM iodo-acetamide and trypsin inhibitor (20μg/ml). To remove non-specifically binding proteins, the lysates were precleared by centrifugation and incubated with normal rat-Ig coupled to mouse-anti-rat-kappa (MARK-1)-Sepharose-4B beads (Pont et al., 1985; Bazin et al., 1984). The ER-MP Ags were immunoprecipitated using rat-mAb linked to MARK-1-Sepharose beads and separated by SDS-PAGE. After drying, the gels were exposed to Fuji RX-NIF film.

RESULTS

In this study we aimed at preparing monoclonal antibodies against macrophage precursor antigens. Antisera from three fusions were screened in our in vitro models of macrophage differentiation and selected on the basis of preferential binding to macrophage precursor stages. Overall, four distinct reactivity patterns were observed that matched the criterion mentioned above. From each type a single monoclonal antibody, represented by ER-MP12 (IgG2a), ER-MP20 (IgG2a), ER-MP54 (IgG1) and ER-MP58 (IgM), respectively, is presented here.

In the first part of this section we show binding patterns of the selected monoclonal antibodies in the various in vitro models of macrophage differentiation. Next, the ability of these antisera to recognize macrophage precursors in bone marrow cell suspensions is examined. Finally, the respective precursor antigens are characterized by immunoprecipitation and biochemical analysis.

I. ER-MP antibody binding in macrophage differentiation models

Macrophage cell lines in differentiation sequence

Figure 6.1 shows the binding patterns of the ER-MP mAbs to the macrophage cell line panel, aligned in a linear differentiation sequence. All ER-MP mAbs showed preferential binding to the macrophage precursor and/or immature macrophage cell lines. ER-MP12 binding was restricted to the macrophage precursor cell lines (M1, RMB-1 and -3). ER-MP20 bound to 2 of 3 macrophage precursor cell lines (RMB-1 and -3) and 1 of 2 immature macrophage cell lines (WEHI-3B), whereas ER-MP54 showed significant binding only to 2 of 3 macrophage precursor cell lines (RMB-1 and -3). ER-MP58 exhibited an exceptional binding pattern in this selection. This mAb also

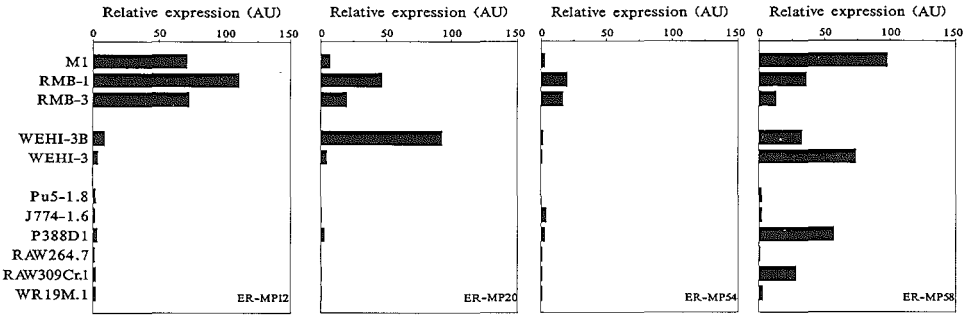

Figure 6.1.

ER-MP antibody binding to macrophage cell lines ordered in a linear differentiation sequence as determined with ELISA. Three groups of macrophage cell lines can be discerned: macrophage precursor cell lines (M1, RMB-1, and -3); immature macrophage cell lines (WEHI-3B and WEHI-3); mature macrophage cell lines (others). Antibody binding is expressed in arbitrary units relative to an internal positive control (see Materials and Methods, and (Leenen et al., 1989d).

recognized some cell lines with mature macrophage characteristics (P388D1 and RAW309Cr.1), in addition to the binding to all examined macrophage precursor and immature macrophage cell lines. The reason for including ER-MP58 in the panel of anti-macrophage precursor mAbs will appear from its reactivity in other macrophage differentiation models (see below).

In summary, ER-MP12, -20, and -54 specifically bound to macrophage precursor- and/or immature macrophage cell lines and lacked recognition of mature macrophage cell-lines. ER-MP58 bound to all tested macrophage precursor- and immature macrophage cell lines as well as to 2 of 6 mature macrophage cell lines.

M-CSF-stimulated bone marrow culture

The reactivity of ER-MP mAbs with immature (BMDM-4) and mature (BMDM-7) bone marrow-derived mononuclear phagocytes is shown in figure 6.2. All ER-MP mAbs bound to immature BMDM to a larger extent, compared to mature BMDM. The antigen recognized by ER-MP20 (= ER-MP20 Ag) is most highly expressed by BMDM-4 and to a much lesser extent by the mature BMDM-7. ER-MP12, -54 and -58 Ags were, in contrast, only present at low levels on BMDM-4 and absent from BMDM-7.

To distinguish reactivity to mononuclear and polymorphonuclear cells in the BMDM-4 population, single cell immuno-ß-galactosidase staining was performed according to methods described previously (Leenen et al., 1987). This analysis learned that ER-MP12, -20, and -54 stained both cell types with similar intensity, whereas ER-MP58 preferen-

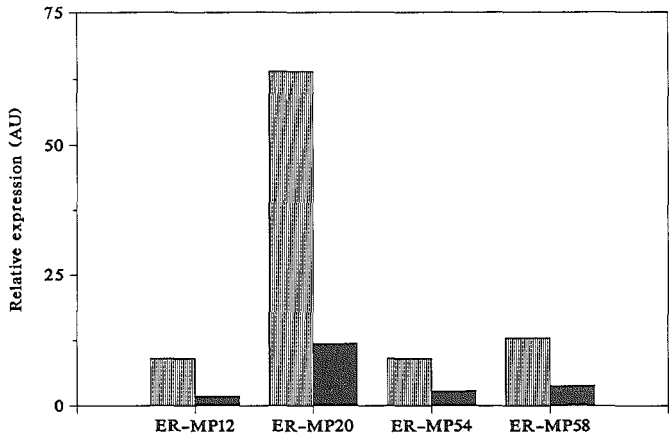

Figure 6.2.
ER-MP antibody binding to immature BMDM-4 (grey bars) and mature BMDM-7 (black bars) as determined with ELISA.

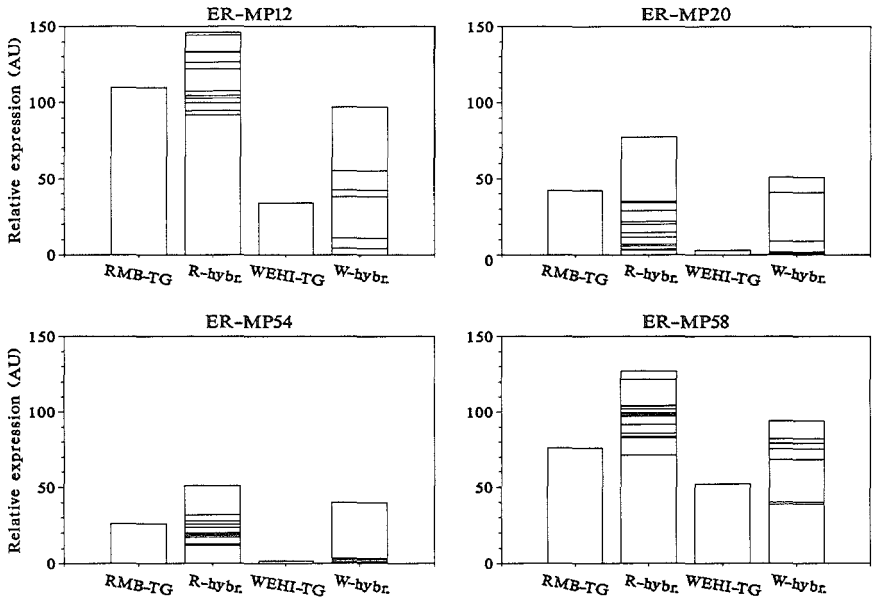

Figure 6.3.
ER-MP antibody binding to the panel of macrophage precursor hybrids and their parental cell lines as determined with ELISA. Each dash in the complex bars represents the mean antibody binding for a single hybrid.

tially bound to mononuclear cells (data not shown).

Macrophage precursor hybrids and parental cell lines

We then tested our mAbs for binding to a panel of macrophage precursor hybrids. This panel represents different macrophage precursor subsets as well as different maturation stages (Leenen et al., 1989d); W-hybrids represent generally more mature stages of differentiation compared to the R-hybrids.

Figure 6.3 shows the reactivity of ER-MP mAbs with the panel of macrophage precursor hybrids and their parental cell lines. ER-MP12 bound at high density to all R-hybrids, whereas only 4 of 6 W-hybrids were clearly positive. ER-MP20 Ag was differentially expressed by both R- and W-hybrids. The majority of R-hybrids was positive for this marker, whereas only 2 of 6 W-hybrids expressed ER-MP20 Ag. A similar distribution was observed for ER-MP54 Ag. All R-hybrids were ER-MP54 positive, though at varying levels of expression. However, only one of the W-hybrids showed ER-MP54 Ag expression. ER-MP58 Ag was expressed by all R-hybrids as well as by all W-hybrids.

Summarizing, ER-MP12, and -58 Ags were expressed by all R- and W-hybrids, though at lower levels by the latter. ER-MP20 Ag was differentially expressed by both R- and W-hybrids. ER-MP54 Ag was expressed by all R-hybrids, but only by 1 of 6 W-hybrids.

Induction of macrophage differentiation in macrophage precursor hybrid R0C2

In the preceding paper we showed that macrophage precursor hybrids can be induced to express mature macrophage characteristics by culturing the cells in the presence of murine post-endotoxin serum (PES) supplemented with IFN-γ. To investigate the changes in expression of the antigens detected by our mAbs in the course of macrophage differentiation, we cultured the macrophage precursor hybrid R0C2 for 5 days in the presence of PES supplemented with recombinant IFN-γ. To monitor macrophage differentiation, the induction of expression of the mature macrophage antigens Mac-1, Mac-2 and F4/80 was analyzed (fig. 6.4). These experiments revealed that, upon differentiation, R0C2 cells show a strong reduction in the expression of ER-MP12, -54, and -58 Ags. Surprisingly, ER-MP20 Ag was expressed to a larger extent upon differentiation.

In conclusion, we have shown in this section that ER-MP12, -20, -54, and -58 mAbs specifically bind to macrophage precursors in several in vitro models of macrophage differentiation. The expression of the antigens, recognized by these mAbs, generally decreases with differentiation of the cells.

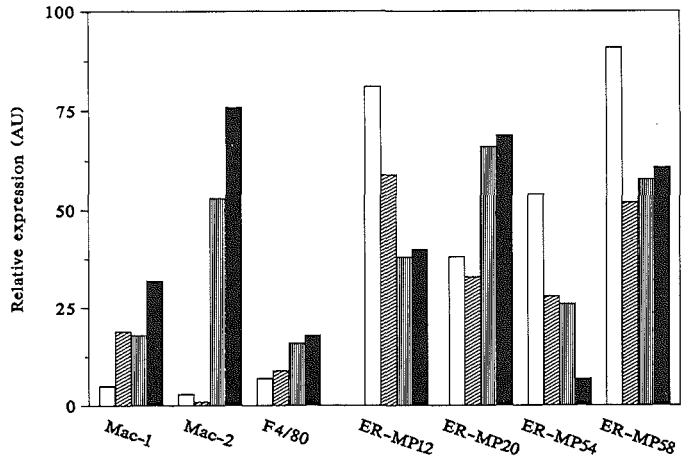

Figure 6.4.

Immunophenotypical changes upon induction of differentiation of the macrophage precursor hybrid R0C2 as determined with ELISA. In three individual experiments, we cultured cells for 5 days with or without 1% PES supplemented with 0.5 (///), 1 (| | |) or 10 U IFN-γ/ml (black bars). Values for control R0C2 cells (open bars) represent the mean of the three experiments.

II. ER-MP antibody binding to bone marrow macrophage precursors

The next set of experiments was designed to answer the question whether the present ER-MP mAbs - which were raised and selected by using immortalized macrophage precursors - also recognize normal bone marrow macrophage precursors. To this purpose, we prepared a bone marrow cell suspension and labeled the cells with ER-MP mAbs and anti-(rat Ig)-FITC. Next, the cells were analyzed by flowcytometry and equal numbers of positive and negative cells were sorted for culture in M-CSF containing medium.

Table 6.I shows the percentages of bone marrow cells labeled with ER-MP mAbs. ER-MP12 Ag is expressed by only a minority (6-9%) of bone marrow cells. ER-MP20 Ag is expressed at different levels by two discrete populations: 31-43% of bone marrow cells showed an intermediate level of ER-MP20 Ag expression (termed 'dull'), whereas 4-6% of the cells highly expressed ER-MP20 Ag ('bright'). In both experiments, ER-MP54 Ag was not detected on bone marrow cells. In contrast, ER-MP58 Ag was expressed by a majority of bone marrow cells (63-65%).

Equal numbers of positive, negative and unseparated cells were sorted and cultured for 7 days in the presence of M-CSF. Progeny of these cells was quantified using the MTT colorimetric assay. The results of these experiments are shown in figure 6.5.

Table 6.I. **Percentages of bone marrow cells stained by ER-MP mAbs and sorted out for culture in M-CSF containing medium**

mAb		net % positive cells exp. 1	exp. 2
ER-MP12		6	9
ER-MP20	bright	6	4
	dull	43	31
ER-MP54		0	0
ER-MP58		65	63

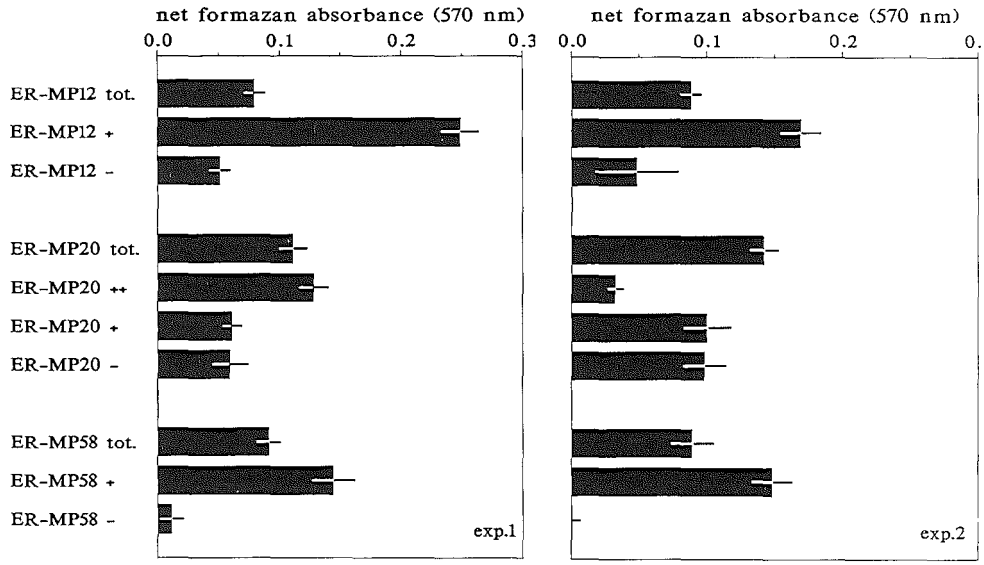

Figure 6.5.

Quantification of mature macrophage progeny, obtained after M-CSF stimulated culture of sorted bone marrow fractions, using the colorimetric MTT assay. Equal cell numbers were sorted in at least 8 wells of a 96 well plate, cultured for 7 days and then incubated for 4 hr with MTT. The resulting formazan product concentration is a measure of the number of cells. The bar represents the net mean formazan absorbance ± SEM.

The ER-MP12$^+$ bone marrow fraction showed an enrichment of macrophage precursors of 2-3 times, compared to unseparated bone marrow. This ER-MP12$^+$ fraction contained only 6-9% of total bone marrow. Relating these data, this implicates that nearly 20% of the bone marrow macrophage precursors express the ER-MP12 Ag, assuming that ER-MP12$^+$ and ER-MP12$^-$ precursors give rise to comparable numbers of mature macrophages.

A similar heterogeneity among bone marrow macrophage precursors was found with respect to the expression of ER-MP20 Ag. Macrophage progeny was found in cultures of both ER-MP20^{bright} and ER-MP20^{dull}, as well as in cultures of ER-MP20$^-$ fractions. In both experiments, however, the sum of progeny of the separated ER-MP20 fractions was less than the progeny of unseparated bone marrow. This may indicate that proliferating macrophage precursors were separated from auxiliary cells, necessary for optimal macrophage precursor proliferation.

Finally, separation of bone marrow in ER-MP58$^+$ and ER-MP58$^-$ fractions showed unambiguously that all macrophage precursors express the ER-MP58 Ag. The enrichment of macrophage precursors in the ER-MP58$^+$ fraction is, however, limited, since this fraction contains 63-65 % of all bone marrow cells.

In conclusion, these experiments show that ER-MP12 and -20 Ags are expressed by a subpopulation of bone marrow macrophage precursors, whereas ER-MP58 Ag is expressed by all bone marrow macrophage precursors.

III. ER-MP antigen characterization

The molecular weight of the ER-MP12, -20, -54, and -58 Ags was assessed by cell surface radio-iodination, followed by immunoprecipitation and SDS-PAGE.

Figure 6.6 shows that ER-MP12 precipitates an antigen of 140 kDa under reducing conditions. The same band is observed under non-reducing conditions, which indicates that a single chain (glyco)protein is recognized by ER-MP12 (data not shown).

The antigen bound by ER-MP20 appears to be a small molecule with apparent molecular weight of 14 kDa under reducing as well as non-reducing conditions.

ER-MP54 precipitates multiple polypeptide chains. Major bands appearing under reducing as well as non-reducing SDS-PAGE show a molecular weight of 90, 80-85 and 70-75 kDa. A 45 kDa protein is precipitated by ER-MP54 in lower amounts from most macrophage precursor cell lines, whereas 27 kDa and 12 kDa bands were observed less frequently.

Finally, ER-MP58 failed to precipitate any ^{125}I-labeled molecules in our hands (data not shown).

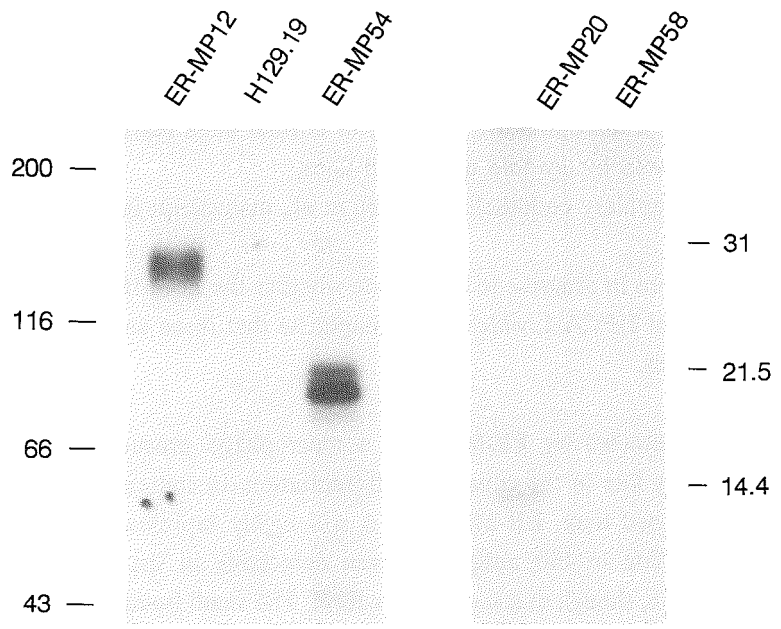

Figure 6.6.

Biochemical analysis of the antigens recognized by ER-MP mAbs. ER-MP12 and ER-MP54 Ags were immunoprecipitated from W1C3 cell lysate and run on a 5-15 % polyacrylamide gel under reducing conditions. ER-MP20 Ag was precipitated from R1B1 cell lysate and run on a 15 % polyacrylamide gel, also under reducing conditions. mAb H129.19 (Pierres et al., 1984), recognizing murine CD4, was used to detect non-specifically binding proteins.

DISCUSSION

In the present paper, we report on the reactivity of 4 rat anti-mouse monoclonal antibodies (mAbs), ER-MP12, -20, -54, and -58, which recognize biochemically distinct molecules on macrophage precursors.

The antigen recognized by **ER-MP12** is expressed by all macrophage precursors in the various models of differentiation used for mAb characterization. This antigen is absent from mature stages and its expression decreases upon induction of differentiation in the macrophage precursor hybrid R0C2. In normal bone marrow only around 20% of the macrophage precursors appears to be ER-MP12$^+$. Nevertheless, the ER-MP12$^+$ bone marrow fraction shows a 2-3 fold enrichment of macrophage precursors compared to unseparated bone marrow. In search of a human homologue, we feel that the mouse ER-MP12 antigen relates to the human progenitor cell antigen HPCA-1 (or My10 Ag,

CD34), since both antigens are:

- single-chain (glyco)proteins of similar size (140 kDa for ER-MP12 vs.115-120 kDa for HPCA-1 (Civin et al., 1984);
- expressed by a small minority (<10%) of normal bone marrow cells;
- highly expressed by myeloid tumor cell lines;
- present on capillary endothelia (Leenen et al., manuscript in preparation; Watt et al., 1987).

Additional studies are in progress to investigate whether the ER-MP12 Ag is the actual murine equivalent of HPCA-1, which would implicate that these antigens are products of homologous genes.

The antigen detected by **ER-MP20** is a macrophage precursor antigen which disappears relatively late in the course of differentiation. In contrast to the other ER-MP antigens described here, ER-MP20 Ag is still highly expressed by the BMDM-4 population. Expression of this antigen is even detectable in the relatively 'mature' BMDM-7 population, though at a much lower level. The small fraction of bone marrow cells (4-6%) with high ER-MP20 Ag expression (ER-MP20^{bright}) consists almost exclusively of monocytes (Leenen et al., manuscript in preparation). This finding further confirms that the ER-MP20 Ag disappears late during macrophage precursor differentiation, since monocytes represent the last stage of macrophage differentiation in the bone marrow compartment (van Furth and Cohn, 1968).

Induction of macrophage differentiation in the macrophage precursor hybrid R0C2 caused an increased expression of the ER-MP20 Ag, instead of the expected decrease. The most likely explanation for this phenomenon is that the ER-MP20 Ag is maximally expressed at the monocytic stage of differentiation (see above), whereas R0C2 cells appear to be arrested around the CFU-GM stage (Leenen et al., 1989d). Induction of differentiation from this stage would thus result in enhanced ER-MP20 expression, until the monocytic stage is reached.

Culture of bone marrow fractions, sorted according to ER-MP20 Ag expression, learned that macrophage precursors are heterogeneous with respect to the expression of this antigen. Both the ER-MP20^{dull} and ER-MP20^{bright}, as well as the ER-MP20^{-} fractions contained cells that proliferated in response to M-CSF. An interesting observation in this context was, that the sum of macrophage progeny derived from bone marrow, fractionated according to ER-MP20 Ag expression, was always less than the progeny derived from unseparated bone marrow. This indicates that auxiliary cells, necessary for optimal macrophage precursor proliferation were separated from the actual macrophage precursors in at least one of the fractions. A likely candidate for the auxiliary cell is the ER-MP20^{bright} monocyte, which is known to produce both IL-1 and

IL-6 (Oppenheim et al., 1986; Aarden et al., 1987). It has been shown recently that these factors in particular support the proliferation of macrophage precursors (Bartelmez and Stanley, 1985; Wong et al., 1988).

Most likely, the Ag recognized by ER-MP20 is identical to Ly-6C, since both Ags are:

- single-chain (glyco)proteins of approximately 14 kDa (Houlden et al., 1986)
- expressed at intermediate levels by bone marrow granulocytes and at high levels by bone marrow monocytes (Leenen et al., manuscript in preparation; Jutila et al., 1988)
- present at small vessel endothelium (Leenen et al., manuscript in preparation; Jutila et al., 1988)
- inducible with IFN-γ (data not shown; Jutila et al., 1988).

Furthermore, ER-MP20 mAb appeared to modulate proliferation of cloned T helper cells, similar to anti-Ly-6C mAbs (Malek et al., 1986). M-CSF stimulated proliferation of macrophage precursors, however, was not significantly altered by ER-MP20. Experiments are in progress to confirm definitely that ER-MP20 recognizes Ly-6C. If this appears to be the case, then our finding that bone marrow macrophage precursors are heterogeneous with respect to ER-MP20 Ag expression is of particular interest, because of the involvement of Ly-6 Ags in cellular activation (Malek et al., 1986).

The **ER-MP54** Ag is expressed by most macrophage precursor stages in the various models of differentiation, though in general at a lower level, compared to the other ER-MP antigens. Induction of differentiation in the R0C2 macrophage precursor hybrid resulted in a strong decrease of ER-MP54 Ag expression.

Using flowcytometry, we could not demonstrate significant ER-MP54 mAb binding to viable bone marrow cells. However, immunoperoxidase staining with ER-MP54 on frozen sections of bone marrow plugs did show positivity (Leenen et al., manuscript in preparation). Furthermore, acetone-fixation of bone marrow cells in suspension, providing access of the mAb to the cytoplasm, does reveal specific ER-MP54 staining as determined by flowcytometry. These observations suggest that, in bone marrow cells, the ER-MP54 Ag is exclusively present in the cytoplasm. Cultured cells, however, express ER-MP54 Ag unambiguously at the cell surface, since it can be detected by ELISA, as well as by surface radio-iodination and subsequent immunoprecipitation. The observation that ER-MP54 precipitates several proteins indicates that either ER-MP54 recognizes a common determinant in multiple related proteins, or, alternatively, that the single protein bound by ER-MP54 is integrated into a multimeric complex.

The **ER-MP58** Ag is expressed, generally at high levels, by all macrophage

precursors in the various differentiation models. Surprisingly, also 2 of 6 mature macrophage cell lines in the cell line panel were found to be ER-MP58$^+$. This is most likely an aberrant phenotype of the particular cell lines, since the expression of ER-MP58 Ag is exclusively restricted to macrophage precursor stages in the other three models.

Virtually all macrophage precursors in the bone marrow express the ER-MP58 Ag, since no progeny was obtained from the M-CSF stimulated ER-MP58$^-$ bone marrow fraction. ER-MP58 Ag is most likely a general marker for immature hemopoietic cells, since it is expressed by the majority of bone marrow cells (63-65%) as well as by a small percentage of spleen cells (< 10%), but is not detected outside hemopoietic compartments (Leenen et al., manuscript in preparation).

Based on the present results, the following conclusions can be reached with respect to the phenotype of M-CSF responsive bone marrow macrophage precursors. Virtually all macrophage precursors express the antigen recognized by ER-MP58. In contrast, ER-MP12 Ag is only expressed by a subpopulation of bone marrow macrophage precursors. With regard to the expression of ER-MP20 Ag, three subpopulations of bone marrow macrophage precursors can be distinguished (ER-MP20$^-$, ER-MP20^{dull}, and ER-MP20^{bright}, resp.). Now the question arises whether this phenotypical heterogeneity reflects the presence of distinct macrophage precursor subsets, or, alternatively, is a consequence of differential expression with maturation of cells along a linear pathway. Unfortunately, on the basis of the present data, we cannot discriminate between either possibility. To confirm whether ER-MP12 and/or ER-MP20 detect macrophage precursor subsets or macrophage precursor maturation stages, we are currently investigating the mature progeny of the various macrophage precursor subpopulations. If distinct macrophage precursor subsets are discerned by either ER-MP12 or ER-MP20, then the mature progeny from these subsets will show functional and/or phenotypical disparity.

As a summary, a schematic representation of ER-MP antigen expression in the course of murine macrophage differentiation is given in Fig. 6.7. Clearly, ER-MP12, -54 and -58 detect macrophage precursor antigens, which disappear at, or shortly after the CFU-M stage of differentiation. ER-MP20 Ag, on the other hand, becomes increasingly expressed from the CFU-M stage on and disappears after the monocytic stage.

We compared the present ER-MP mAbs with a large panel of known anti-leukocyte mAbs (partially presented in table 5.I) with respect to molecular weight and tissue

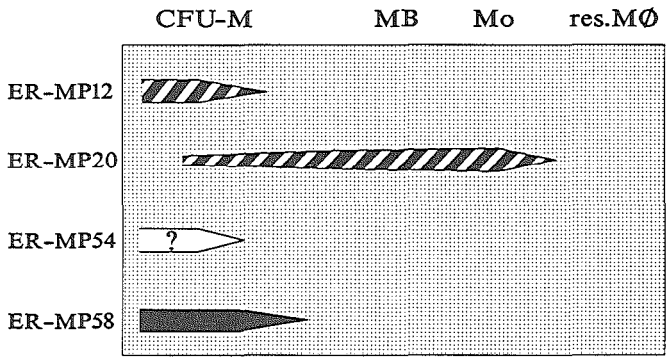

Figure 6.7.

Schematic representation of ER-MP Ag expression during murine macrophage differentiation. The hatched bars for ER-MP12 and -20 indicate that possibly separate lineages of macrophage differentiation are recognized, whereas the closed bar for ER-MP58 indicates that all bone marrow macrophage precursors bind this mAb. It could not be determined whether all macrophage precursors or only a subpopulation express ER-MP54 Ag. For the construction of this diagram, the results obtained from the in vitro models of differentiation as well as from the sorting of bone marrow macrophage precursors have been taken into account. CFU-M = colony forming unit - macrophage; MB = monoblast; Mo = monocyte; res.Mϕ = resident macrophage.

distribution of the recognized Ags. On the basis of these studies, we conclude that ER-MP12, -54 and -58 recognize hitherto unknown antigens on murine macrophage precursor cells. As outlined above, ER-MP20 most likely binds to the Ly-6C antigen.

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CHAPTER 7

DIFFERENTIAL INHIBITION OF MACROPHAGE PROLIFERATION BY ANTI - TRANSFERRIN RECEPTOR ANTIBODY ER-MP21. CORRELATION TO MACROPHAGE DIFFERENTIATION STAGE

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SUMMARY

Monoclonal antibodies (mAbs) directed against the transferrin receptor are known to inhibit proliferation of cells due to iron deprivation. Some cell types, however, escape from growth inhibition by a mechanism which is unclear at present. This mechanism is the subject of the present study. We investigated the differential growth inhibition caused by anti-transferrin receptor mAb ER-MP21 in connection to the differentiation of murine macrophages (macrophage). Therefore, we applied two models of macrophage differentiation, namely culture of bone marrow cells in the presence of M-CSF and a panel of macrophage cell lines ordered in a linear differentiation sequence. In both models we observed that proliferation of macrophage precursors was strongly inhibited by ER-MP21. In contrast, proliferation of more mature stages of macrophage differentiation was hardly affected. Remarkably, iron uptake by macrophage precursor and mature macrophage cell lines was inhibited by ER-MP21 to the same extent. However, mature macrophage cell lines showed a 2 to 3-fold higher iron uptake compared to macrophage precursor cell lines. These observations strongly suggest that mature macrophages escape from ER-MP21 mediated growth inhibition, because these cells take up more iron than is actually needed for proliferation. Furthermore, we found that enhanced iron uptake by mature macrophages is not necessarily accompanied by a higher cell surface expression of transferrin receptors, thus suggesting an increased recycling of transferrin receptors in mature macrophages.

INTRODUCTION

In previous studies we reported on the phenotypical differentiation of murine macrophages, using mAbs directed against cell surface molecules (Leenen et al., 1986a; 1986b; 1989b). In search of functionally important epitopes, involved in the regulation of macrophage differentiation, we produced new mAbs against macrophage precursor antigens (Leenen et al., 1989b) and screened these mAbs for interference with macrophage colony development in M-CSF stimulated bone marrow cultures. One of these mAbs, termed ER-MP21, appeared to inhibit the in vitro development of macrophages by hindering macrophage precursor proliferation. Moreover, the proliferation of macrophage precursor cell lines was strongly inhibited by ER-MP21. In contrast, mature macrophage cell line proliferation was hardly affected. Pilot studies using ^{59}Fe-loaded transferrin indicated that mAb ER-MP21 inhibited the iron uptake by macrophages and is therefore most likely directed against the murine transferrin receptor.

Using other anti-transferrin receptor mAbs, a similar differential inhibitory effect on the proliferation of different cell lines has been observed by other investigators as well (Trowbridge et al., 1982b; Lesley and Schulte, 1985). Additionally, different protocols for induction of normal T- and B-lymphocyte proliferation show different degrees of sensitivity towards the inhibitory action of an anti-transferrin receptor mAb (Kemp et al., 1987). The mechanism of differential growth inhibition by a particular anti-transferrin receptor mAb remains, however, unclear at present (Trowbridge and Shackelford, 1986). In general, inhibitory anti-transferrin receptor mAbs cause iron deprivation and thus withhold cells from a vital element in their metabolism (Trowbridge et al., 1982b; Lesley and Schulte, 1985; Taetle et al., 1986). However, it remains to be established whether insensitive cell types are deprived from iron to a lower extent upon culture with a particular anti-transferrin receptor mAb, or, alternatively, employ additional mechanisms for iron uptake other than the transferrin receptor.

Our initial finding of differential inhibition of macrophage proliferation by ER-MP21 prompted us to a detailed analysis of the functional properties of this anti-transferrin receptor mAb. In the present study we substantiate the differential inhibition of macrophage proliferation by ER-MP21 and show that growth inhibition is inversely correlated to macrophage maturation. Furthermore, we investigate the mechanism of differential sensitivity for growth inhibition by ER-MP21, using a panel of macrophage cell lines arrested in different, previously defined stages of macrophage differentiation (Leenen et al., 1986b). The results show that ER-MP21 equally inhibits iron uptake by macrophage precursor and mature macrophage cell lines. However, mature macrophage cell lines exhibit a significantly higher level of iron uptake compared to macrophage precursor cell lines. These data suggest that mature macrophages escape from ER-MP21 mediated growth inhibition because these cells take up iron in greater amounts than merely needed for proliferation.

MATERIALS AND METHODS

Mice. Male and female BALB/c mice were purchased from Olac (Bicester, Oxon, UK). Animals were kept under clean, routine laboratory conditions with free access to food and water.

Cells and culture conditions. The macrophage precursor cell lines R1B1 and R2C4 were hybrids obtained by fusion of RMB-TG myeloid cells and macrophage derived from M-CSF stimulated bone marrow culture (Leenen et al., 1989d). WEHI-3 cells were previously characterized as immature macrophages, whereas RAW309Cr.1 and

WR19M.1 cells exhibited mature macrophage features (Warner et al., 1969; Raschke et al., 1978; Leenen et al., 1986b). As non-macrophage control cells, we used SP2/0 myeloma cells and RLD-1 pre-T cells (Shulman et al., 1978; de Both et al., 1983). All cell lines were cultured in DMEM (Flow Laboratories, Irvine, Scotland), supplemented with 10% SERUM-PLUS™ (KC Biological, Lenexa, KS) and antibiotics.

M-CSF stimulated bone marrow cultures were performed in 24-wells culture plates (Costar, Cambridge, MA) with L-cell conditioned medium as source of M-CSF, essentially as described by van der Meer et al. (1983).

Quantification of cell proliferation. Cell lines were set into culture in 96-wells culture plates in low density, ensuring exponential growth after 3 days. Then, after 3 days of culture in the presence or absence of mAbs, resulting cell numbers were quantified using the colorimetric MTT assay as described by Mosmann (1983). For M-CSF stimulated bone marrow cultures, resulting progeny was quantified similarly after 7 days.

Antisera and conjugates. The IgG2a anti-transferrin receptor mAb ER-MP21, as well as the control IgG2a mAbs ER-MP20, ER-MP39 and ER-MP42, were produced by hybridomas, obtained after fusion of Y3 myeloma cells with spleen cells from a Lewis rat that had been immunized with macrophage precursor hybrid cells (Leenen et al., 1989b). Hybridoma cells, secreting the anti-transferrin receptor mAb H129.121 (Van Agthoven et al., 1984) were kindly provided by Dr. Michel Pierres (INSERM-CNRS, Marseille, France). For application in culture, mAbs were purified from ascitic fluid (1:1 diluted in PBS) by 18% Na_2SO_4 precipitation (w/v; final concentration), desalted using Sephadex G-25M columns (Pharmacia, Uppsala, Sweden), and filter-sterilized. In flowcytometric experiments, we used FITC-coupled $F(ab)_2$ fragments of rabbit anti-(rat-IgG) (Cappel, Malvern, PA) to detect rat mAb binding to cells.

MAb and transferrin purification and radiolabeling. ER-MP21 mAb purification for binding affinity studies was achieved by specific adsorption and subsequent elution from a column of mouse anti-rat kappa (MARK-1) coupled Sepharose-4B beads (Bazin et al., 1984).

Murine transferrin was purified from pooled normal mouse serum by affinity chromatography over rabbit anti-rat transferrin coupled to Sepharose-4B (van Eijk and van Noort, 1976), taking advantage of immunological crossreactivity between rat and mouse transferrin.

Iron removal from purified transferrin and re-loading with ^{59}Fe was performed as described before, using the nitrilotriacetic acid method (van der Heul et al., 1978).

Radio-iodination of ER-MP21 mAb and transferrin was performed with $Na^{125}I$

(Amersham Radiochemical Centre, UK) using Iodogen reagent (Pierce Chemical, Rockford, IL) as catalyst (Markwell and Fox, 1978).

^{59}Fe uptake studies. Cellular iron uptake from ^{59}Fe-loaded transferrin was determined essentially as described by Verhoef and Noordeloos (1977). Briefly, cells were harvested, washed at least twice with serum-free DMEM and resuspended in DMEM supplemented with 0.5% (w/v) bovine serum albumin. 1 - 4.10^6 cells/ml were incubated in siliconized vials for 30 min at 37°C in presence or absence of mAb (20 μg/ml). Then, ^{59}Fe-loaded transferrin (5 μg/10^6 cells) was added. At various times, duplicate samples of 0.5 ml were taken and iron uptake was stopped immediately by adding 5 ml ice-cold PBS. Cells were washed three times in ice-cold PBS and cell-bound radioactivity was measured in a Packard Autogamma 500-C spectrometer. In all experiments, non-specific cell-bound radioactivity - usually less than 1 pmole / 10^6 cells - was subtracted from the obtained experimental values.

Radiolabeled mAb and Tf binding studies. For determination of binding of ^{125}I-labeled ER-MP21 or ^{125}I-labeled transferrin, all steps were performed on ice. Cells were washed with DMEM and resuspended at a concentration of 5.10^6 cells / 200 μl in DMEM containing 0.5% BSA as described above. In competitive binding studies, pre-incubation was performed with 100 μl potentially blocking non-radioactive reagents for 30 min. Next, 100 μl ^{125}I-labeled ER-MP21 or ^{125}I-labeled transferrin, diluted in DMEM + 0.5% BSA to the concentration indicated, was added to the cells. In binding affinity determination, dilution series were used with and without 100-fold excess of unlabeled ligand to assess non-specific binding. Cells were incubated under regular mixing for 3 hr to equilibrate ligand binding. Then, cell-bound and free radioactivity from 100 μl aliquots were separated by spinning the cells through 300 μl of an oil mixture of dinonylphtalate and dibutylphtalate (3:7.5) in 0.4 ml reaction vessels (Eppendorf, Hamburg, FRG), as adapted from Klausner et al. (1983). The tubes were frozen in liquid nitrogen and cut using a heated chirurgical blade. Cell bound and free radioactivity were measured as indicated. For affinity determination, binding data were analyzed according to Scatchard (1949).

Antigen characterization. The molecular mass of the antigens recognized by ER-MP21 and H129.121 mAb was determined by surface iodination of cells, followed by specific immunoprecipitation and SDS-PAGE analysis, essentially as described by Pont et al. (1985).

Immunofluorescence labeling and flowcytometric analysis. Cells were labeled for

immunofluorescence as described previously (Leenen et al., 1986b). Analysis was performed on a FACScan™ (Becton and Dickinson, Sunnyvale, CA) with logarithmic amplification. Antibody binding was quantified by interpolation on a calibration scale, using quantitative fluorescent micro beads standards (Beckton and Dickinson). Thus, antibody binding is expressed as soluble FITC molecular equivalents (mol.eq. FITC).

RESULTS

I. ER-MP21 recognizes the murine transferrin receptor but does not compete with transferrin binding

In order to discover epitopes that are of functional importance in the course of macrophage differentiation, we screened several anti-macrophage precursor mAbs for interference with macrophage differentiation in M-CSF stimulated bone marrow cultures. The rat IgG2a mAb, termed ER-MP21, appeared to inhibit the development of mature bone marrow-derived macrophages in a dose-dependent manner (fig. 7.1). Maximal inhibition was reached at ER-MP21 concentrations higher than 5 μg/ml. In contrast, control rat IgG2a mAbs (ER-MP39 shown as example), which recognize other molecules on macrophage precursor cells, caused no inhibition of macrophage development, even at the highest concentration tested (30 μg/ml).

Inhibition of iron uptake by anti-transferrin receptor mAbs is a well known cause of inhibition of proliferation (Trowbridge and Shackelford, 1986). Therefore, we examined the effect of ER-MP21 on cellular iron uptake. Figure 7.2 shows that ER-MP21 indeed inhibits iron uptake from ^{59}Fe-loaded transferrin in a dose-dependent manner.

The question arises whether inhibition of iron uptake is mediated through competition between transferrin and ER-MP21 for the same binding site at the transferrin receptor. To investigate this possibility, RAW309Cr.1 cells were incubated with ^{125}I-labeled transferrin or ^{125}I-labeled ER-MP21, with or without pre-incubation with excess unlabeled transferrin, ER-MP21, or irrelevant IgG2a mAb ER-MP20. The results, presented in table 7.I, clearly show that ER-MP21 and transferrin have different binding sites on the transferrin receptor, since pre-incubation of cells with ER-MP21 caused no significant inhibition of ^{125}I-labeled transferrin binding, and vice versa. Similar results were obtained when R2C4 macrophage precursor cells were used (data not shown). We have no reasonable explanation for the relatively high non-specific binding of ^{125}I-labeled

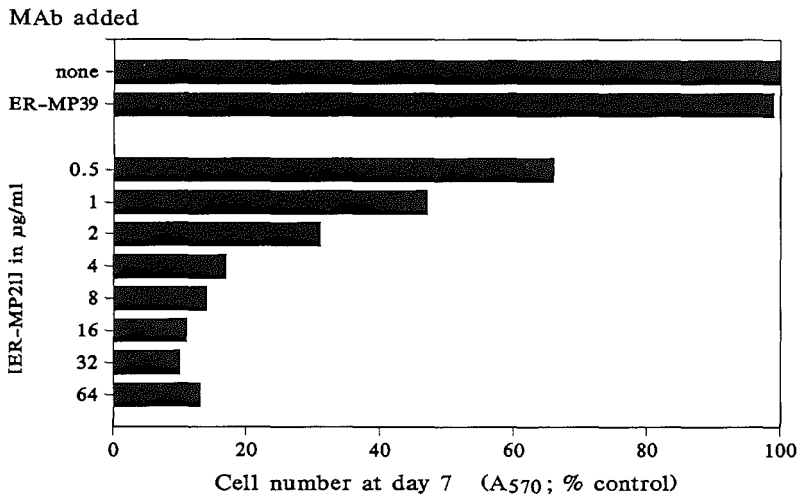

Figure 7.1.

Inhibition of macrophage development in M-CSF stimulated bone marrow culture by ER-MP21. Murine bone marrow was cultured with M-CSF in the presence or absence of different concentrations mAb. After 7 days the obtained mature macrophage progeny was quantified using the colorimetric MTT assay.

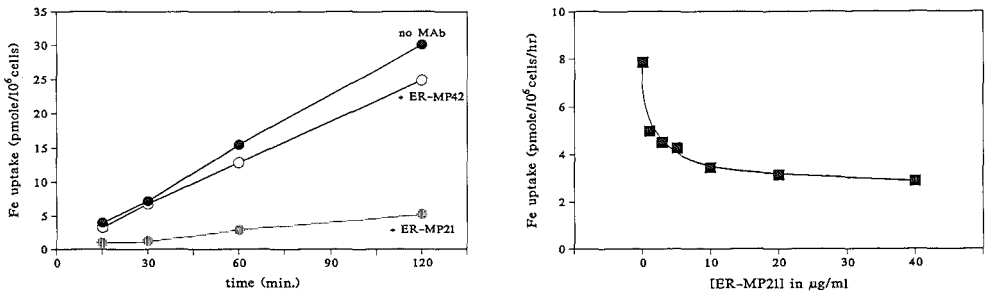

Figure 7.2.

Inhibition of iron uptake by ER-MP21. (LEFT). Specific ^{59}Fe-uptake by the mature macrophage cell line WR19M.1 in absence and presence of 25 µg/ml ER-MP21 or 25 µg/ml ER-MP42 control mAb. The slight inhibition of iron uptake in the presence of ER-MP42 was not observed in other experiments.
(RIGHT). Dose-dependency of ER-MP21-mediated inhibition of ^{59}Fe-uptake by the mature macrophage cell line RAW309Cr.1.
Cells were incubated with mAb for 30 min at 37°C prior to the addition of ^{59}Fe-transferrin.

Table 7.I. ER-MP21 and transferrin have different binding sites on transferrin receptors

preincubation	^{125}I-labeled ligand	bound pmole / 3.10^6 cells exp. I	exp. II
-	Mu-Tf (1×10^{-6} M)	0.62	0.52
rat-Tf or NMS (1.3×10^{-5} M) (33%)	Mu-Tf	0.48	0.30
ER-MP21 (1.3×10^{-5} M)	Mu-Tf	0.63	0.45
ER-MP20 (1.3×10^{-5} M)	Mu-Tf	0.60	0.43
-	ER-MP21 (1×10^{-7} M)	0.45	0.44
rat-Tf or NMS (1.3×10^{-6} M) (33%)	ER-MP21	0.41	0.45
ER-MP21 (1.3×10^{-6} M)	ER-MP21	0.07	0.08
ER-MP20 (1.3×10^{-6} M)	ER-MP21	0.42	0.40

RAW309Cr.1 cells were assayed for competitive binding of murine transferrin (Mu-Tf), anti-transferrin receptor mAb ER-MP21, rat transferrin (rat-Tf) or normal mouse serum (NMS) on ice, using the concentrations indicated. Rat mAb ER-MP20 served as irrelevant IgG2a control.

transferrin in these experiments.

To confirm the notion that ER-MP21 recognizes the murine transferrin receptor, we immunoprecipitated the ER-MP21 antigen from ^{125}I-labeled cells and assessed its molecular mass by SDS-PAGE followed by autoradiography. Figure 7.3 shows that ER-MP21 precipitates an antigen of the same apparent molecular mass as the murine transferrin receptor, precipitated by mAb H129.121 (Van Agthoven et al., 1984). Both mAbs recognize a complex of 200 kDa, consisting of two identical chains of 100 kDa.

In summary, rat mAb ER-MP21 inhibits murine macrophage development in M-CSF stimulated bone marrow culture. ER-MP21 recognizes the murine transferrin

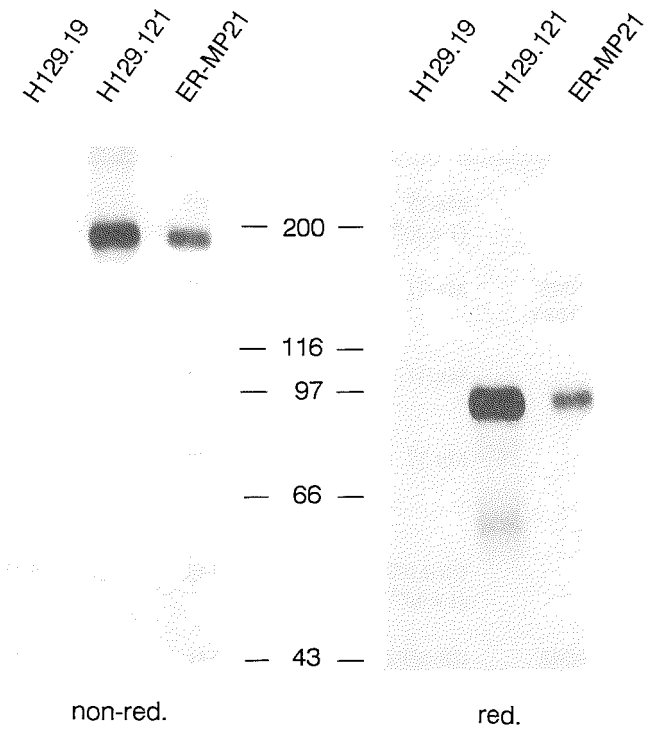

Figure 7.3.

Immunoprecipitation of transferrin receptors by mAbs ER-MP21 and H129.121 from ^{125}I-labeled WR19M.1 cells. Precipitated transferrin receptors were run on a 10 % polyacrylamide gel under reducing and non-reducing conditions. MAb H129.19, directed against murine CD4, was used to detect non-specifically binding proteins.

receptor, since the mAb inhibits cellular uptake of ^{59}Fe-transferrin and precipitates aglycoprotein of identical molecular mass as the murine transferrin receptor. However, ER-MP21 does not interfere with transferrin binding to the receptor.

II. ER-MP21 inhibits proliferation of macrophage precursors, but not of mature macrophages

We then approached the question whether proliferation of different macrophage differentiation stages is equally affected by ER-MP21. This was investigated in 2 in vitro models of macrophage differentiation, viz. M-CSF stimulated bone marrow culture and a panel of macrophage cell lines, which previously has been ordered in a differentiation sequence on the basis of functional and phenotypical characteristics (Leenen et al.,

1986b).

In M-CSF stimulated bone marrow cultures, macrophages develop from macrophage precursors by the action of the lineage specific growth factor M-CSF. The relative maturity of the proliferating cells steadily increases with time during the first days of culture (van der Meer et al., 1983). In order to assess the inhibitory effect of ER-MP21 on proliferation of cells in different stages of macrophage differentiation, we added mAb (30 μg/ml) to bone marrow cultures at different days and quantified the resulting macrophage progeny at day 7 after onset of culture. Parallel cultures were irradiated at the same days with 1500 rad γ-radiation as 100% growth inhibition controls. This radiation dose prevented further proliferation of the cells, but allowed terminal differentiation. When ER-MP21 was added at day 0, the total cell count at day 7 was less than 10% of the untreated controls (fig. 7.4, left). In contrast, addition of mAb at day 4 of culture hardly affected the magnitude of resulting progeny at day 7 (93% of control). For all irradiated and ER-MP21 treated cultures, the number of cell cycles was calculated that treated cultures were left behind compared to untreated controls. The retarded cell cycle numbers thus obtained for the irradiated cultures were taken as 100% growth inhibition controls. Then, the retarded cell cycle numbers obtained for ER-MP21 treated cultures were expressed relative to the radiation-treated controls, thus revealing a relative growth inhibition for the macrophage precursors present on the respective days of mAb addition (fig. 7.4, right). Clearly, bone marrow macrophage precursors (assayed

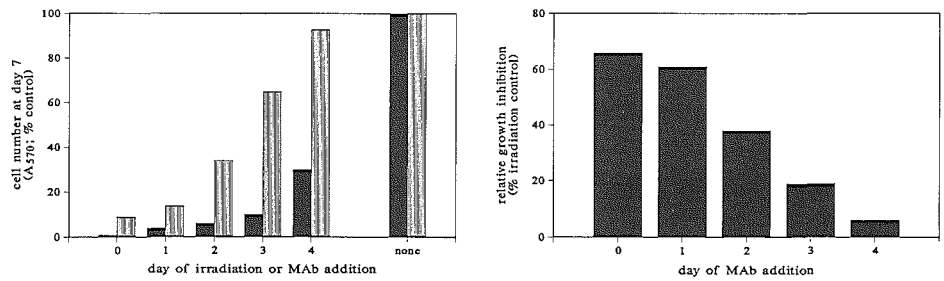

Figure 7.4.

Differentiation stage dependent inhibition of macrophage proliferation by ER-MP21 in M-CSF stimulated bone marrow cultures. (LEFT). Macrophage progeny at day 7 in cultures, treated in parallel at various days by irradiation (1500 rad ^{60}Co γ-radiation)(black bars) or addition of ER-MP21 (30μg/ml final concentration) (hatched bars). Cell numbers were quantified using the MTT assay and expressed relative to untreated controls. (RIGHT). Relative growth inhibition calculated from data represented in figure 7.4-left. For mAb- or radiation-treated cultures, the number of cell cycles needed to reach the cell number in untreated controls was calculated. Values obtained for irradiated cultures were taken as 100% inhibition controls and values for ER-MP21 treated cultures were expressed relative to these.

at day 0) were very sensitive to growth inhibition by ER-MP21, whereas the more mature cells present in culture at day 4 were hardly affected.

The second model of macrophage differentiation, in which the inhibitory effect of ER-MP21 was examined, was a panel of macrophage cell lines aligned according to their differentiation stage (Leenen et al., 1986b), supplemented with the macrophage precursor hybrids R2C4 and R1B1 (Leenen et al., 1989d). Figure 7.5 shows that proliferation of macrophage precursor (R2C4, R1B1 and RMB-TG) and immature macrophage (WEHI-3) cell lines was strongly inhibited. Most R2C4 cells died in the presence of ER-MP21. In contrast, proliferation of the mature macrophage cell lines RAW309Cr.1 and WR19M.1 was only marginally influenced by ER-MP21. A similar differential growth inhibition was obs rved for non-macrophage cell lines as well: RLD-1 pre-T cells died during 3 days of culture in the presence of ER-MP21, whereas proliferation of SP2/0 myeloma cells was not inhibited.

Thus, in two models of macrophage differentiation we observed that anti-transferrin receptor mAb ER-MP21 strongly inhibited proliferation of macrophage precursors, whereas proliferation of more mature stages of macrophage differentiation was hardly affected.

III. ER-MP21 equally inhibits Fe uptake of macrophage precursor and mature macrophage cell lines, but the latter show a higher Fe uptake

We then approached the question what the mechanism was of differentiation-stage correlated inhibition of macrophage proliferation by ER-MP21. Therefore, we investigated the iron uptake by the various cell lines in absence and presence of ER-MP21 (fig. 7.6, left). The presence of ER-MP21 (20 μg/ml) reduced the uptake of ^{59}Fe for all tested cell lines to an average of 35% (range 28-51%) of untreated control value, irrespective of differentiation stage or cell type. However, mature macrophage cell lines showed a 2 to 3-fold higher ^{59}Fe uptake compared to macrophage precursor and immature macrophage cell lines, both in the absence and presence of ER-MP21. This difference can not be ascribed to significant differences in cell size of the various cell lines as determined by flowcytometric forward light scatter analysis (data not shown). Hence, for the cell lines of the macrophage lineage, the proliferation in presence of ER-MP21 correlates highly to the residual iron uptake in the presence of the mAb (fig. 7.6, right; $r = 0.86$). This relationship appears to be strongly cell type dependent. SP2/0 myeloma cells, namely, grew undisturbedly on a level of residual iron uptake as low as 1 pmole/10^6 cells/hr, whereas proliferation of R2C4 macrophage precursor cells was completely inhibited at the same low level of iron uptake.

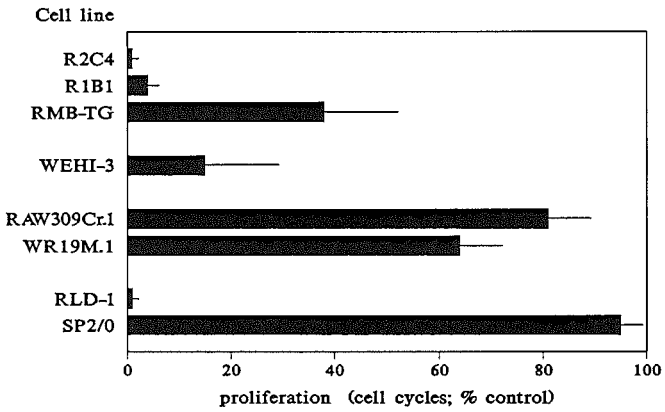

Figure 7.5.

Differentiation stage dependent inhibition of macrophage proliferation by ER-MP21 in a panel of macrophage cell lines. Macrophage precursor (R2C4, R1B1, RMB-TG), immature macrophage (WEHI-3), mature macrophage (RAW309Cr.1, WR19M.1), pre-T (RLD-1) and myeloma (SP2/0) cell lines were cultured in the presence or absence of ER-MP21 (20 μg/ml) or control IgG2a mAb (not shown). After 3 days, cell numbers in parallel cultures were quantified using the MTT assay. Proliferation is given as number of cell cycles performed relative to untreated controls ± SD.

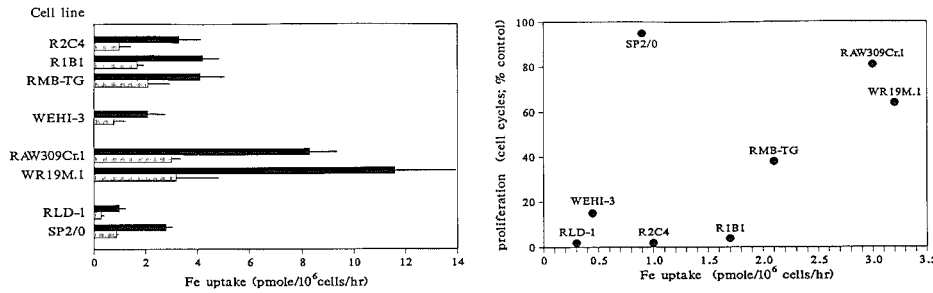

Figure 7.6.

(LEFT). **ER-MP21-mediated inhibition of iron uptake by macrophage and non-macrophage cell lines.** ^{59}Fe-uptake was measured in macrophage precursor (R2C4, R1B1, RMB-TG), immature macrophage (WEHI-3), mature macrophage (RAW309Cr.1, WR19M.1), pre-T (RLD-1) and myeloma (SP2/0) cell lines in the absence (black bars) or presence (hatched bars) of ER-MP21. Data represent the mean of at least 4 determinations per cell line ± SEM.

(RIGHT). **Correlation between residual iron uptake by the various cell lines and proliferation in the presence of ER-MP21.** Data represented in figures 7.5 and 7.6-left are combined in this figure.

IV. Macrophage precursor and mature macrophage cell lines express similar numbers of slightly different transferrin receptors

The most likely explanation for increased iron uptake by mature macrophage cell lines, compared to macrophage precursor and immature macrophage cell lines, is an increased cell surface expression of transferrin receptors. To affirm this supposition, we examined the transferrin receptor expression by the macrophage cell line panel semi-quantitatively using the flowcytometer. Simultaneously, ^{59}Fe-uptake was determined for another part of the same cell preparation. In different experiments, transferrin receptor expression on macrophage precursor and immature macrophage cell lines varied from approximately 1 to 4 x 10^5 mol.eq. FITC, depending on cell line and culture density (fig. 7.7). Indeed, the mature macrophage cell line WR19M.1 showed an increased transferrin receptor expression (5 - 6 x 10^5 mol.eq. FITC), which related to an increased iron uptake. Surprisingly, the mature RAW309Cr.1 cells showed a transferrin receptor expression comparable to the expression found for macrophage precursor and immature macrophage cell lines (2 x 10^5 mol.eq. FITC). Iron uptake by RAW309Cr.1 cells, however, was 3-fold higher. Apparently, RAW309Cr.1 cells endocytosed as much as 3 times the amount of transferrin-bound iron compared to macrophage precursor cells, using the same number of cell surface transferrin receptors.

This observation raised the question whether transferrin receptors on mature macrophage cell lines, in particular on RAW309Cr.1 cells, are structurally different from transferrin receptors on macrophage precursor and immature macrophage cell lines. From binding affinity studies using ^{125}I-labeled ER-MP21 we obtained circumstantial evidence that this is indeed the case. Figure 7.8 (left) shows that ER-MP21 bound to R2C4 transferrin receptors with a single affinity (K_d = 19 - 23 x 10^{-9} M). R2C4 cells expressed 2 to 13 x 10^4 ER-MP21 binding sites per cell, depending on culture density of the cells (Musgrove et al., 1984). Remarkably, RAW309Cr.1 cells showed two types of receptors with different affinity for ER-MP21 (fig. 7.8, middle). High affinity receptors on RAW309Cr.1 cells bound the mAb more avidly compared to R2C4 transferrin receptors (K_d = 7.3 x 10^{-9} M; range 5.7 - 9.0 x 10^{-9} M) and were present at 3 to 10.10^4 receptors per cell. A likely candidate for the ER-MP21 low affinity receptor on RAW309Cr.1 cells ($K_d \approx$ 4.5 x 10^{-7} M) is the receptor for the Fc part of IgG, which is known to be expressed by mature macrophage cell lines (Morahan, 1980). Therefore, we performed the same binding affinity studies in the presence of 1.3 x 10^{-6} M irrelevant rat IgG2a mAb ER-MP20. This concentration is about equimolar to the highest concentration ^{125}I-ER-MP21 used (1.10^{-6} M). Clearly, the binding pattern of ER-MP21, revealing two affinities, was not significantly altered by the addition of irrelevant rat mAb (fig. 7.8, right). It is therefore unlikely that the low affinity ER-MP21 receptor on

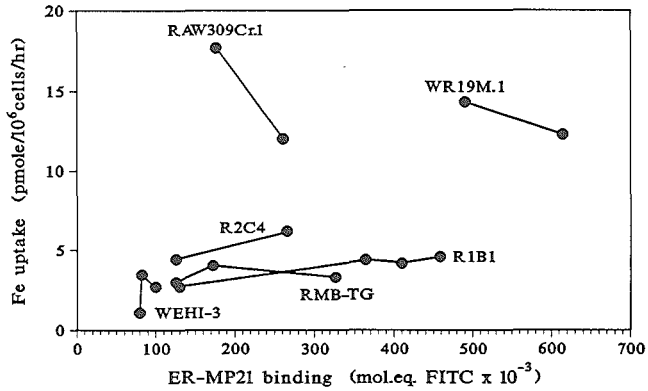

Figure 7.7.

Relationship between cell surface transferrin receptor expression and iron uptake for different macrophage cell lines. Part of a cell preparation was processed for immunofluorescence and used to quantify ER-MP21 binding by flowcytometry. For another part of the same preparation ^{59}Fe uptake was measured. Each point represents the ER-MP21 binding and ^{59}Fe uptake determined in a single experiment. Data from different experiments on the same cell line are connected.

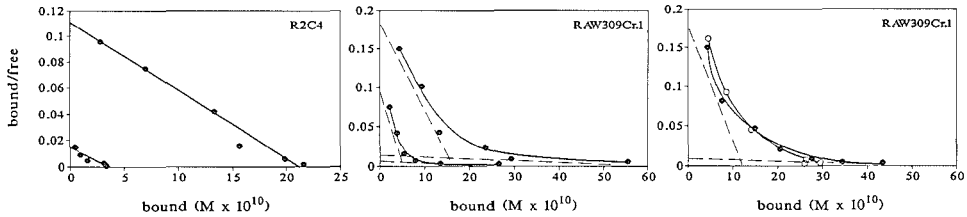

Figure 7.8.

Scatchard analysis of ER-MP21 binding to R2C4 and RAW309Cr.1 cells. (LEFT). Scatchard analysis of ER-MP21 binding to R2C4 macrophage precursor cells. Results from two representative experiments, revealing different numbers of binding sites, but similar affinities, are given. (MIDDLE). Scatchard analysis of ER-MP21 binding to RAW309Cr.1 mature macrophage cells in two different experiments. (RIGHT). Scatchard analysis of ER-MP21 binding to RAW309Cr.1 cells in the presence (O) and absence (●) of irrelevant rat IgG2a mAb ER-MP20.

RAW309Cr.1 cells is identical to the Fc receptor.

Thus, the different binding affinities of ER-MP21 to transferrin receptors on RAW309Cr.1 and R2C4 cells suggest that transferrin receptors on mature and immature macrophages are structurally different.

DISCUSSION

In search of mAbs directed against functionally important epitopes on macrophage precursor cells, we selected IgG2a mAb ER-MP21 for its inhibitory capacity of macrophage development in M-CSF stimulated bone marrow cultures. ER-MP21 appeared to be directed against the murine transferrin receptor, since this mAb inhibited uptake of transferrin-bound ^{59}Fe and precipitated, as the anti-transferrin receptor mAb H129.121, a homodimer of 200 kDa (Van Agthoven et al., 1984). Like other IgG anti-transferrin receptor mAbs (Takei, 1983; Van Agthoven et al., 1984; Trowbridge and Shackelford, 1986; Kemp et al., 1987), ER-MP21 bound to an epitope different from the transferrin-binding site.

In general, growth inhibition by anti-transferrin receptor mAbs is caused by iron starvation of cells (Trowbridge et al., 1982b; Lesley and Schulte, 1985; Taetle et al., 1986). The underlying mechanism, proposed for IgG anti-transferrin receptor mAbs, is that mAb binding decreases transferrin receptor recycling between the cell surface and intracellular compartments (Taetle et al., 1986; Schmidt et al.,1986; Lesley et al., 1989). Furthermore, transferrin receptor degradation is enhanced, possibly by inappropriate intracellular routing of the complex consisting of transferrin, transferrin receptor and mAb (Lesley and Schulte, 1985; Lesley et al., 1989). Both decreased recycling and enhanced degradation cause a reduced cell surface expression of transferrin receptors, leading to reduced iron uptake with concomitant growth inhibition in the presence of anti-transferrin receptor mAb.

For macrophage precursors, we indeed observed a strong inhibition of proliferation by anti-transferrin receptor mAb ER-MP21. Most likely, this inhibition is mediated by the mechanism described above. However, proliferation of more mature stages of macrophage differentiation was hardly affected by ER-MP21. We observed this differentiation-stage related inhibition of macrophage proliferation in two independent models of macrophage differentiation, namely M-CSF stimulated bone marrow culture and a panel of macrophage cell lines, aligned in differentiation sequence. Thus, with increasing maturity, proliferating macrophages become refractory to inhibition by anti-transferrin receptor mAb. The highly sensitive macrophage precursor cells are found around the CFU-GM/CFU-M stage of differentiation: CFU-M are the main M-CSF responsive cells in the bone marrow, and these were shown to be strongly inhibited (fig. 7.4, left). Furthermore, the equally sensitive macrophage precursor cell lines appear to be arrested around the CFU-GM stage of differentiation (Leenen et al., 1986b). More mature, insensitive stages of differentiation, occurring at day 4 of bone marrow culture and represented by the mature macrophage cell lines in the panel, are around the monoblast / monocyte stage (Leenen et al., 1986b; van der Meer et al., 1983).

A comparable lack of growth inhibition by an anti-transferrin receptor mAb, inhibitory for other cell types, has been reported previously for murine L cells (Trowbridge et al.,1982b) as well as for different lymphocyte subsets (Kemp et al., 1987). The mechanism of differential growth inhibition, however, remained largely obscure (Trowbridge and Shackelford, 1986). Our cell line model of macrophage differentiation offered a unique opportunity to study this mechanism, since large numbers of cells with different sensitivity are available from a single hemopoietic lineage. In ^{59}Fe-uptake studies, we observed that ER-MP21 equally inhibited iron uptake by sensitive and insensitive macrophage cell lines. Thus, the mechanism by which ER-MP21 blocks iron uptake is most likely similar for sensitive and insensitive cell lines. However, insensitive mature macrophage cell lines showed a 2 to 3 times higher iron uptake both in presence and absence of ER-MP21, compared to immature macrophage cell lines. Apparently, the diminished iron uptake by mature macrophage cell lines, in the presence of the mAb, is still sufficient for undisturbed proliferation. In contrast, reduction of the lower level of iron uptake in macrophage precursor cell lines caused by the action of the mAb, leads to severe inhibition of proliferation or even cell death. This indicates that macrophage precursor cell lines apply virtually all endocytosed iron for growth and proliferation, whereas mature macrophage cell lines normally take up more iron than merely needed for growth. A similar explanation is, most likely, valid for the differential growth inhibition of bone marrow-derived macrophages: insensitive monoblasts / monocytes at day 4 of culture may take up a multitude of the amount of iron taken up by CFU-M.

How do mature macrophages take up more iron, compared to macrophage precursors? The most likely mechanism is the expression of an increased number of transferrin receptors (Andreesen et al., 1988). In this study, we indeed observed for the mature WR19M.1 cells a higher transferrin receptor expression, compared to the investigated macrophage precursor cell lines. This might serve as an explanation for increased iron uptake by WR19M.1 cells. However, RAW309Cr.1 cells showed a 2 to 3 times increased iron uptake, compared to macrophage precursor cell lines, and yet expressed similar numbers of transferrin receptor on the cell surface. Thus, increased iron uptake is not necessarily accompanied by increased transferrin receptor expression. Our binding affinity analyses of ER-MP21 revealed that RAW309Cr.1 transferrin receptors are presumably structurally different from transferrin receptors on macrophage precursors. ER-MP21 bound with single affinity to transferrin receptors on R2C4 cells, whereas two affinities were found for receptors on RAW309Cr.1 cells. The high affinity binding - to the transferrin receptor - was more avid, compared to the binding to R2C4 cells, thus indicating a structural difference between the binding sites on both cell types. The low affinity ER-MP21 receptor on RAW309Cr.1 cells appeared to be distinct from the receptor for the Fc part of IgG. Unfortunately, we have no further clues to its nature

and relationship to the transferrin receptor.

Interestingly in this context, Andreesen et al. (1988) showed that mature human macrophage transferrin receptors bind their natural ligand transferrin with different affinity compared to other cell types, thus supporting the view that mature macrophages express structurally different transferrin receptors. The present results suggest that structurally different transferrin receptors on mature macrophages account for a more efficient endocytosis of transferrin-bound iron. Increased efficiency in this respect is presumably mediated by an increased receptor recycling of transferrin receptor between cell membrane and intracellular compartments. Noteworthy, a similar increase of transferrin receptor recycling appears to occur during erythroid differentiation (Mulford and Lodish, 1988).

An alternative explanation for the increased iron uptake by mature macrophages - using the same number of transferrin receptors - might be the existence of additional pathways of iron uptake, independent from the transferrin receptor. For example, receptors for glycan-moieties on transferrin, such as the asialo-glycoprotein receptor or the mannose receptor, might mediate endocytosis of transferrin (van Berkel et al., 1987). Moreover, fluid phase endocytosis would be a highly feasible mechanism of alternative iron uptake for the actively endocytosing mature macrophages (Steinman and Cohn, 1972). Such alternative mechanisms would not be blocked by anti-transferrin receptor mAb ER-MP21. To investigate this possibility, we studied whether the competitive protein asialo-fetuin or the competitive saccharides mannan and N-acetyl-glucosamine affected ^{59}Fe-uptake by mature macrophage cell lines or caused increased inhibition of ^{59}Fe-uptake in the presence of ER-MP21 (unpublished results). Unequivocally, these agents had no effects on cellular iron uptake. Furthermore, the level of fluid phase endocytosis in mature macrophages, measured as uptake of ^{59}Fe-citrate, could by no means account for a 2 to 3 times increased iron uptake, compared to macrophage precursor cells (data not shown). These alternative pathways of iron uptake have to be excluded therefore as mechanisms of enhanced iron uptake by mature macrophages. Moreover, the similar relative inhibition of iron uptake by ER-MP21 for macrophage precursor and mature macrophage cell lines (Fig. 7.6, left) indicates that the same mechanism, i.e. transferrin receptor mediated iron uptake, is active in both cell types.

Why do mature macrophages endocytose as much as 2 to 3 times the amount of iron needed for proliferation? Iron is incorporated in a number of vital proteins, such as heme containing globins, cytochromes and iron-containing enzymes. Furthermore, iron is stored intracellularly in ferritin (Aisen and Listowski, 1980; Jacobs, 1985). Macrophages are known to be an important link in the iron metabolism by the phagocytosis of senescent erythrocytes (Finch and Huebers, 1982; Deiss, 1983). In connection to this, macrophages constitute a considerable iron storage in the body. The development of this

function is reflected by the increasing intracellular ferritin content during terminal differentiation of macrophages (Andreesen et al., 1984). Both the present results and those obtained by Andreesen et al. (1984; 1988) suggest that mature macrophages can accumulate iron for storage purposes, not only by phagocytosis of erythrocytes, but also by means of transferrin receptor mediated iron uptake.

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CHAPTER 8

DETECTION OF MURINE CONNECTIVE TISSUE MACROPHAGES BY MONOCLONAL ANTIBODY ER-MP23

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SUMMARY

ER-MP23 is a novel rat monoclonal antibody directed against a 38 kDa polypeptide exclusively expressed by a subpopulation of murine macrophages located in various connective tissues. ER-MP23 positive connective tissue macrophages (classically termed histiocytes) are most abundant in the vicinity of several epithelia, such as the epidermis or the submandibular gland. ER-MP23 positive cells are virtually absent from common mononuclear phagocyte populations such as monocytes, or peritoneal or alveolar macrophages. Among a panel of mature macrophage cell lines, a marked heterogeneity is observed with respect to the expression of ER-MP23 antigen (Ag). Absence or presence of the ER-MP23 Ag appears to be an intrinsically determined feature of mature macrophages, rather than imposed by differentiation stage or environmental conditions. The heterogeneity in ER-MP23 Ag expression thus observed among macrophage cell lines as well as among macrophages in situ suggests that this antigen is a marker for a separate lineage in macrophage differentiation.

INTRODUCTION

From all hemopoietic stem cell derived lineages, the mononuclear phagocytes undoubtedly constitute the most heterogeneous cell population. The various tissues harbour different macrophage populations, each with distinguishing features (Gordon et al., 1988b). Moreover, within a single organ such as the spleen, multiple distinct macrophage populations can be discerned (van Rooyen et al., 1989, Leenen et al., 1989b).

The origin of the observed macrophage heterogeneity is presently obscure. Differences in macrophage differentiation and activation stages obviously contribute to the generation of macrophage diversity (Gordon et al., 1988). However, it is not conclusively established whether separate lineages of macrophage differentiation exist (cf. Treves, 1984).

In studies which deal with the origin of macrophage heterogeneity, tools that define particular subpopulations or differentiation stages are indispensable. Therefore, we produced a panel of monoclonal antibodies (mAbs), recognizing different subpopulations of immature and mature murine mononuclear phagocytes (Leenen et al., 1986a; Leenen et al., 1989a; Leenen et al., 1989b). In the present study, we describe a novel mAb, ER-MP23, which exclusively recognizes a subpopulation of murine macrophages present in connective tissues.

MATERIALS AND METHODS

Animals. BALB/c mice were raised in the animal colony of our department and kept under clean, routine conditions with free access to food and water.

Antisera and conjugates. Monoclonal antibody ER-MP23 (rat IgG2a) was raised against murine macrophage precursor hybrids, as described elsewhere (Leenen et al., 1989b). Furthermore, mAbs were applied against T-200 (Ledbetter and Herzenberg, 1979), Mac-1 (Springer et al., 1979), Mac-2 (Ho and Springer, 1982), F4/80 (Austyn and Gordon, 1981), BM8 (Malorny et al., 1986), ER-TR9 (van Vliet et al., 1985) and ER-HR3 antigens (de Jong et al., 1987).

Routinely, mAbs were used as undiluted hybridoma culture supernatants. As second step conjugates, we applied in the micro-ELISA and immuno-ß-galactosidase assay anti-rat-Ig coupled to E.coli-ß-galactosidase from Radiochemical Centre (Amersham, UK) and Zymed Laboratories (San Fransisco, CA), respectively.

For immunohistological detection, we used anti-rat Ig coupled to horseradish peroxidase (DAKO, Copenhagen, Denmark).

Cells. The macrophage cell lines, used for mAb screening, have been characterized extensively (Leenen et al., 1986b). ER-MP23 binding was also determined on various isolated macrophage populations. Resident peritoneal and alveolar macrophages were obtained by lavage using serum-free DMEM (Flow Laboratories, Irvine, Scotland) and PBS supplemented with 0.6 mM EDTA, respectively. Different populations of peritoneal exudate cells were isolated at 8 hr and at 4 days after i.p. injection of 1 ml Brewer's thioglycollate medium (Difco Laboratories, Detroit). Nucleated cells from peripheral blood were isolated after specific lysis of erythrocytes by incubation for 10 min at 4°C in 17 mM Tris.HCl pH 7.2 containing 144 mM NH_4Cl.

ELISA. The binding of ER-MP23 to a panel of macrophage cell lines was semi-quantitatively assessed in a micro-ELISA as described previously (Leenen et al., 1989a).

Immuno-ß-galactosidase staining of cell suspensions. Antibody binding at single cell level was detected using an immuno-ß-galactosidase staining procedure (Leenen et al., 1987).

Immunohistological analysis. Immunohistological characterization of ER-MP23 was performed on cryostat sections essentially as described previously (van Ewijk et al., 1988).

Biochemical analysis. The antigen recognized by ER-MP23 was immunoprecipitated from radio-iodinated WR19M.1 macrophage tumor cells and analyzed by SDS-PAGE according to methods described elsewhere (Leenen et al., 1989b).

RESULTS

The purpose of the present study is the characterization of a novel phenotypical marker, recognized by mAb ER-MP23, for a unique subpopulation of murine macrophages.

In the screening for mAbs against differentiation-stage related macrophage antigens, we applied a cell line model of macrophage differentiation (Leenen et al., 1986b). This model comprises a panel of macrophage tumor cell lines, ordered in a linear differentiation sequence (fig. 8.1). Three groups of cell lines can be discerned, viz. (i) macrophage precursor lines (M1, RMB-1, RMB-3), (ii) immature macrophage cell lines (WEHI-3B, WEHI-3) and (iii) macrophage cell lines with mature characteristics (Pu5-1.8, J774-1.6, P388D1, RAW264.7, RAW309Cr.1, WR19M.1). General macrophage mar-

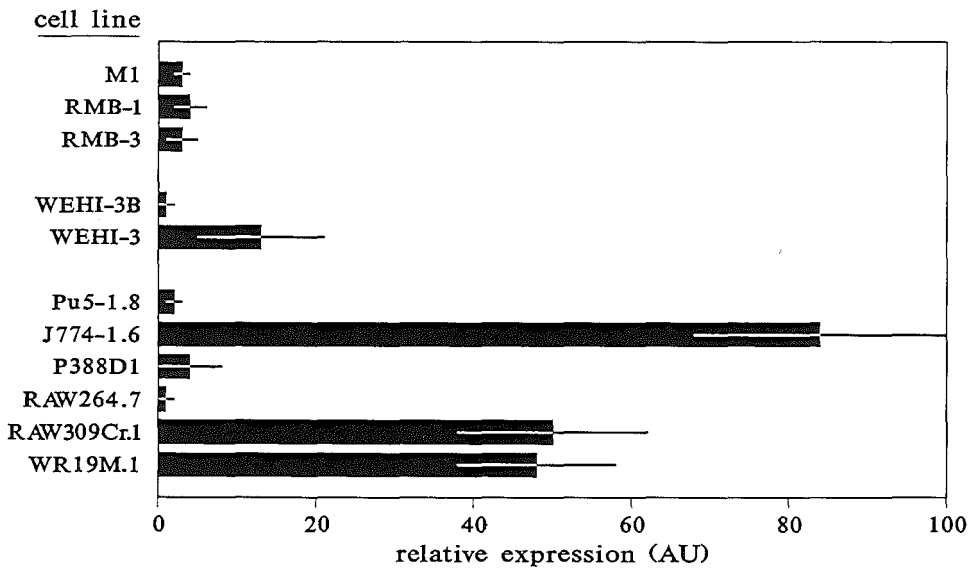

Figure 8.1.

ER-MP23 antigen expression by a panel of macrophage cell lines ordered in a linear differentiation sequence. Antibody binding was determined by ELISA and expressed in arbitrary units (AU) relative to an intra-experimental positive control. Data represent the mean ± SD of at least three determinations.

kers such as Mac-1, Mac-2, or F4/80 antigen (Ag) show a gradual increase in expression along with the proposed sequence (Leenen et al., 1986b).

The present antigen recognized by ER-MP23 is not expressed beyond background level by the macrophage precursor cell lines (fig. 8.1). Of the immature macrophage cell lines, only WEHI-3 is weakly positive. In contrast, expression of the ER-MP23 Ag by mature macrophage cell lines appears to be an all-or-none phenomenon. Three of 6 mature macrophage cell lines highly express ER-MP23 Ag, whereas the other 3 are negative.

The specificity of ER-MP23 in the recognition of no other cells than macrophages is most clearly demonstrated in immunohistological staining of frozen sections (fig. 8.2). In general, ER-MP23 +ve macrophages occur as irregularly shaped or stretched cells

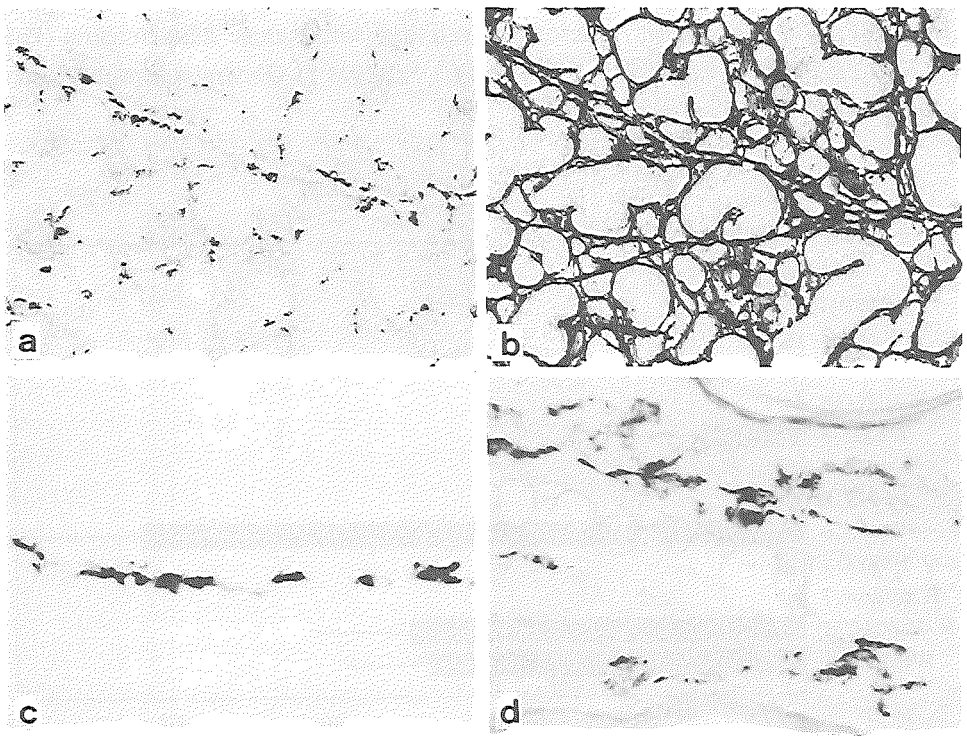

Figure 8.2.
Immunohistological distribution of ER-MP23 Ag. Immunoperoxidase staining of the submandibular gland showing ER-MP23 +ve connective tissue macrophages (a) and ER-TR7 +ve reticular fibroblasts and fibres (b). ER-MP23 +ve macrophages in septa of the thymus (c) and in the dermis of the ear (d). Original magnifications x 125 (a and b), x 310 (c and d).

in especially those connective tissues which are situated in the vicinity of epithelia.

Figures 8.2a and 8.2b show the staining of the submandibular gland with ER-MP23 and ER-TR7, respectively. ER-TR7 clearly delineates the framework of the gland, as it heavily labels the fibroblasts and reticular fibres in connective tissue (van Vliet et al., 1986). Evidently, the ER-MP23 +ve macrophages are located in connective tissue septa that separate the various lobules. Similarly, in the thymus, ER-MP23 +ve macrophages are located in, or just under the capsule, and in interlobular septa (fig. 8.2c). A high density of ER-MP23 +ve macrophages was further observed in the dermis of the ear (fig. 8.2d). The notion that the ER-MP23 +ve cells are macrophages and not, for instance, fibroblasts was confirmed in immunofluorescence double labeling of ear sections (data not shown). ER-MP23 +ve cells in the dermis of the ear appeared to co-express T-200, Mac-1, Mac-2, F4/80, BM8, ER-TR9 and ER-HR3, thus unequivocally indicating the macrophage identity of these cells. Furthermore, a significant part of the ER-MP23 +ve macrophages expressed Ia antigens.

In order to investigate the expression of ER-MP23 Ag by other well-defined macrophage populations, we screened resident alveolar and peritoneal cells, and peripheral blood nucleated cells (table 8.I). Furthermore, thioglycollate-elicited granulocytes and macrophages were tested for ER-MP23 Ag expression. Clearly, ER-

Table 8.I. ER-MP23 Ag expression by isolated macrophage populations

population	% Mϕ	% ER-MP23 +ve
peripheral blood nucleated cells	4 ± 2[a]	0
resident peritoneal cells	53 ± 15[a]	5 ± 5
resident alveolar cells	74 ± 7[b]	1 ± 1
thioglycollate-elicited cells		
8 hr after i.p. injection	4 ± 3[a]	1 ± 1
4 days - - -	87 ± 8[b]	1 ± 1

Percentages of positive cells are determined in at least 3 assays and expressed ± SD

[a] determined as % F4/80 +ve cells

[b] determined as % non-specific esterase +ve cells, using naphthyl acetate as substrate

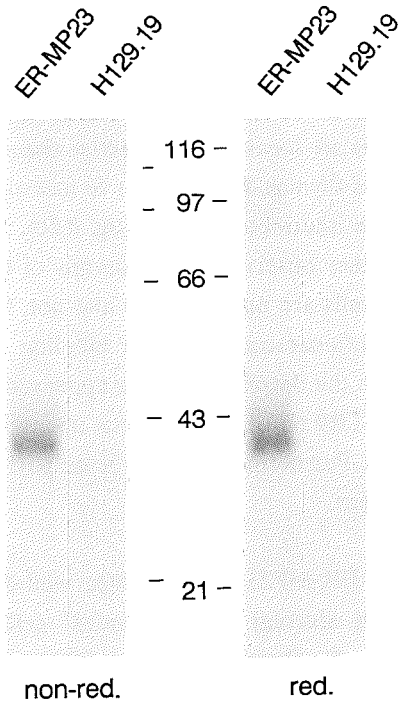

Figure 8.3.
Biochemical analysis of the ER-MP23 Ag. WR19M.1 macrophage tumor cells were radioiodinated, lysed, and subjected to specific immunoprecipitation. The precipitate was run on a 10 % polyacrylamide gel under reducing and non-reducing conditions. Control precipitations were carried out using an irrelevant antibody (H129.19, detecting murine CD4 (Pierres et al., 1984)).

MP23 +ve macrophages were only scarcely present in these isolated macrophage populations, whereas blood monocytes were never found to express ER-MP23 Ag.Furthermore, granulocytes (the main cell type present at 8 hr after thioglycollate injection) nor other cells in the peripheral blood bound ER-MP23. Together, these findings indicate that ER-MP23 Ag is specifically expressed by connective tissue-associated macrophages.

In order to determine the biochemical nature of the ER-MP23 Ag, we radioiodinated WR19M.1 cells and immunoprecipitated the antigen. Analysis by SDS-PAGE followed by autoradiography, revealed that ER-MP23 binds to a (glyco)protein of 38 kDa apparent molecular mass under reducing and non-reducing conditions (fig. 8.3). This indicates that the ER-MP23 Ag consists of a single chain polypeptide.

DISCUSSION

In the present study, we characterize the reactivity of a novel mAb which may serve as an important tool for the study of murine macrophage differentiation and heterogeneity. This mAb, ER-MP23, specifically recognizes a subpopulation of macrophages located in connective tissues (histiocytes).

ER-MP23 +ve macrophages are especially abundant in connective tissues in the vicinity of particular epithelia, such as the epidermis, the thymic epithelium or the submandibular gland. However, not all connective tissues - either supporting epithelia or not - harbour ER-MP23 +ve macrophages. For instance, the macrophages that are present in the lamina propria of the small intestine (Hume et al., 1984) do not significantly express ER-MP23 Ag (data not shown). Similarly, ER-MP23 +ve macrophages are hardly detectable in lymph node or splenic connective tissue. The reason for this inconsistent occurrence of ER-MP23 +ve macrophages in the various connective tissues is unclear at present. Obviously, the type of connective tissue is not of significant importance, as ER-MP23 +ve macrophages occur in the loose connective tissue of the dermis, as well as in the reticular type present in the submandibular gland, or the dense connective tissue of the thymic capsule and septa.

The heterogeneity of macrophage ER-MP23 Ag expression in situ is likewise reflected in the mature lines of the macrophage cell line panel. Three of 6 mature macrophage cell lines strongly express the ER-MP23 Ag, whereas the other 3 mature cell lines are ER-MP23 -ve. The absence or presence of ER-MP23 Ag appears to be a stable characteristic of the particular cell line, as similar ER-MP23 Ag phenotypes were observed 2 years after initial screening of the mAb. The possibility that ER-MP23 Ag is polymorphic can be excluded, since both positive and negative cell lines occur which are originally derived from the same mouse strain (e.g. Pu5-1.8 and J774-1.6 both originate from BALB/c). Moreover, connective tissue macrophages from several mouse strains tested (BALB/c, DBA/2, CBA, C3H, C57Bl/6), similarly expressed the ER-MP23 Ag.

What is then the origin of the mature macrophage heterogeneity in ER-MP23 Ag expression as observed in situ as well as in the cell line panel? The following, alternative explanations are feasible (cf. Treves, 1984): (1) ER-MP23 Ag expression is determined by the macrophage maturation stage; (2) ER-MP23 Ag expression is induced by environmental stimuli and thus determined by the macrophage activation stage; (3) ER-MP23 Ag expression is a characteristic feature of a separate macrophage differentiation lineage or independently maintained macrophage population.

Most likely, the observed heterogeneity in ER-MP23 Ag expression can not be ascribed to different macrophage maturation stages. Previously, the macrophage cell lines have been ordered in a linear differentiation sequence on the basis of expression of multiple differentiation-related characteristics (Leenen et al., 1986b). ER-MP23 Ag expression shows no correlation with the established differentiation sequence. Furthermore, expression of ER-MP23 Ag by mature macrophage cell lines appears to be an all-or-none phenomenon rather than showing a fine-tuned increase with cellular maturation as observed with the general macrophage markers Mac-1 or F4/80 Ag.

Modulation of ER-MP23 Ag expression by particular environmental conditions is also no likely explanation for the observed heterogeneity. All cell lines are kept under identical conditions and yet show a stable positive or negative ER-MP23 phenotype. In addition, the ER-MP23 Ag phenotype of a negative and a positive cell line (RAW264.7 and RAW309Cr.1, resp.) is not significantly altered by culture in the presence of cytokine mixtures, such as conA-stimulated spleen cell supernatant or post-endotoxin serum (data not shown). Hence, the ER-MP23 phenotype appears to be a stable, intrinsically determined characteristic of mature macrophages. Possibly, the differential expression of ER-MP23 Ag by the mature macrophage cell lines reflects the heterogeneous origin of the various cell lines: ER-MP23 +ve cell lines may be derived from connective tissue macrophages, whereas ER-MP23 -ve cell lines originate from other macrophage populations.

The unlikeliness of correlation of ER-MP23 Ag expression with macrophage differentiation- or activation stage leaves ER-MP23 Ag as a putative marker for a separate macrophage differentiation pathway or independently maintained macrophage population. This deduced assumption is, however, difficult to affirm experimentally. Bone marrow cultures in the presence of M-CSF revealed so far no colonies or individual cells expressing ER-MP23 Ag (data not shown). Either precursors of ER-MP23 +ve macrophages are not triggered under the conditions used, or these precursors are forced into an alternative differentiation pathway, leading to ER-MP23 -ve macrophages.

In summary, the present mAb ER-MP23 detects a hitherto unrecognized 38 kDa polypeptide, which is specifically expressed by a murine macrophage subpopulation occurring in various connective tissues. The heterogeneous ER-MP23 Ag expression observed among macrophages in situ as well as among a panel of macrophage cell lines suggests that ER-MP23 Ag is a marker for a separate macrophage differentiation pathway or independently regulated macrophage population. The highly restricted expression of the ER-MP23 Ag by a connective tissue macrophage population may make this a valuable marker in the analysis of murine macrophage heterogeneity.

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CHAPTER 9

MONOCLONAL ANTIBODY ER-BMDM1 RECOGNIZES A MACROPHAGE AND DENDRITIC CELL DIFFERENTIATION ANTIGEN WITH AMINOPEPTIDASE ACTIVITY

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SUMMARY

In this paper we describe the reactivity of monoclonal antibody (mAb) ER-BMDM1, directed against a 160 kDa cell membrane-associated aminopeptidase present on various macrophage populations. The antigen recognized by ER-BMDM1 appears to be increasingly expressed upon maturation of macrophages, as shown in various in vitro models of macrophage differentiation. In vivo, the expression of the ER-BMDM1 aminopeptidase arises after the monocytic stage of differentiation, since bone marrow cells and peripheral blood monocytes are ER-BMDM1 negative. Analysis of isolated - resident and elicited - macrophage populations revealed that ER-BMDM1 recognizes a subpopulation of mature macrophages: only a minority of the resident peritoneal and alveolar macrophages are ER-BMDM1 positive, whereas virtually all thioglycollate elicited peritoneal exudate macrophages bind the mAb. In situ, a subpopulation of macrophages in lymphoid organs is recognized. Furthermore, interdigitating cells in T cell areas are ER-BMDM1 positive. Phenotypical analysis of isolated dendritic cells - the in vitro equivalents of interdigitating cells - from spleen and lymph node confirmed that the majority of this important antigen-presenting cell population expresses the ER-BMDM1 antigen. In non-lymphoid organs, ER-BMDM1 binding is primarily associated with the presence of microvilli.

INTRODUCTION

Mature mononuclear phagocytes comprise a heterogeneous bone marrow derived cell population. The various representatives perform divergent functions, such as immune stimulation by means of antigen presentation, immune suppression, stimulation of hemopoiesis, phagocytosis of senescent cells, or killing of harmful microorganisms and tumor cells (reviewed by Unanue and Allen, 1987; Johnston, 1988; Gordon et al., 1988). Most of these functions have been determined using in vitro assays on isolated populations and/or cultured cells. It remains, however, difficult to ascribe these functions to specific macrophage subpopulations, located in different tissue compartments. Therefore, a detailed characterization of macrophage heterogeneity in situ is needed, using markers that are specifically indicative of particular macrophage functions.

Monoclonal antibodies directed against macrophage antigens have greatly facilitated the definition of different macrophage subpopulations. For instance, ER-TR9 and MOMA-1 specifically recognize two macrophage subpopulations in the spleen, located at the marginal zone and at the inner border of the marginal sinus, respectively (van Vliet et al., 1985; Kraal et al., 1986). Other monoclonal antibodies, such as F4/80, BM8

or MOMA-2, show broader anti-macrophage specificity, yet fail to detect particular macrophage subpopulations (Witmer and Steinman, 1984; Malorny et al., 1986; Kraal et al., 1987). Such phenotypical differences between macrophage subpopulations suggest different functions for these cell types. This supposition, however, lacks convincing evidence as long as the functions of the mentioned antigens remain unknown.

In this paper we define a new subpopulation of mature murine macrophages, characterized by the binding of monoclonal antibody ER-BMDM1. Moreover, a significant subpopulation of dendrocytes - i.e. Langerhans cells, veiled cells, interdigitating cells and their in vitro equivalents, dendritic cells (Austyn, 1987) - is ER-BMDM1 positive. Biochemical analysis of the ER-BMDM1 antigen reveals that an 160 kDa protein is recognized, which exhibits aminopeptidase activity.

MATERIALS AND METHODS

Animals. Male and female BALB/c mice were bred at our facility under clean, routine conditions and sacrificed at 10 - 20 weeks of age. For the production of post-endotoxin serum (PES) we used C57BL/6J mice. A female F1 (Louvain x Lewis) rat was immunized (see below) and sacrificed as donor of immune spleen cells.

Monoclonal antibodies and conjugates. As sources of monoclonal antibodies (mAbs) we used either undiluted hybridoma culture supernatants, or optimally diluted ascitic fluid. In addition to the ER-BMDM1 mAb, described below, we used BM8 (anti-macrophage; Malorny et al., 1986), ER-MP20 (anti-macrophage precursor; Leenen et al., manuscript submitted), F4/80 (anti-macrophage; Austyn and Gordon, 1981), H129.19 (anti-CD4; Pierres et al., 1984), M3/38 (anti-Mac-2 macrophage antigen; Ho and Springer, 1982), MOMA-1 (anti-macrophage; Kraal and Janse, 1986), MOMA-2 (anti-macrophage; Kraal et al., 1987), and NLDC-145 (anti-interdigitating / dendritic cell; Kraal et al., 1986).

For detection of mAb binding in ELISA and immuno-ß-galactosidase procedures, anti-rat Ig coupled to ß-galactosidase (Radiochemical Centre, Amersham, UK and Zymed Laboratories, San Francisco, CA, resp.) was applied. Anti-rat Ig coupled to horseradish peroxidase was obtained from DAKO (Copenhagen, Denmark).

Generation of ER-BMDM1 producing hybridoma. As immunogen we used mononuclear phagocytes obtained from bone marrow after culture for 7 days with M-CSF (see below). A priming immunization was given i.p. with 4 x 10^6 cells, emulsified in complete Freund's adjuvant (Difco, Detroit, MI). After 4 weeks the rat was boosted

i.p. with 1 x 10^7 cells, suspended in incomplete Freund's adjuvant. After four days the rat was sacrificed and the spleen was taken out aseptically. A spleen cell suspension was made in RPMI-1640 (Flow). The HAT-sensitive rat myeloma cell line Y3-Ag1.2.3 (abbr. Y3), used as fusion partner for hybridoma production (Galfre et al., 1979), was kindly donated by Dr. G. Galfre, MRC, Cambridge, UK. For fusion, Y3 cells were mixed with immune spleen cells at a ratio of 1:10. This mixture was washed three times with serum-free RPMI. Cell fusion was induced by addition of 1 ml 71% polyethylene glycol (PEG-4000; Merck, Darmstadt, FRG) in serum-free RPMI supplemented with 5% DMSO. After 2 min, the PEG was gradually diluted with 10 ml RPMI. Next, the cells were centrifuged (5 min, 50 x g) and resuspended in DMEM (α-modification) supplemented with 10% fetal calf serum (FCS), hybridoma growth factor (40 U/ml) as present in human endothelium culture supernatant (Aarden et al., 1985), hypoxanthine (10^{-4} M), azaserine (1 μg/ml), 2-mercaptoethanol (5 x 10^{-5} M) and antibiotics. The cells were plated in 24-well culture plates (Costar, Cambridge, MA) at a density of 3 x 10^5 cells / well. After selection, the ER-BMDM1 (Erasmus University Rotterdam - bone marrow derived macrophages) mAb secreting hybridoma was subcloned by limiting dilution. The secerned immunoglobulins appeared to be of the IgG2a subclass, as determined by standard Ouchterlony immunodiffusion using subclass-specific antisera from Nordic (Tilburg, The Netherlands) and Miles (Kankakee, IL).

Target cells for hybridoma screening. Monoclonal antibodies obtained from the fusion described above were screened in three models of macrophage differentiation for the recognition of differentiation-stage related antigens.

The first model comprised a panel of macrophage cell lines, ordered in a linear differentiation sequence. This cell line panel has been characterized extensively elsewhere (Leenen et al., 1986b).

A second model of macrophage differentiation was the comparison of immature and mature bone marrow-derived mononuclear phagocytes (BMDM), isolated from M-CSF stimulated bone marrow cultures, performed essentially according to van der Meer et al. (1983). Immature BMDM were isolated as non-adherent cells after 4 days of culture, whereas relatively mature BMDM were harvested after 7 days.

A third model of macrophage differentiation was provided by the induction of differentiation of the macrophage precursor hybrid R0C2 (Leenen et al, 1989d). Briefly, R0C2 cells, which showed macrophage precursor characteristics, were induced to express mature macrophage features by culture of the cells for 5 days in DMEM + 10% SERUM-PLUS™ (KC Biological, Lenexa, KS) supplemented with 1% post-endotoxin serum (PES) and interferon (IFN)-γ. For the production of PES, C57Bl/6J mice were injected i.v. with 400 μg Salmonella typhosa LPS-W (Difco Laboratories, Detroit, MI)

in PBS, and blood was collected 6 hr later by cardiac puncture of CO_2-anasthesized animals. Recombinant IFN-γ was applied as supernatant from transfected CHO-cells, kindly provided by Dr. Jan Trapman from the dept. Pathology in our faculty (Dijkmans et al., 1985).

Furthermore, ER-BMDM1 mAb was characterized by binding to several isolated macrophage populations. Resident peritoneal and alveolar macrophages were obtained by peritoneal and alveolar lavage, using serum-free DMEM and PBS supplemented with 0.6 mM EDTA, respectively. Different populations of peritoneal exudate cells were isolated at 8 hr and at 4 days after i.p. injection of 1 ml Brewer's thioglycollate medium (Difco Laboratories, Detroit). We obtained nucleated cells from peripheral blood after specific lysis of erythrocytes by 10 min incubation at 4°C in 17 mM Tris.HCl pH 7.2 containing 144 mM NH_4Cl. Lymph node and splenic dendritic cells were isolated as described by Breel et al. (1987).

ELISA. The expression of ER-BMDM1 antigen by macrophage cell lines and BMDM was quantitatively assessed by means of a fluorescence micro-ELISA on target cells coated to Terasaki trays using 0.05% glutaraldehyde (Leenen et al., 1985). Application of this low concentration of fixative has no deleterious influence on the detectability of any investigated cell surface antigens, including ER-BMDM1 antigen (Leenen et al., 1985). Briefly, cells were incubated with mAb for 1 hr, rinsed with PBS supplemented with Tween-20 (0.05% v/v; PBS-Tw), incubated for 1hr with optimally diluted anti-rat Ig-ß-galactosidase conjugate, rinsed again, and finally incubated for 1 hr at 37°C with the fluorogenic substrate 4-methylumbelliferyl galactopyranoside. The amount of fluorescent product was then measured using a scanning microfluorometer. We related the resulting fluorescence data to an internal standard (described below) in order to enable direct comparison of antigen expression by different cell types as well as comparison of data obtained in different experiments. Variations in cell density on different Terasaki trays were excluded by coating equal amounts of cells, measured as protein content, to the wells. An internal standard was obtained for each experiment by measuring the conjugate activity on a separate Terasaki tray, maximally coated with purified ER-BMDM1 mAb. By relating fluorescence data to this standard, inter-experimental variations in specific enzyme activity, conjugate dilution and amplification of fluorescence signal did not influence the final data. Thus, antigen expression is given in arbitrary units (AU), implying that for 100 AU a fluorescence signal was obtained that equalled the internal standard.

Immuno-ß-galactosidase and -peroxidase staining of cell suspensions. ER-BMDM1 antigen expression was assessed at the single cell level using an immuno-ß-galactosidase

or immunoperoxidase assay on cells coated to multi-well microscopic slides or on cytocentrifuge preparations, respectively. These assays were performed as described previously (Leenen et al., 1987; Breel et al., 1988b).

Immunohistology. Immunohistological characterization of ER-BMDM1 was performed on cryostat tissue sections of lymphoid and non-lymphoid organs, essentially as described by van Ewijk et al. (1988).

Antigen immunoprecipitation. The molecular weight of the antigen recognized by ER-BMDM1 mAb was determined by surface radio-iodination of cells, followed by specific immunoprecipitation and SDS-PAGE analysis, essentially as described by Pont et al. (1985).

Demonstration of aminopeptidase activity. Aminopeptidase activity was demonstrated in lysates from the mature macrophage cell line P388D1 as well as in lysates from small intestinal cells, which were obtained by scraping. After isolation, the cells were washed twice in serum-free DMEM and lysed in 1 or 2 ml 30 mM Tris.HCl pH 8.0, 1% Triton X-100, 150 mM NaCl for 30 min on ice. The lysates were cleared by centrifugation for 15 min at 15,000 x g. Antigens were specifically immunoprecipitated using mAb coupled to anti-rat kappa chain (MARK-1)-coated Sepharose-4B beads (Pont et al., 1985). Prior to immunoprecipitation, the lysates were incubated for 2 hr with plain MARK-1 beads to remove non-specifically binding proteins. The beads were washed three times in lysate buffer and incubated for 90 min with 0.21 mM leucyl-ß-naphthylamide as aminopeptidase substrate in 200 mM phosphate buffer, pH 8.0, supplemented with 0.2% Triton X-100. The reaction was stopped by adding 400 μl 1 M Na_2CO_3 to 10 μl reaction mixture. Generation of the product ß-naphthylamine was monitored directly on a fluorometer, using an excitation wavelength of 340 nm and an emission wavelength of 410 nm.

RESULTS

I. Biochemical characterization of the ER-BMDM1 antigen

The aim of the present study was the development of monoclonal antibodies (mAbs) against mature macrophages in order to get more insight in the functional and phenotypical heterogeneity of these cells. One of the obtained mAbs, ER-BMDM1, was found to recognize a cell surface antigen present on various macrophage subpopulations (see

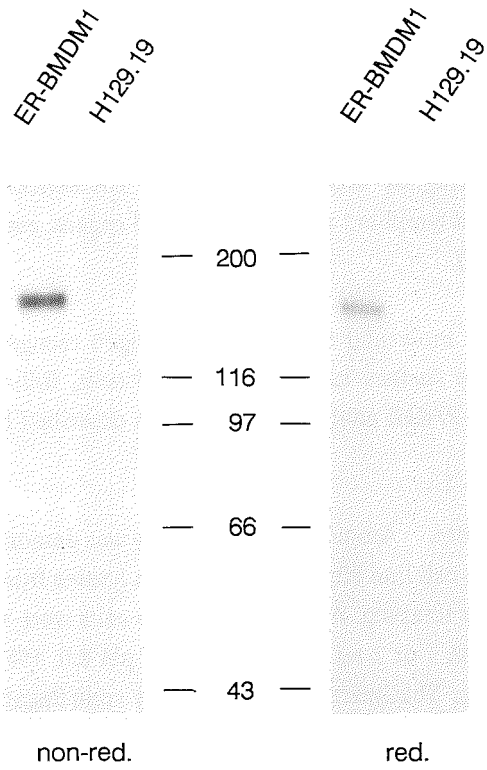

Figure 9.1.
ER-BMDM1 precipitates a 160 kDa antigen from the mature macrophage cell line WR19M.1. Cells were surface iodinated and lysed. ER-BMDM1 antigens were precipitated and run on a 10 % polyacrylamide gel under reducing and non-reducing conditions. mAb H129.19, with specificity for murine CD4, was used to detect non-specifically binding proteins.

below). Furthermore, the antigen appeared to be highly expressed on the microvillous brush border of the small intestine. These findings prompted us to investigate whether the antibody was directed against a proteolytic enzyme.

First, the molecular mass of the ER-BMDM1 antigen (Ag) was determined by immunoprecipitation from radio-iodinated WR19M.1 mature macrophage tumor cells. Figure 9.1 shows that ER-BMDM1 recognizes a polypeptide of approximately 160 kDa apparent molecular mass, both under reducing and non-reducing conditions. This indicates that the macrophage-derived ER-BMDM1 antigen consists of a single polypeptide chain.

Thereafter, we investigated whether ER-BMDM1 precipitated any proteolytic activity, using substrates for enzymes with a molecular mass of approximately 160 kDa. The results, shown in table 9.I, indicate that ER-BMDM1 Ag, isolated either from the P388D1 macrophage cell line or from small intestinal cells, exhibits leucine aminopeptidase activity. In contrast, isotype matched control mAbs did not precipitate any aminopeptidase activity. The ER-BMDM1 mAb is, most likely, directed against an epitope distinct from the catalytic site of the enzyme, since addition of the antibody to lysate from P388D1 cells did not influence the observed aminopeptidase activity.

Table 9.I. **Precipitation of aminopeptidase activity by ER-BMDM1**

	cells	sample	APA[a]
exp. 1	P388D1	untreated lysate (10^5 cells)	231
		ER-BMDM1 precipitate[b]	175
		H129.19 precipitate[b,c]	4
exp. 2	small intestine	untreated lysate	153
		ER-BMDM1 precipitate[b]	111
		NLDC-145 precipitate[b,c]	0
exp. 3	P388D1	untreated lysate (10^5 cells)	161
		lysate + ER-BMDM1[d]	163
		lysate + ER-MP20[d,c]	164

[a] APA = aminopeptidase activity determined as generation of fluorescent β-naphthylamine (emission at 410 nm) from L-leucyl-β-naphthyl amide; fluorescence is expressed in arbitrary units

[b] MARK-1-coupled Sepharose-4B beads (40 μl wet volume), to which rat mAb was linked, were incubated with excess cell lysate, washed and assayed for precipitated aminopeptidase activity

[c] H129.19, NLDC-145 and ER-MP20 served as IgG2a-isotype matched control mAbs

[d] mAb was added as 5 μl ascites (1:1 diluted in PBS) per 50 μl lysate (corresponding to 10^6 cells); after 30 min incubation on ice, an aliquot from this mixture corresponding to 10^5 cells was taken for determination of aminopeptidase activity

II. Distribution of ER-BMDM1 aminopeptidase in situ

To investigate the ER-BMDM1 specificity for different cell populations in situ, we examined its reactivity on frozen tissue sections of lymphoid and non-lymphoid organs.

Lymphoid organs

Spleen In the splenic white pulp different ER-BMDM1 positive cell types can be discerned (fig. 9.2a). Strongly labeled cells surround the central arteriole. Such cells are presumably macrophages (Hume et al., 1983). In the T cell area (peri-arteriolar lymphocyte sheath, PALS), interdigitating cells (IDC) are moderately stained, whereas scattered strongly positive cells most likely represent PALS-macrophages. Furthermore, scattered irregularly shaped cells are present at the inner border of the marginal sinus (fig. 9.2b) and scarcely in B cell areas (fig. 9.2a). Immunofluorescence double labeling (not shown) revealed that ER-BMDM1 co-localized with the interdigitating cell mar-

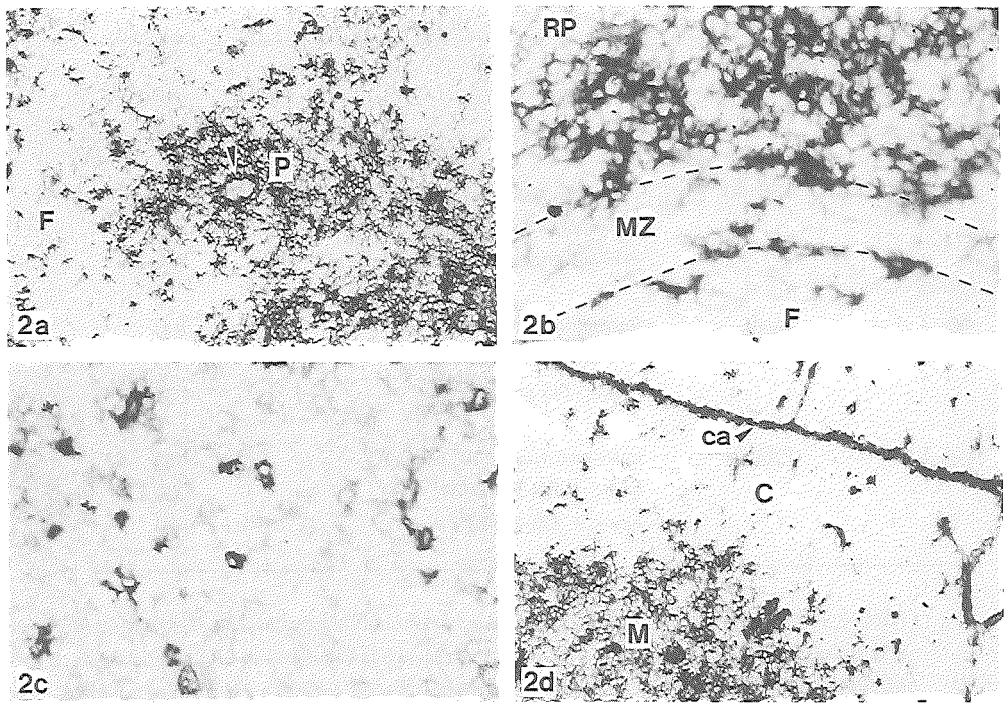

Figure 9.2.

ER-BMDM1 recognizes interdigitating cells as well as several macrophage subpopulations in frozen sections of murine spleen and thymus. **(a).** IDC and PALS-macrophages in the splenic white pulp. The arrow indicates the location of the central arteriole; P = periarteriolar lymphocyte sheath; F = follicle. Original magnification x 125. **(b).** A rim of positive cells in the splenic red pulp lines the marginal zone discontinuously. We propose the designation "marginal red pulp macrophages" for these cells. RP = red pulp; MZ = marginal zone; F = follicle. Original magnification x 310. **(c).** A subpopulation of classical splenic red pulp macrophages. Original magnification x 310. **(d).** In the thymus, ER-BMDM1 positive macrophages are integrated in the capsule and present in the cortex, cortico-medullary junction, and mainly associated with blood vessels in the medulla. Also medullary IDC are ER-BMDM1 positive. ca = capsule; C = cortex; M = medulla. Original magnification x 125.

ker NLDC-145 in the PALS, except for the strongly ER-BMDM1 positive PALS-macrophages. Furthermore, ER-BMDM1 positive cells along the marginal sinus appeared to be distinct from the MOMA-1 positive marginal metallophilic macrophages. In the splenic red pulp, two ER-BMDM1 positive cell types are present. Firstly, patches of irregularly shaped cells are observed at the periphery of the marginal zone (fig. 9.2b). In addition to ER-BMDM1 antigen, these cells express BM8, F4/80, MOMA-2, and Mac-2 antigens as well as a low level of acid phosphatase, but no detectable non-specific esterase activity (de Jong et al., 1989c). Based on the cellular morphology, phenotype

and localization, we propose to designate these cells as "marginal red pulp macrophages". The second population of ER-BMDM1 positive cells in the red pulp is a subpopulation of "classical" red pulp macrophages, located deeper in the red pulp (fig. 9.2c).

Thymus ER-BMDM1 positive cells are situated in virtually all thymic compartments (fig. 9.2d). Adjacent to the inner side of the capsule and lining the connective tissue septa, a strong continuous staining pattern is observed. Most likely, macrophages, which are known to be located in this area (Hume et al., 1983), are ER-BMDM1 positive. In addition, reaction product appears to occur unassociated with particular cell surfaces, suggesting secretion of the ER-BMDM1 antigen. In the cortex and corticomedullary junction, ER-BMDM1 stains macrophages. However, not all acid phosphatase positive macrophages are recognized by ER-BMDM1 (data not shown). Furthermore, positivity is observed in association with cortical and medullary capillaries. A fine reticular pattern can be seen in the thymic medulla caused by staining of interdigitating cells. Medullary lymphocytes are not stained by ER-BMDM1, since virtually no positive cells were observed in a suspension of thymocytes, isolated over a nylon sieve (data not shown). Hence, ER-BMDM1 positive cells are most likely integrated within the thymic stroma.

Mesenteric lymph node Similar cell types are stained in the mesenteric lymph node as observed in spleen and thymus. Stretched macrophages, at the inner side of the capsule are clearly ER-BMDM1 positive (fig. 9.3a). In the B cell follicles, only a few irregularly shaped cells, distinct from tingible body macrophages, are labeled by the

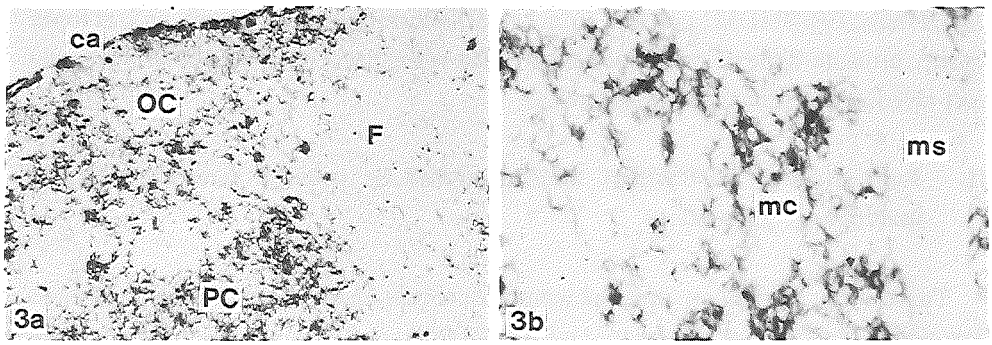

Figure 9.3.

ER-BMDM1 positive macrophages and IDC in the mesenteric lymph node. (a). The cortical area shows positive macrophages in the capsule, positive macrophages mainly outside the B cell follicles as well as positive IDC in the paracortex. ca = capsule; OC = outer cortex; PC = paracortex; F = follicle. Original magnification x 125. (b). In the medulla, macrophages in the medullary cords (mc) are ER-BMDM1 positive. Note that medullary sinus (ms) macrophages do not express ER-BMDM1 Ag. Original magnification x 310.

mAb. Furthermore, a rim of ER-BMDM1 positive cells surrounds the follicles at the paracortical side. ER-BMDM1 positive interdigitating cells are easily discerned in the paracortex (T cell area). In the lymph node medulla (fig. 9.3b), ER-BMDM1 positive macrophages are located within the medullary cords. Apparently, medullary sinus macrophages are not recognized by ER-BMDM1. Sinus macrophages highly express acid phosphatase activity, whereas ER-BMDM1 positive cord macrophages are only weakly acid phosphatase positive (not shown).

Peyer's patches Staining with ER-BMDM1 in the Peyer's patches is primarily associated with the interdigitating cells in the interfollicular T cell area (not shown). Furthermore, some scattered non-lymphoid cells in the follicles are ER-BMDM1 positive.

Non-lymphoid organs

Intestine A clear distinction is found between the small and large intestine, with regard to binding of ER-BMDM1. In the small intestine, villous epithelial cells are heavily labeled, especially at the brush border (fig. 9.4a). The lamina propria in the villi contains only a few scattered positive cells. In contrast, epithelial cells in the large intestine are ER-BMDM1 negative, whereas the numerous macrophages in the lamina propria are easily distinguished (fig. 9.4b).

Liver Strong ER-BMDM1 staining in the liver appears to be associated with the bile capillaries (fig. 9.4c). However, acid phosphatase positive Kupffer cells are ER-BMDM1 negative (not shown).

Lung In the lung, two ER-BMDM1 positive cell types can be distinguished (fig. 9.4d). Firstly, ER-BMDM1 positive, stretched cells surround blood vessels, and, at lower density, bronchi. Type II pneumocytes are also recognized by ER-BMDM1, whereas most alveolar macrophages are negative.

III. Expression of ER-BMDM1 aminopeptidase by isolated dendritic cell and macrophage populations

To confirm the notion that, in lymphoid organs, both macrophages and interdigitating cells (IDC) are recognized by ER-BMDM1, we stained the in vitro equivalents of IDC, dendritic cells, as well as various isolated macrophage populations.

Figure 9.5 shows that lymph node dendritic cells - identified by positive NLDC-145 staining and acid phosphatase activity located in a cytoplasmic spot - are ER-BMDM1 positive. When isolated from the spleen, approximately 50% of the NLDC-145 positive dendritic cells stain with ER-BMDM1 (data not shown).

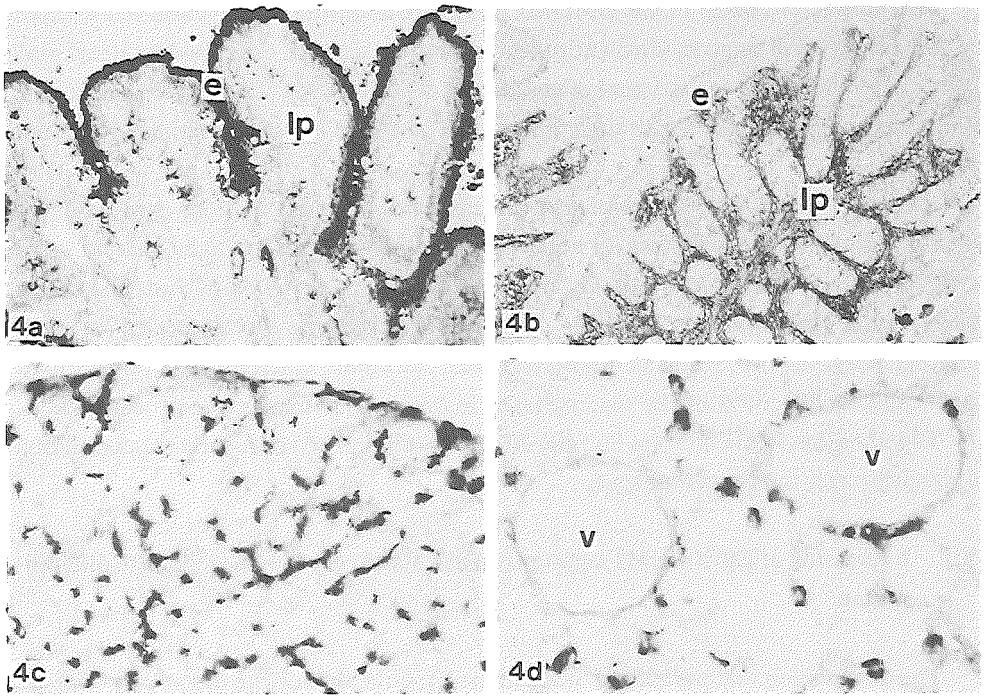

Figure 9.4.

ER-BMDM1 aminopeptidase is present in the brush border of small intestine, but not in large intestine. In addition, microvilli in liver and lung are ER-BMDM1 positive. (a). Small intestinal villous epithelium. Note that crypts are virtually negative. e = epithelium; lp = lamina propria. Original magnification x 125. **(b).** Large intestinal epithelium is ER-BMDM1 negative. Macrophages in the lamina propria are clearly positive. e = epithelium; lp = lamina propria. Original magnification x 125. **(c).** ER-BMDM1 staining of bile capillaries in the liver. Original magnification x 310. **(d).** In the lung, ER-BMDM1 stains type II pneumocytes as well as stretched cells, presumably macrophages, that surround blood vessels and bronchi. v = venule. Original magnification x 310.

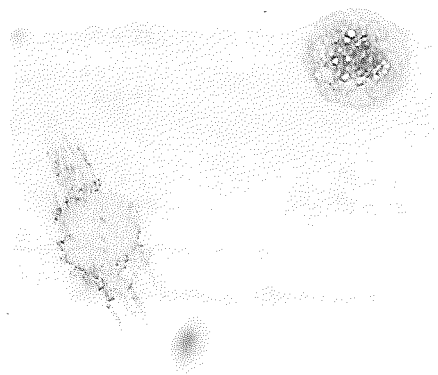

Figure 9.5.

Isolated lymph node dendritic cells are recognized by ER-BMDM1. DC were isolated, spun down on cytocentrifuge slides and detected using immunoperoxidase staining for ER-BMDM1 and endogenous acid phosphatase staining. Original magnification x 630.

Potential interference of ER-BMDM1 mAb with dendritic cell function was assayed by addition of mAb in an in vitro antigen-presentation assay, as described by Breel etal. (1988c). Under these experimental conditions, ER-BMDM1 exerted no significant effects, whereas anti-Ia mAb strongly inhibited T cell proliferative responses (data not shown).

Binding of ER-BMDM1 to various isolated macrophage populations is represented in table 9.II. Clearly, monocytes do not express the ER-BMDM1 antigen, nor do other peripheral blood cells. In contrast, virtually all thioglycollate elicited peritoneal exudate macrophages are strongly positive. ER-BMDM1 labeling demonstrates a clear heterogeneity in the resident peritoneal and alveolar macrophages populations: approximately 20 - 30% of the macrophages in both populations express this marker.

Together these data indicate that ER-BMDM1 recognizes a particular subpopulation of dendritic cells and macrophages.

IV. Expression of ER-BMDM1 aminopeptidase in the course of macrophage differentiation

The observation that peripheral blood monocytes, the immediate precursors of tissue macrophages, did not express the ER-BMDM1 antigen, prompted us to investigate the onset of ER-BMDM1 aminopeptidase expression in macrophage differentiation in more

Table 9.II **ER-BMDM1 binding to various mononuclear phagocyte populations as determined by immuno-β-galactosidase assay**

cell type	% macrophages	% ER-BMDM1 positive
peripheral blood nucleated cells	4 ± 2[a]	0
resident peritoneal cells	53 ± 15[a]	15 ± 7
alveolar lavage cells	74 ± 7[b]	14 ± 8
thioglycollate elicited peritoneal exudate cells		
8 hr after injection	4 ± 3[a]	1 ± 1
4 d after injection	87 ± 8[b]	82 ± 6

Percentages of positive cells are determined in at least 3 assays and expressed ± standard deviation

[a] based on positive staining for F4/80 Ag

[b] based on positive staining for non-specific esterase, using naphthyl acetate as substrate

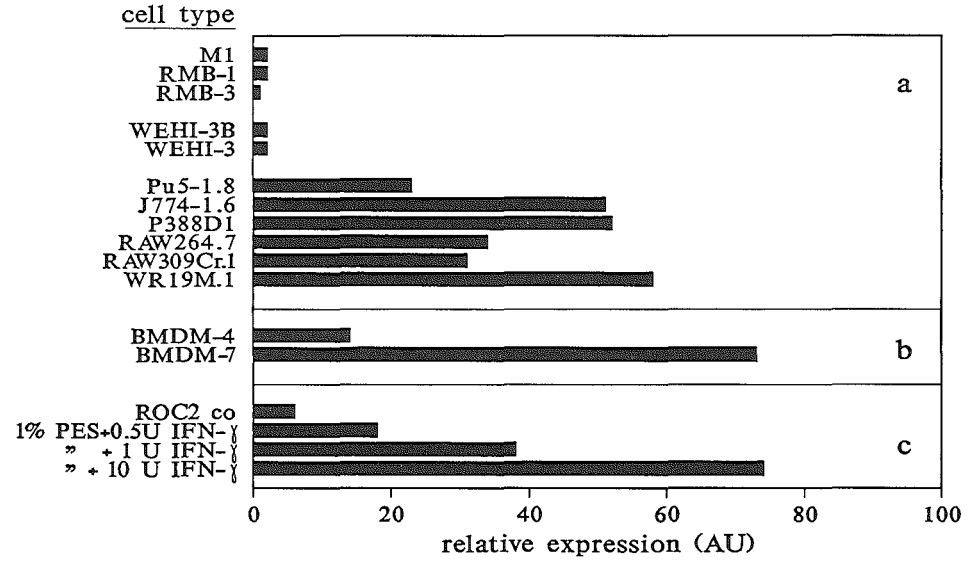

Figure 9.6.

ER-BMDM1 aminopeptidase is increasingly expressed in the course of macrophage differentiation.

(a). A panel of macrophage cell lines ordered in a linear differentiation sequence: macrophage precursor (M1, RMB-1, RMB-3), immature macrophage (WEHI-3B, WEHI-3) and mature macrophage cell lines (Pu5-1.8, J774-1.6, P388D1, RAW264.7, RAW309Cr.1, WR19M.1). (b). Immature non-adherent macrophage precursor cells, isolated after 4 days of M-CSF stimulated bone marrow culture (BMDM-4) are compared to mature adherent bone marrow derived macrophage, isolated after 7 days of culture (BMDM-7). (c). Induction of differentiation in the macrophage precursor hybrid R0C2 by culture of the cells during 5 days in the presence of 1% post-endotoxin serum supplemented with 0.5 U, 1 U or 10 U IFN-γ/ml.
The level of antibody binding is determined by ELISA and expressed in arbitrary units (AU), relative to the internal standard (see Materials and Methods).

detail.

Screening of ER-BMDM1 reactivity on a panel of macrophage cell lines - arrested in distinct, characterized stages of differentiation (Leenen et al., 1986b) - revealed that only cell lines with mature macrophage characteristics were recognized (fig. 9.6a). In contrast, macrophage precursor and immature macrophage cell lines did not bind ER-BMDM1.

M-CSF stimulated bone marrow cultures, which were harvested at different time points, provided a second model of macrophage differentiation. Non-adherent cells, isolated after 4 days of culture (BMDM-4), consist of macrophage precursors and polymorphonuclear cells in approximately equal amounts. After 7 days of culture, most cells from M-CSF stimulated bone marrow cultures exhibit mature macrophage features

(BMDM-7). Clearly, BMDM-7 strongly expressed the ER-BMDM1 antigen, whereas binding was hardly detectable on BMDM-4 (fig. 9.6b).

A third model of macrophage differentiation was provided by the induction of differentiation in the macrophage precursor cell line R0C2 using post-endotoxin serum supplemented with different concentrations of interferon-γ (Leenen et al., 1989d). Control R0C2 cells hardly expressed the ER-BMDM1 antigen, whereas cells cultured with increasing doses of differentiation inducing agent, increasingly expressed the ER-BMDM1 antigen (fig. 9.6c).

Taking these results together, we conclude that ER-BMDM1 aminopeptidase is increasingly expressed beyond the monocytic stage of macrophage differentiation in a strict maturation-related manner.

DISCUSSION

The initial purpose of the present study was the development of monoclonal antibodies against mature stages of macrophage differentiation. One specifically interesting antibody, ER-BMDM1, is shown in this paper. The data indicate that ER-BMDM1 binds to an aminopeptidase on the cell surface of macrophages and dendritic cells. In various differentiation models, ER-BMDM1 recognizes an antigen that is increasingly expressed upon maturation of murine macrophages. Expression of the ER-BMDM1 Ag arises after the monocytic stage.

Phenotypical analysis of macrophages in various isolated populations revealed that ER-BMDM1 recognizes a subpopulation of mature macrophages: virtually all peritoneal macrophages elicited by thioglycollate are ER-BMDM1 positive, but only about 20 - 30% of the resident peritoneal and resident alveolar macrophage populations expresses the ER-BMDM1 aminopeptidase. These observations can be interpreted in different ways:

1. ER-BMDM1 positive macrophages in resident populations represent a separate macrophage differentiation lineage, or alternatively
2. ER-BMDM1 Ag is expressed by macrophages in particular differentiation or activation stages.

Previously, similar heterogeneity of peritoneal macrophages has been reported with respect to the expression of other markers, such as endogenous peroxidase activity (Hoefsmit et al., 1986), 5'-nucleotidase activity (Bursuker and Goldman, 1979), or concanavalin A binding (de Water et al., 1982). Also for the latter markers, however, it is not yet established whether the observed macrophage heterogeneity is to be ex-

plained in terms of various differentiation / activation stages or of separate differentiation lineages.

Also in lymphoid organs, only a subpopulation of macrophages is recognized by ER-BMDM1. For instance, in the mesenteric lymph node, medullary cord macrophages clearly bind ER-BMDM1, whereas medullary sinus macrophages are ER-BMDM1 negative. Furthermore, in the spleen a characteristic subpopulation of red pulp macrophages, located in foci at the periphery of the marginal zone, is recognized by ER-BMDM1. Based on the simultaneous expression of the macrophage antigens recognized by BM8 (Malorny et al., 1986), F4/80 (Austyn et al., 1981), MOMA-2 (Kraal et al., 1987), and M3/38 (Ho et al., 1982), these cells undoubtedly belong to the mononuclear phagocyte system. Since these cells have not been recognized as a distinct subpopulation previously, we propose the designation "marginal red pulp macrophages" for these cells. The strategic localization of this macrophage subpopulation at the junction of the immunological and hemopoietic compartments of the spleen points to an important - yet unknown - function of these cells.

Interdigitating cells (IDC) occurring in T-cell areas in lymphoid organs are also recognized by ER-BMDM1. The in vitro equivalents of IDC, dendritic cells (DC; Breel et al., 1987), have been shown to play a principal role as accessory cells in primary T-cell dependent immune responses (reviewed by Austyn, 1987). Staining of isolated DC revealed that virtually all lymph node DC are ER-BMDM1 positive, whereas only around 50% of the splenic NLDC-145 positive DC are recognized. This staining pattern confirms the notion that DC from different organs exhibit phenotypical heterogeneity (Crowley et al., 1989).

In general, DC and macrophages show a considerable phenotypical overlap. DC express, though in varying amounts, several markers characteristic for macrophages, such as MOMA-2 Ag and F4/80 Ag (Kraal et al., 1987; Crowley et al., 1989). Conversely, macrophages may express markers which are characteristic for DC. Breel et al. (1988b) showed the expression of the DC Ag NLDC-145 by a subpopulation of alveolar macrophages. Our present finding, that - within the hemopoietic lineage - only DC/IDC and a macrophage subpopulation express the ER-BMDM1 Ag, contributes to the phenotypical evidence of a close relationship between these cell types. Interestingly, the only immunohistological staining with the anti-DC antibody 33D1 is found in foci of non-lymphoid cells at the border area between splenic red and white pulp (Witmer et al., 1984). Exactly in this area the marginal red pulp macrophage subpopulation is located. Because of the similarity in staining pattern, it is tempting to speculate that 33D1 and ER-BMDM1 recognize the same marginal red pulp macrophage population, which might represent an intermediate cell type between macrophagess and dendritic cells.

In non-lymphoid organs, the staining pattern of ER-BMDM1 strongly correlates to

the presence of microvilli: the small intestinal brush border, bile capillaries in the liver, type II pneumocytes in the lung as well as tubules in the kidney are stained by ER-BMDM1. This specific association with microvilli suggests that ER-BMDM1 recognizes a proteolytic enzyme. We substantiated this notion by the demonstration of aminopeptidase activity specifically associated with immunoprecipitated ER-BMDM1 Ag. Although the nomenclature concerning aminopeptidases is not clear at present, we assume that ER-BMDM1 Ag is identical to leucine aminopeptidase, as described by Wachsmuth and Wüst (1982) or aminopeptidase N, as described by Bowes and Kenny (1987). We base this assumption on the suggested linkage between macrophage differentiation stage and expression of leucine aminopeptidase (Wachsmuth, 1975) as well as on the similarity in tissue distribution of aminopeptidase N and the present ER-BMDM1 aminopeptidase (Bowes and Kenny, 1987).

Recently, it was recognized that aminopeptidase N is identical to the human CD13 antigen (Look et al., 1989). The similarity in both molecular mass and enzyme activity suggests that the antigen recognized by ER-BMDM1 is the murine homologue of human CD13. However, CD13 is expressed by immature human myeloid cells and granulocytes (Griffin et al., 1983), in contrast to the murine ER-BMDM1 Ag. Additional studies are in progress to investigate the putative homology between the ER-BMDM1 aminopeptidase and CD13.

Macrophages are well-known producers of a large variety of proteolytic enzymes like the present aminopeptidase (Nathan, 1987). Most macrophage proteolytic enzymes are involved in digestion of endocytosed material. However, the specific association of aminopeptidase N with the macrophage cell surface - instead of the lysosomal compartment - suggests a role in the "communication" of the macrophage with its environment, rather than a digestive function of the enzyme. In this respect, it has been reported that aminopeptidase N is involved in the inactivation of peptide hormones, such as the immunostimulatory tetrapeptide tuftsin (Mathe, 1987). Thus, cells expressing aminopeptidase may have a protective function against exaggerated hormonal stimulation of the immune system. A confirmation of this putative "surveyor" function can be found in the preferential location of ER-BMDM1 positive macrophages at sites of entry in lymphoid organs, e.g. associated with capillaries and beneath the thymic capsule.
A specific function for ER-BMDM1 aminopeptidase on DC remains more speculative. Similar to macrophages, DC aminopeptidase may play a role in hormonal regulation of immune responses. On the other hand, it may be envisaged that DC aminopeptidase is involved in the accurate sizing of peptides, which subsequently become associated with class II MHC antigens and are presented to T cells (Brown et al., 1988). Our finding that ER-BMDM1 mAb does not inhibit the antigen-presenting capacity of isolated DC seemingly argues against such an antigen sizing function of aminopeptidase on DC. In

this respect, however, it must be realized that ER-BMDM1 mAb is not inhibitory for aminopeptidase activity. Hence, a role for ER-BMDM1 aminopeptidase in the DC antigen presenting function remains possible.

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CHAPTER 10

IMMUNOPHENOTYPICAL CHARACTERIZATION OF MACROPHAGE AND DENDROCYTE SUBPOPULATIONS IN THE MURINE SPLEEN

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Submitted for publication.

SUMMARY

In this study we utilized a number of macrophage and dendritic cell-specific monoclonal antibodies to unravel the macrophage and dendrocyte heterogeneity in the murine spleen. We demonstrate that these non-lymphoid cells exhibit unique, characteristic phenotypes and that phenotypically distinct cells occupy distinct microanatomical sites. These phenotypical differences are suggestive of a functional diversity of the various subpopulations. Furthermore, we observed a considerable overlap in marker expression between macrophages and dendrocytes. Based on these findings, we argue that macrophages and dendrocytes belong to the same hemopoietic lineage.

INTRODUCTION

Recent studies indicate that macrophages and dendrocytes - i.e. Langerhans cells, veiled cells and interdigitating or dendritic cells (Austyn, 1987) - occupy specific anatomical locations within the various peripheral lymphoid organs (Veerman and van Ewijk, 1975; Witmer and Steinman, 1984; Buckley et al., 1987). This non-random spatial distribution is most likely indicative of a functional specialization of the various subpopulations.

Particularly, the spleen has been chosen by many investigators as a model to study this assumption (Claassen et al., 1986, Groeneveld et al., 1986; van Vliet et al., 1985). This organ contains many macrophages and is well compartmentalized (Veerman and van Ewijk, 1975; van Ewijk and Nieuwenhuis, 1985). The splenic red pulp is densely occupied with stromal macrophages. Part of these cells function as scavengers in the bloodstream (Hume et al., 1983; Gordon et al., 1988). Other red pulp macrophages perform essential functions as central macrophages in hemopoietic islands (Crocker et al., 1988) or as regulators of cellular migration (Weiss and Sakai, 1984).

Like the macrophages of the red pulp, the splenic white pulp macrophages may also vary in their function when present at distinct locations. For example, tingible body macrophages, present in the germinal centers, are thought to be involved in the elimination of aberrantly programmed B lymphocytes (Nieuwenhuis and Opstelten, 1984). Furthermore, marginal zone macrophages are highly phagocytic (Matsuno et al., 1986) and were initially thought to play a specific role in presenting thymus-independent type 2 antigens (TI-2) to specific B cells located in the marginal zone (Humphrey and Grennan, 1981; van Vliet et al., 1985). However, it has recently been demonstrated that specific elimination of marginal zone macrophages did not influence TI-2 immune responses (Kraal et al., 1989). Marginal metallophils, at the border of the marginal sinus

and the periarterial lymphatic sheath (PALS), are likely involved in antigen presentation (Kraal and Janse, 1986; Kraal et al., 1988). These cells may also direct lymphocyte traffic through the spleen (Brelinska and Pilgrim, 1982).

Dendrocytes also vary in their function depending on their localization in distinct compartments. In the PALS, interdigitating cells (IDC) reside (Veldman, 1970), which in vitro give rise to the dendritic cells (DC) (Breel et al., 1987; Austyn, 1987). These cells are specialized as accessory cells for induction of immune responses in resting T cells.

Taken together, these data indicate that macrophages and dendrocytes play pivotal roles in various processes taking place in the spleen. Most important, these accessory functions appear to be related to particular macrophage and dendrocyte subpopulations present at specific lymphopoietic and hemopoietic microenvironments. Unraveling the accessory cell heterogeneity in the spleen thus aids to gain insight in the different functions performed by these cells in situ.

Recently, we extended the available panel of monoclonal antibodies (mAbs) against DC and macrophage antigens (de Jong et al., 1989a, Leenen et al., 1989a; 1989c). Moreover, we developed an improved fixation procedure to detect cellular determinants in frozen tissue sections (de Jong et al., 1989b). These new mAbs and fixation procedure are applied in the present paper to study the heterogeneity of the mononuclear phagocytes in the spleen and to further elucidate the relation between macrophages and dendrocytes.

MATERIALS AND METHODS

Mice. Male and female BALB/c mice were bred at our facility, and kept under conventional conditions. Mice were sacrificed by exposure to carbon dioxide and the spleen was removed.

Monoclonal antibodies. Monoclonal antibodies used in the present study and their specificities and references are given in table 10.I. MAbs were applied either as undiluted hybridoma culture supernatants or as optimally diluted purified antibody.

Tissue preparation and immunostaining. Organs were embedded in Tissue-Tek II (Miles Laboratories, Inc., Kankakee, IL, USA) on a specimen stub and frozen immediately. Five μm sections were cut on a cryostat (model 1720, Leitz, FRG) and collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium potassium sulphate. Slides were air-dried and used immediately or

Table 10.I. Specificities of the monoclonal antibodies (mAbs) used for antigen detection

MAb	Antigen	Specificity	References
BM8	BM8 Ag	Mϕ	Malorny et al., 1986
ER-BMDM1	Mϕ amino-peptidase	Mϕ, dendrocytes, microvilli	Leenen et al., 1989a
ER-HR3	ER-HR3 Ag	Mϕ	de Jong et al., 1987; 1989a
ER-MP23	ER-MP23 Ag	Mϕ	Leenen et al., 1989c
ER-TR9	ER-TR9 Ag	Mϕ	van Vliet et al., 1985
F4/80	F4/80 Ag	Mϕ	Austyn and Gordon, 1981
MIDC-8	MIDC-8 Ag	dendrocytes	Breel et al., 1987
MOMA-1	MOMA-1 Ag	Mϕ	Kraal and Janse, 1986
MOMA-2	MOMA-2 Ag	Mϕ	Kraal et al., 1987
Monts-4	Monts-4 Ag	Mϕ, IDC	Jutila (pers. commun.)
M1/70	Mac-1, CR3	non-fixed Mϕ, granulocytes, NK cells, DC	Springer et al.,1979
M3/38	Mac-2	Mϕ, dendrocytes, epithelium	Ho and Springer, 1982 Flotte et al., 1983
M5/114	H-2 Ia	immunologically reactive cells	Bhattacharya et al., 1981
NLDC-145	NLDC-145 Ag	dendrocytes	Kraal et al., 1986 Breel et al., 1987

abbreviations:
CR3 - complement receptor type 3; DC - dendritic cells; IDC - interdigitating cells; Mϕ - macrophages

stored at -20 °C. Prior to use, sections were fixed for 2 min. in hexazotized pararosanilin (de Jong et al., 1989b) prepared as follows: one volume of a 4% (w/v) pararosanilin solution in 2M HCl was added to one volume of 4% (w/v) $NaNO_2$ in distilled water. From this mixture 0.8 ml was immediately diluted with 10 ml distilled water.

After fixation, sections were washed with phosphate-buffered saline, pH 7.8 (PBS) supplemented with 0.05% (v/v) Tween-20 (PBS-Tw). The sections were overlaid with 70 µl of the first stage monoclonal antibody and incubated for 1h at room temperature. During incubations the sections were kept in a moist chamber to prevent air drying. After rinsing the sections with PBS-Tw, they were overlaid with 70µl of optimally diluted rabbit-anti-rat Ig conjugated to horseradish peroxidase (DAKOPATTS, Copenhagen, Denmark) and incubated for 1h at room temperature. Normal mouse serum (2%; v/v) was added to avoid non-specific binding of the conjugate. Sections were rinsed with PBS-

Tw and binding of the conjugate was visualized by incubation with a diaminobenzidine (Sigma, St. Louis, USA) solution (1 mg DAB/ml PBS supplemented with 20 µl 1% (v/v) H_2O_2). The sections were briefly counterstained with hematoxylin and embedded in Entallan mounting medium (Merck, FRG) after dehydration and cover-slipped.

Enzyme histochemistry. Acid phosphatase and non-specific esterase activities were demonstrated as described by Burnstone (1962) with Naphthol-AS-BI phosphate (Sigma) and α-naphthyl acetate (Sigma) as substrates, respectively. In both procedures hexazotized pararosanilin was used as diazonium salt.

RESULTS

In order to facilitate the identification of distinct macrophage and dendrocyte subpopulations, we defined a number of microanatomical sites at which these cell types are present. This definition is primarily based on the compartmentalization of the spleen as described previously (van Ewijk and Nieuwenhuis, 1985).

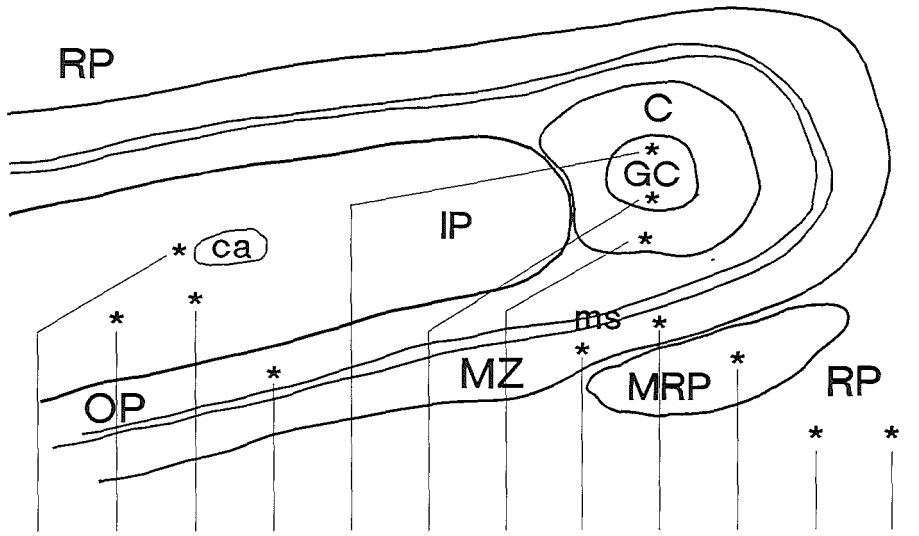
In this study, the location of the various subpopulations of macrophages and dendrocytes in four splenic compartments - i.e. PALS, follicle, marginal zone and red pulp - is described. The localizations and complete phenotypes of the distinctive mononuclear phagocyte subpopulations are presented in figure 10.1. Individual subpopulations are shown in the figures 10.2 and 10.3 by immunostaining with a characteristic mAb.

Localization of macrophages and dendrocytes in the splenic PALS

In close association with the central arteriole, 'peri-arteriolar macrophages' are recognized by mAb ER-BMDM1 (fig. 10.2a). Furthermore, two other non-lymphoid cell types in the inner PALS are recognized, i.e. the NLDC-145-positive interdigitating cells (fig. 10.2b) and the MOMA-2-positive macrophages (fig. 10.2c). In the outer PALS, strongly non-specific esterase-positive marginal metallophils are the predominant non-lymphoid cell type. They are located at the follicular site of the marginal sinus and merge between the coronal B lymphocytes. Marginal metallophils are specifically stained by mAb MOMA-1 (fig. 10.2d).

Localization of macrophages and dendrocytes in the splenic follicle

The large tingible body macrophages of the germinal centre are easily recognized by their content of effete lymphocytes. Moreover, these cells stain clearly with mAb



mAb												
NLDC-145	-	+	-	-	-/±?	-	-	+	-	-	-	+
MIDC-8	-	+	-	-	-	-	-	±	-	-	-	+
ER-BMDM1	++	+	++	-?	-	+	+	+	-?	+	s++	?
ER-TR9	-	-	-	-	-	-	-	-	+	-	-/s+?	-
Monts-4	-	+	+	++	+	+	+	+?	++	+	±	?
ER-MP23	-	-	-	-	-	-	-/s+?	-	-	-	-/s+?	-
M5/114	±	+	+	+	+	+?	+?	+	-	?	s±	+
M1/70	-	-	-	-	-	-	-	-	s±	-	-	-
M3/38	+	+	+?	+	?	+	+	?	+	+	+	?
MOMA-1	-	-	-	++	-	-	?	?	-	-	±	?
MOMA-2	±	-	+	+	+	+?	?	?	-	+	+	?
BM8	-	-	-	-	-	-	-	-	-	+	++	?
F4/80	-	-	-	-	-	-	-	-	-	+	+	?
ER-HR3	-	-	-	-	-	-	-	-	-	?	s++	?
Enzyme												
NSE	-	-	±	++	+	±	±	?	±	-	s±	-
AcPh	s±?	+	+	+	+	+	+	?	-	±	s±	?

Figure 10.1.

Localization and phenotype of the macrophages and dendrocytes present at specific microanatomical sites. Summary of phenotypical data. ++ - strongly positive; + - moderately positive; ± - weakly positive; s - subpopulation; ? - difficult to determine conclusively. Abbreviations: ca - central arteriole; IP - inner PALS; OP -outer PALS; C- corona; GC - germinal centre; ms - marginal sinus; MZ - marginal zone; MRP - marginal red pulp; RP - red pulp; PA Mϕ - peri-arteriolar macrophages; IDC - interdigitating cells; PALS Mϕ - Mϕ located in the peri-arteriolar lymphocyte sheath; MM - marginal metallophils; TGB - tingible body Mϕ; DDC - dendrocytes; NSE - non-specific esterase; AcPh - acid phosphatase.

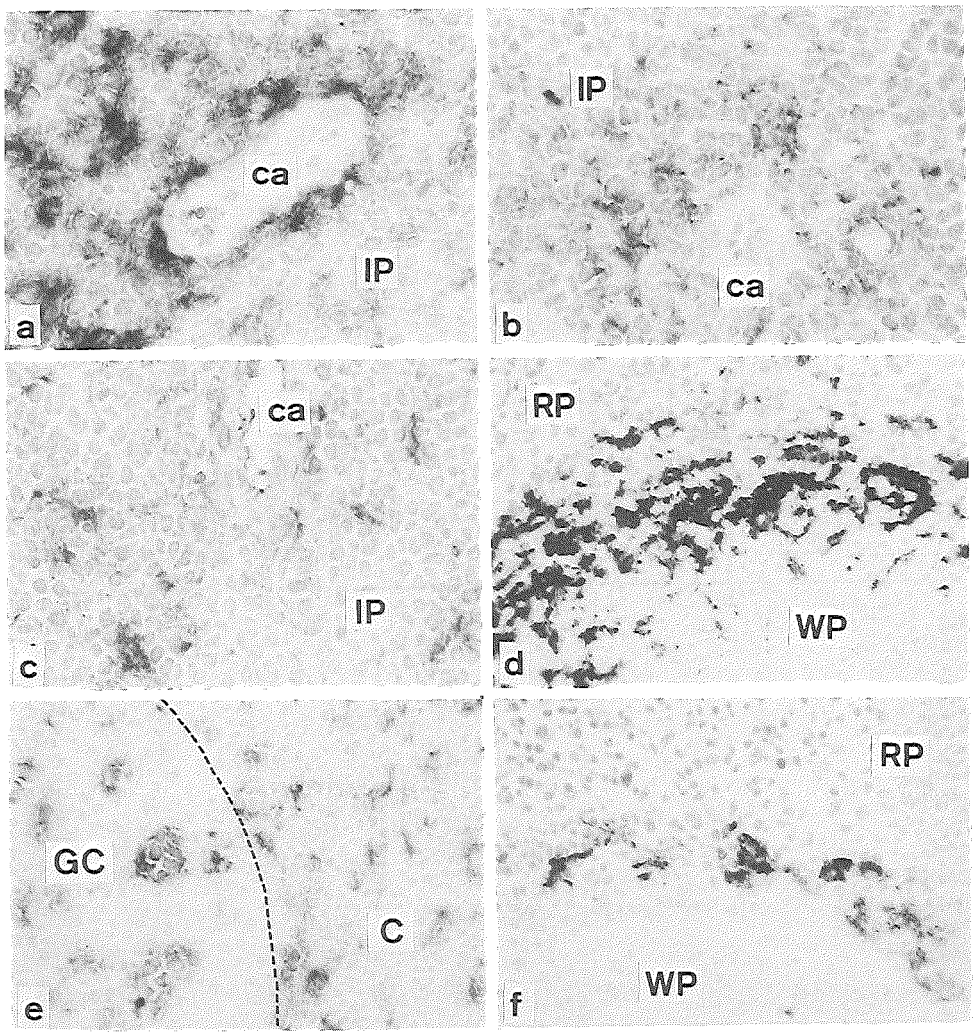


Figure 10.2.

Macrophage and dendrocyte subpopulations in the splenic white pulp. (a) peri-arteriolar macrophages stained with mAb ER-BMDM1, (b) IDC stained with mAb NLDC-145, (c) PALS macrophages stained with mAb MOMA-2, (d) marginal metallophils stained with mAb MOMA-1, (e) tingible body macrophages, follicular macrophages and corona macrophages stained with mAb Monts-4, (f) marginal zone macrophages stained with mAb ER-TR9. Original magnifications x 320.

Abbreviations: ca - central arteriole; IP - inner PALS; WP - white pulp; RP - red pulp; GC - germinal centre; C - corona.

Monts-4 (fig. 10.2e). Another macrophage population in the germinal centre consisting of smaller cells is also recognized by this mAb. Cells of the latter population are acid

phosphatase-positive and termed follicular macrophages. The coronal macrophages, present within the surrounding B cell corona exhibit a comparable phenotype, which also includes the Monts-4 marker (fig. 10.2e).

Localization of macrophages and dendrocytes in the marginal zone

The very large marginal zone macrophages, located at the red pulp-side of the marginal sinus are specifically stained with mAb ER-TR9 (fig. 10.2f). In addition to these cells, a small NLDC-145-positive population of cells is observed which are referred to as marginal dendrocytes (data not shown).

Localization of macrophages and dendrocytes in the splenic red pulp

Using our mAb panel, the non-lymphoid cells of the splenic red pulp can be subdivided into at least four subpopulations. One of these subpopulations is located in close association with the marginal zone. Therefore, cells assigned to this population are termed marginal red pulp macrophages. They are recognized for example by mAb ER-BMDM1 (fig. 10.3a). Other macrophages are scattered throughout the red pulp. They are considered as a single macrophage population by other investigators but staining with the various mAbs revealed that distinct subpopulations consist within the red pulp macrophage population. Thus, F4/80 (fig. 10.3b) and BM8 (fig. 10.3c) recognize all red pulp macrophages, whereas ER-HR3 (fig. 10.3d) and ER-BMDM1 (fig. 10.3e) recognize a major and a minor subpopulation, respectively. ER-BMDM1-positive cells are present in the central red pulp, but the majority of the ER-HR3-positive cells is located in a zone just beneath the splenic capsule. In addition, a very small population of NLDC-145 positive cells is demonstrated in the red pulp (fig. 10.3f).

DISCUSSION

In this paper we report on the phenotypical heterogeneity of macrophages and dendrocytes present in the murine spleen. For the characterization of these cells we utilized a number of macrophage and dendritic cell-specific monoclonal antibodies.

Our results, summarized in figure 10.1, show that macrophages and dendrocytes present at different microanatomical sites partially overlap in their marker expression. However, phenotypical differences between both cell types are also noticed. Nearly all dendrocytes are recognized by mAb NLDC-145 and MIDC-8, and macrophages by mAb MOMA-2 and/or F4/80. Although we were unable to determine the exact location of some markers due to overstaining of adjacent cells, we conclude from figure 10.1 that,

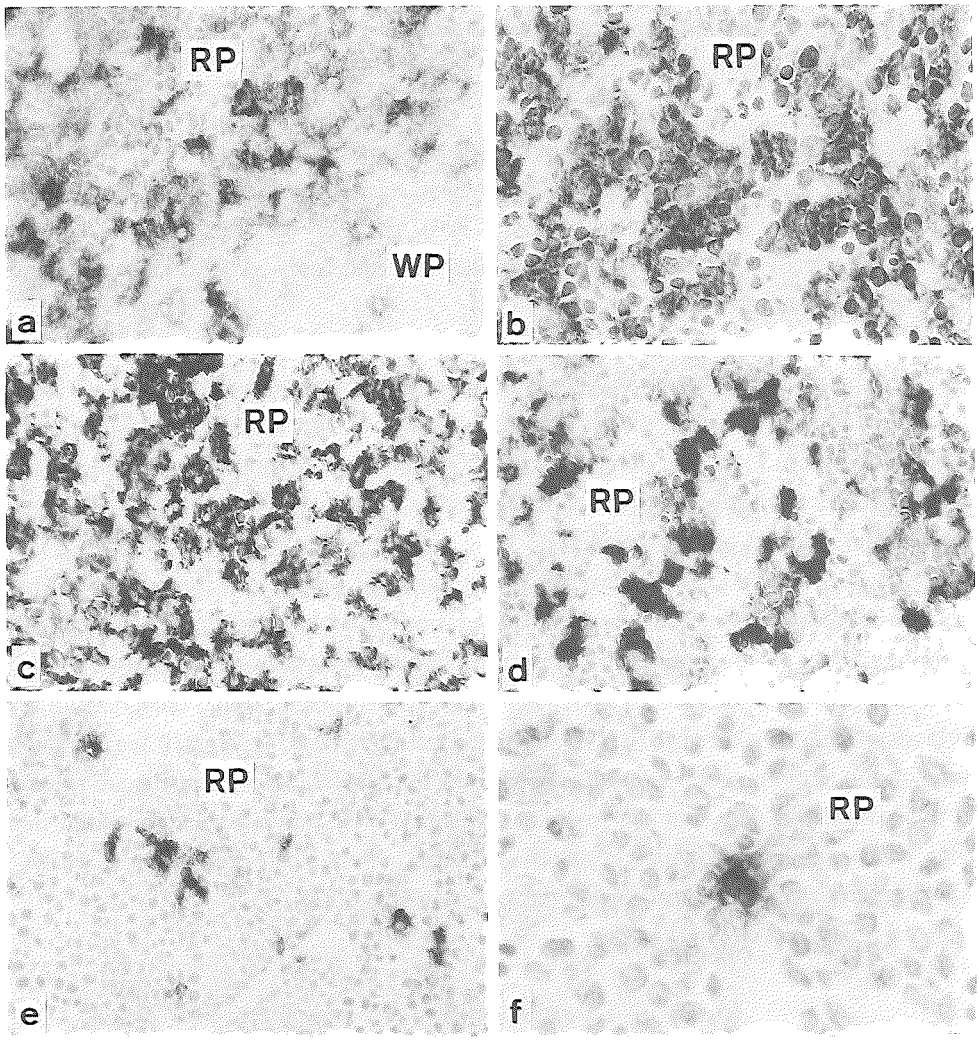


Figure 10.3.

Macrophage and dendrocyte subpopulations in the splenic red pulp. (a) marginal red pulp macrophages stained with mAb ER-BMDM1, (b) red pulp macrophages stained with F4/80, (c) red pulp macrophages stained with BM8, (d) red pulp macrophages stained with ER-HR3, (e) red pulp macrophages stained with ER-BMDM1, (f) red pulp dendrocyte stained with NLDC-145. Original magnifications: x 320 (a, c, d, e); x 510 (b); x 800 (f).

Abbreviations: RP - red pulp; WP - white pulp.

when compared to macrophages, the marker expression of dendrocytes at distinct microanatomical sites is rather constant.

The present study especially refines the information on the presence of macrophage subpopulations in the red pulp. In former studies, positively stained red pulp macrophages have been observed with F4/80 (Hume et al., 1983), BM8 (Malorny et al., 1986), MOMA-2 (Kraal et al., 1987) and ER-HR3 (de Jong et al., 1989a). However, the distinct subpopulations were not identified in these studies. In the present study, we observe at least four subpopulations: (i) a major F4/80+, BM8+, ER-BMDM1+, M3/38+ population in close association with the marginal zone, (ii) a smaller F4/80+, BM8+, ER-HR3+ population mainly located near the capsule, (iii) a minor F4/80+, BM8+, ER-BMDM1+ population in the central red pulp and (iv) a remaining population of F4/80+, BM8+, MOMA-2+ cells scattered throughout the red pulp. Double staining procedures are presently ongoing in order to determine a further immunophenotypical distinction between these populations. Our study further indicates that the expression of the markers detected by F4/80, BM8 and ER-HR3 is confined to cells of the red pulp suggesting that these antigens are involved in functions performed exclusively in this compartment.

The absence of F4/80+ macrophages in the white pulp contrasts with the results of studies by other investigators (Hume et al., 1983; Witmer and Steinman, 1984). They observed F4/80+ cells around the central arteriole of the inner PALS. In our study we identified these peri-arteriolar macrophages with mAbs ER-BMDM1, MOMA-2 and M5/114, but not F4/80. Possibly, differences in the methodology explain the observed discrepancy with regard to F4/80 detection. However, acetone fixation as applied by Witmer and Steinman (1984) instead of pararosanilin fixation of the tissue sections did not alter our results (data not shown). Another explanation for the observed discrepancy could be the existence of local differences in the concentration of microenvironmental cytokines. In this context, it has been demonstrated that interferon-γ-activated macrophages show a decreased expression of the F4/80 antigen after exposure to these factors (Berton and Gordon, 1983).

Noteworthy, follicular dendritic cells were not recognized by any of the mAb from our panel. This finding supports the notion that follicular dendritic cells have an origin and function different from dendrocytes. Such cells are not bone marrow-derived; rather they originate from fibroblastic reticulum cells in situ (Humphrey and Grennan, 1981).

Our study also indicates that macrophages and dendrocytes share a number of antigens such as ER-BMDM-1 Ag, Monts-4 Ag, Ia and Mac-2 (fig. 10.1). Therefore, both cell types are most probably closely related and may originate from the same progenitor cell. So far, the classification of macrophages and dendrocytes has been a matter of considerable debate. The results of ontogenetic studies (Janossy et al., 1986; van Rees et al., 1988) indicating that both cell types must be regarded as separate lineages, have been contradicted by other observations. A direct relationship between the two cell types

has originally been suggested by Hoefsmit et al. (1982). These investigators observed Birbeck granule-containing veiled cells and IDC within the draining lymph and lymph node of the skin, respectively. They proposed that these cells originate from epidermal Langerhans cells (LC). This proposal is strengthened by the observations that both LC (Katz et al., 1979) and IDC (Barclay, 1981) are bone marrow-derived, a feature which they have in common with monocytes. Noteworthy, there are several other indications that LC have a monocytic origin: both cells exhibit ATPase and non-specific esterase activity (Wolff and Stingl, 1983). Moreover, these cells possess type II Fc- and C3bi receptors (Austyn, 1987) and express the macrophage markers Mac-2 and Mac-3 (Haines et al., 1983), F4/80 Ag (Hume et al., 1983), BM8 Ag (Malorny et al. 1986) and ER-HR3 Ag (de Jong et al., 1989a). Analogous to the epidermis, a similar relationship between monocytes, veiled cells and IDC in the intestine has been proposed (Sminia et al., 1983). Additional arguments for a monocytic origin of dendrocytes come from the observation that functional dendrocytes can be obtained from cultured blood monocytes (Peters et al., 1987). Furthermore, under some specific experimental conditions, IDC have been shown to become phagocytic (Duijvestijn et al., 1982; Fossum et al., 1984).

In conclusion, we demonstrated that mononuclear phagocytes present at distinct microanatomical sites in the spleen exhibit distinctive phenotypes. This compartmentalization of dendrocyte and macrophage subpopulations within the spleen is most likely critical for their function. Furthermore, the phenotypical overlap between dendrocytes and macrophages is indicative of the close relationship between these cell types.

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CHAPTER 11

GENERAL DISCUSSION

11. GENERAL DISCUSSION

The differentiation of macrophages is a complex process leading to a wide variety of cell types with divergent functions and phenotypes. At present, the origins of macrophage diversity are largely unknown. There are now four different views that provide explanations for the generation of macrophage heterogeneity (schematically represented in figure 2.5). As already discussed in chapter 2, macrophage heterogeneity might be explained in terms of differences in maturation stage, activation stage, differentiation lineage or macrophage compartment. From the literature it is evident that maturation and activation of macrophages by environmental factors greatly contribute to the generation of diversity. However, the existence of separate differentiation lineages or independently maintained macrophage compartments is still disputed. In this discussion I will try to fit our present data in these four different views dealing with the generation of macrophage heterogeneity.

To identify particular stages in macrophage differentiation, mAbs directed against cell surface molecules are invaluable tools. Therefore, we produced mAbs against antigens expressed by various macrophage differentiation stages. The rationale behind this approach is the notion that analysis of the reactivity of these mAbs on different macrophage populations would reveal more insight in the origins of macrophage heterogeneity. However, at the onset of this study, definition of the reactivity of a particular mAb in terms of macrophage differentiation stages appeared to be hampered by the paucity of pure populations of defined differentiation stages, useful as targets for mAb screening. To overcome this problem, we first developed a screening model of macrophage differentiation by arranging a panel of macrophage tumor cell lines in a linear differentiation sequence (see chapter 4). This cell line screening model appeared of great value in the screening and characterization of the mAbs produced against various macrophage differentiation stages (chapters 6 - 9). Further independent support for the validity of our macrophage cell line model came recently from studies by other investigators. Cross and co-workers (1988) reported that the level of lysozyme M gene expression increased in different cell lines with increasing maturation stage. Moreover, a similar correlation with the proposed cell line maturation sequence was observed for the expression of the homeobox gene Hox 2.3 (L. de Laaf and F. Meijlink, personal communication). This gene is known to be increasingly expressed with macrophage differentiation (Kongsuwan et al., 1988).

As particularly the phenotype of the first stages in macrophage differentiation are unexplored, we decided to focus on the macrophage precursor population. Putatively, separate differentiation lineages - if these exist - would be initiated at the precursor cell level. In order to test this hypothesis, we developed a second screening model of

macrophage differentiation, enabling the clonal analysis of such precursors. To this purpose, we immortalized macrophage precursors by somatic cell hybridization with myeloid tumor cell lines (see chapter 5). A panel of 22 hybrid macrophage cell lines was obtained using this approach.

One should keep in mind, however, that application of tumor cell lines for the clonal analysis of macrophage precursor heterogeneity bears the risk of observing artificial phenomena, caused by chromosomal aberrations connected to the malignant status of the cells. However, two findings strengthened our view about the valid applicability of tumor cell lines for the analysis of normal population heterogeneity. Firstly, as delineated above, the panel of macrophage tumor cell lines ordered in a differentiation sequence appeared to be a reliable and invaluable screening model of differentiation. Secondly, *mature* macrophage hybrids produced by other investigators appeared to express stable, divergent phenotypes, which accurately reflect mature macrophage heterogeneity (Tzehoval 1984; 1989; Uchida et al., 1985). Our macrophage hybrids exhibited a rather uniform precursor cell phenotype. Furthermore, both phenotype and chromosomal content of the precursor hybrids appeared to be stable over long periods in culture, similar to the stability observed for mature macrophage hybrids. Based on these observations, we feel that the various macrophage precursor hybrids resemble normal macrophage precursors with respect to phenotype and differentiation potential.

Remarkably, an extensive heterogeneity among the different hybrids was observed in the response to a single differentiation inducing stimulus (chapter 5). Thus, different hybrids displayed divergent patterns of mature macrophage marker acquirement. This heterogeneous response most likely reflects the heterogeneity within the normal bone marrow macrophage precursor population. This observation therefore argues in favour of the existence of distinct macrophage differentiation lineages in the bone marrow. As representatives of different macrophage precursor subsets, our precursor hybrids constitute a second model of differentiation.

An additional application of the precursor hybrids is found in a third screening model of macrophage differentiation: the hybrids as such represent immature stages, whereas induction of differentiation reveals cells of increased maturity. Thus, in this third model, macrophage maturation can be followed along a single differentiation lineage.

The developed screening models of macrophage differentiation were of particular value during the production and identification of mAbs against macrophage precursor stages (chapter 6). Here, the reactivity of four different mAbs is reported: ER-MP12, -20, -54, and -58. These antibodies were selected on the basis of specific recognition of precursor stages of macrophage differentiation in the various models. Most importantly,

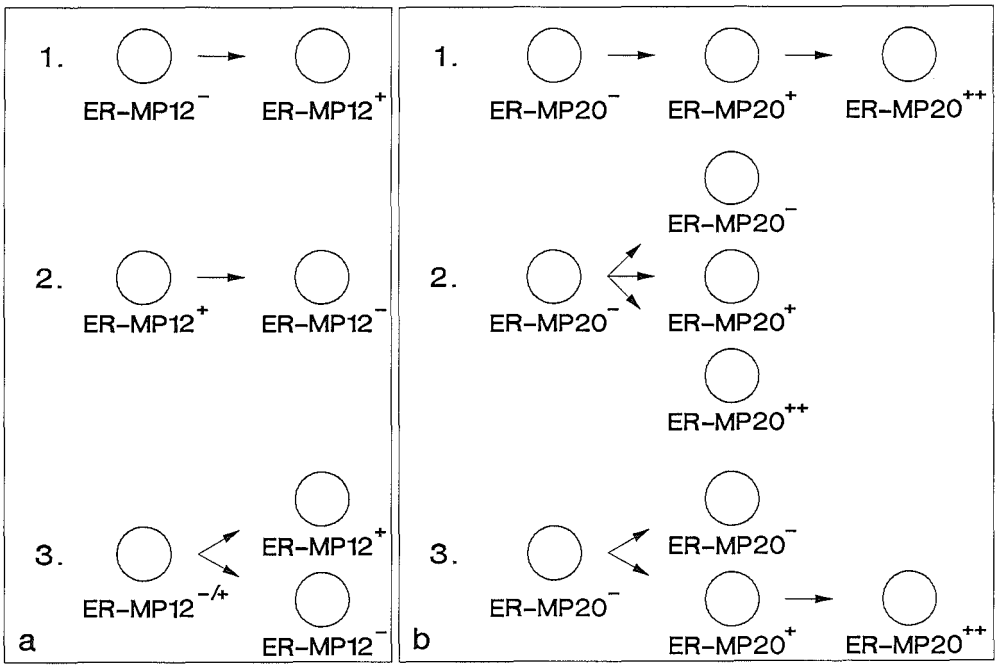

Figure 11.1.

Alternative explanations for the observed heterogeneous ER-MP12 and ER-MP20 antigen expression among M-CSF responsive macrophage precursors in the bone marrow. (a). Bone marrow macrophage precursors are either ER-MP12 positive or ER-MP12 negative. Both phenotypes may occur as different maturation stages along the same lineage (1 or 2). Alternatively, distinct - ER-MP12 positive and ER-MP12 negative - lineages may develop (3). (b). ER-MP20 recognizes three distinct subpopulations among bone marrow macrophage precursors: ER-MP20 negative, ER-MP20 'dull' (+), and ER-MP20 'bright' (++) cells. Similar to ER-MP12, the different ER-MP20 phenotypes may represent different maturation stages of the same lineage (1), or distinct differentiation lineages (2). In addition, combinations of both explanations are feasible (3).

ER-MP12, -20 and -58 were successfully applied for the separation of macrophage precursors from *normal* bone marrow. ER-MP58 appeared to recognize all bone marrow macrophage precursors, whereas ER-MP12 and -20 recognized subpopulations. The observed heterogeneity of macrophage precursors with respect to the expression of ER-MP12 Ag and ER-MP20 Ag may be explained in different ways (see fig. 11.1). ER-MP12 positive and ER-MP12 negative cells may represent different maturation stages of the same lineage. Alternatively, ER-MP12 positive and ER-MP12 negative macrophage precursors may belong to separate differentiation lineages. A similar reasoning is applicable to explain the observed heterogeneity in ER-MP20 Ag expression. However, additional alternative explanations are feasible because of the fact

that macrophage precursors may either be ER-MP20 negative, ER-MP20 'dull', or ER-MP20 'bright'. Experimental evidence for one of these possible developmental pathways likely will come from progeny analysis of the various macrophage precursors separated according to their ER-MP12 and ER-MP20 phenotype. If precursors with different ER-MP12 or -20 phenotypes belong to separate differentiation lineages, then mature macrophages with divergent functions and/or phenotypes will develop.

Another mAb isolated on the basis of its reactivity in the cell line models is ER-MP23 (chapter 8). In situ, this mAb specifically recognizes a subpopulation of macrophages primarily present in connective tissues (chapter 8). ER-MP23 positive macrophages most likely constitute a separate differentiation lineage. Reactivity of ER-MP23 with the macrophage cell line panel shows a striking dichotomy: 3 of 6 mature cell lines show a high level of ER-MP23 Ag expression, whereas the other 3 are ER-MP23 negative. One may argue that this diversity reflects the heterogeneous origin of the various cell lines; ER-MP23 positive cell lines may be derived from ER-MP23 positive connective tissue macrophages, whereas ER-MP23 negative lines may originate from other populations. A lineage-dependency of ER-MP23 Ag expression is supported by the finding that the ER-MP23 Ag expression of two mature macrophage cell lines - RAW264.7 (ER-MP23 negative) and RAW309Cr.1 (ER-MP23 positive) - is not significantly altered by various cytokine mixtures. Hence, differences in maturation or activation stage are no likely explanations for the observed heterogeneity in ER-MP23 Ag expression. The fact that ER-MP23 was evoked by immunization with a precursor cell line indicates that ER-MP23 Ag already must be expressed at early differentiation stages. Indeed, low levels of ER-MP23 Ag are detectable on some precursor cell lines. In view of the lineage-specificity of ER-MP23, this finding suggests that early differentiation stages may already express lineage-specific markers.

Expression of the antigen recognized by ER-BMDM1 is clearly correlated to the maturation stage of macrophages, since this marker is absent from precursors and increasingly expressed upon maturation of the cells (chapter 9). This conclusion is based on results obtained from the in vitro models of differentiation, as well as from analysis of isolated cells from bone marrow, blood and periphery. However, ER-BMDM1 detects not all mature macrophages. Only 20 - 30% of the resident peritoneal and alveolar macrophages appears to be ER-BMDM1 positive, whereas virtually all thioglycollate elicited macrophages express the ER-BMDM1 Ag. At least three explanations are feasible for this phenomenon (summarized in fig. 11.2). Firstly, ER-BMDM1 positive exudate macrophages may develop into ER-BMDM1 negative resident macrophages. In that case, expression of ER-BMDM1 Ag would reflect a recent entry of macrophages from the circulation. Secondly, monocyte-derived macrophages might be ER-BMDM1 positive, whereas the ER-BMDM1 negative cells belong to independent, locally

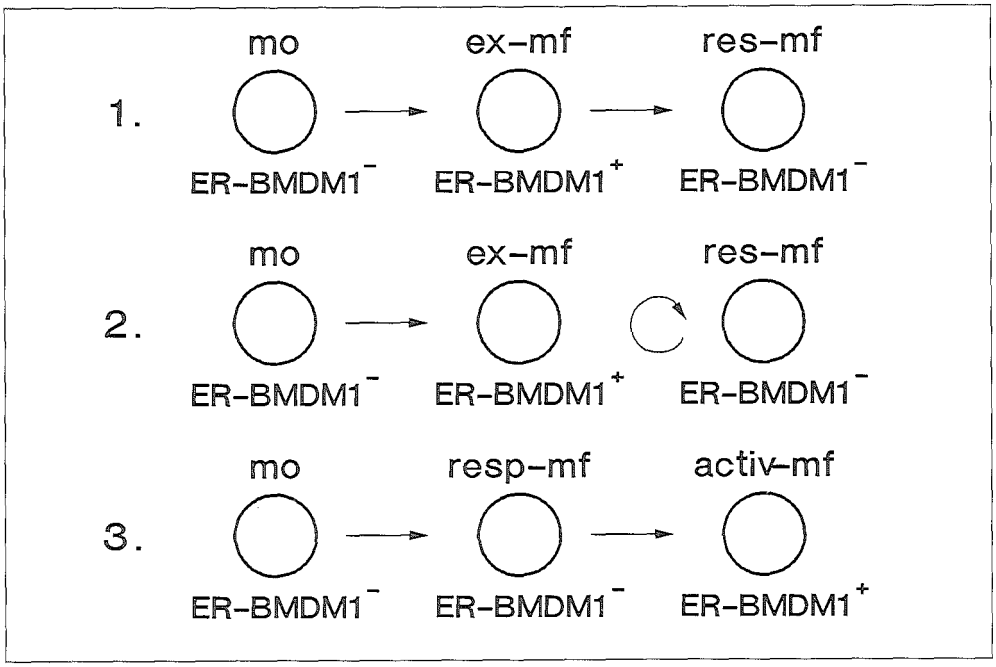

Figure 11.2.

Alternative explanations for the observed heterogeneous ER-BMDM1 antigen expression among mature macrophage populations. 1. ER-BMDM1 Ag may be transiently expressed during the maturation of monocytes (mo) into resident macrophages (res-mf). 2. ER-BMDM1 Ag expression may be induced upon maturation of monocytes into exudate macrophages (ex-mf), whereas the resident macrophages constitute an autonomous population. 3. ER-BMDM1 Ag expression may be induced in the development of activated macrophages (activ-mf) via a responsive macrophage (resp-mf) stage.

maintained macrophage compartments. Thirdly, ER-BMDM1 Ag might be expressed only in response to particular activation signals, which are mimicked by thioglycollate elicitation. At present, none of these alternatives can be excluded on the basis of the available data. Experimentally, this issue might be approached by transplantation of ER-BMDM1 positive exudate macrophages in recipient animals with a congenic marker difference. Development of ER-BMDM1 negative resident macrophages - traceable on the basis of the simultaneous expression of the congenic marker and a resident macrophage phenotype - would indicate that ER-BMDM1 negative cells originate from ER-BMDM1 positive exudate macrophages.

Interestingly, dendrocytes - Langerhans cells, veiled cells, interdigitating cells and their in vitro equivalents, dendritic cells - are the only leukocytes which also express ER-BMDM1 Ag. This and other phenotypical resemblances strengthen the idea that

dendrocytes belong to the mononuclear phagocyte lineage. As outlined before, arguments in favour of this view are the observed phagocytic capacity of dendrocytes after tissue damage (Duijvestijn et al., 1982; Fossum et al., 1984) and the in vitro differentiation of monocytes into dendrocytes (Peters et al., 1987; Kabel et al., 1989). An apparently strong argument against the view that dendrocytes belong to the mononuclear phagocyte system is the observation that dendrocytes and 'classical' macrophages develop separately during ontogeny (Janossy et al., 1986; van Rees et al., 1988). However, the validity of this argument is questionable, since also well-known members of the mononuclear phagocyte system appear to develop as separate lineages in ontogeny. Already in the yolk sac two different macrophage lineages have been recognized. Recent data indicate that, early in ontogeny, hemopoietic stem cells develop directly into so-called primitive macrophages, which subsequently give rise to fetal macrophages (Takahashi et al., 1989; Naito et al., 1989). However, recognizable precursor cells of the common monocyte-macrophage lineage arise separately in a later stage. Similarly, alveolar macrophages and Kupffer cells appear to develop as distinct lineages in ontogeny, independently from the appearance of monocytes in the circulation (Sorokin et al., 1984; 1989; Deimann and Fahimi, 1978; Pino and Bankston, 1979). On the other hand, in adult life monocytes appear to give rise to alveolar macrophages and Kupffer cells (van Furth and Cohn, 1968; van Furth et al., 1985a). Although difficult to imagine, the separate ontogenetic development of a macrophage population - independent from the regular monocyte lineage - apparently does not prohibit their development from monocytes in adult life. Similarly, dendrocytes might originally develop separately during ontogeny, and yet be maintained by monocytic differentiation in adult life. The observed distinct ontogenetic development (Janossy et al., 1986; van Rees et al., 1988), therefore provides no conclusive evidence for the view that dendrocytes and macrophages belong to different hemopoietic lineages. Instead, dendrocytes can most likely be regarded as a specialized lineage within the mononuclear phagocyte system.

In the last study described in this thesis (chapter 10), ER-BMDM1 and other recently available anti-macrophage mAbs have been used in an attempt to unravel the complexity of the mononuclear phagocyte composition of the murine spleen. Clearly, several phenotypically distinct macrophage and dendrocyte subpopulations can be discerned. Most splenic microenvironments appear to be occupied by a predominant, characteristic subpopulation. Examples of such distinctive subpopulations are the ER-TR9 positive macrophages in the marginal zone or the newly discovered ER-BMDM1 positive marginal red pulp macrophages located in foci at the periphery of the white pulp. However, in other microenvironments multiple subpopulations are intermingled, for example in the T cell area of the white pulp. Here, the observed heterogeneity can not be explained in terms of activation by different local conditions, since distinct

subpopulations occupy the same microenvironment. Also differences in maturation stage can not explain this diversity, as the distinct subpopulations display no signs of functional or phenotypical immaturity. Consequently, these subpopulations must either belong to different monocyte-derived lineages, or to separate, independently maintained compartments. Support for the separate lineage option comes from the observation that different splenic macrophage subpopulations return at different time points after elimination with drug-loaded liposomes (van Rooijen et al., 1989). This observation strongly suggests that the macrophage subpopulations in the spleen originate from different precursors. In this context, van Furth and Diesselhoff-den Dulk (1984) have previously shown that splenic macrophages have a dual origin: about half of the macrophage content originates from monocyte influx, whereas the other half arises from local proliferation. Possibly, this proliferative activity is not equally distributed among the distinct subpopulations: particular splenic macrophage subpopulations might be maintained by local proliferation, whereas other subpopulations are supplied by monocyte influx.

In summary, different mechanisms appear to contribute to the extensive heterogeneity observed among mononuclear phagocytes. **Maturation** of cells induces many phenotypical and functional changes (chapters 5 - 7 and 9). Similarly, local **activation** affects many facets of macrophages (primarily discussed in chapter 2). **Separate differentiation lineages** are indicated by data in chapters 5 and 8. Finally, the existence of **independently maintained macrophage compartments** is fully compatible with the results presented in chapters 9 and 10.

Based on our findings outlined above and those reported by other investigators, a model can be drawn up that integrates the various views on the generation of macrophage diversity. This model might be designated as a "flexible lineage hypothesis" (see fig. 11.3).

The basic features of this hypothesis are :

1. In the **steady state** (fig. 11.3 - left), different macrophage lineages arise in the bone marrow at the macrophage precursor level. Monocytes that belong to a certain lineage show a preferential development into particular peripheral macrophage populations. This implies, for instance, that monocytes are heterogeneous with regard to the tendency to differentiate into alveolar or connective tissue macrophages. Some distinct peripheral macrophage populations might be self-maintaining by local proliferation.
2. Under **immunological stress conditions** (fig. 11.3 - right), the regular differentiation pathways of multiple lineages can be overruled by extreme environmental conditions. This situation leads to the preferential production of those mature

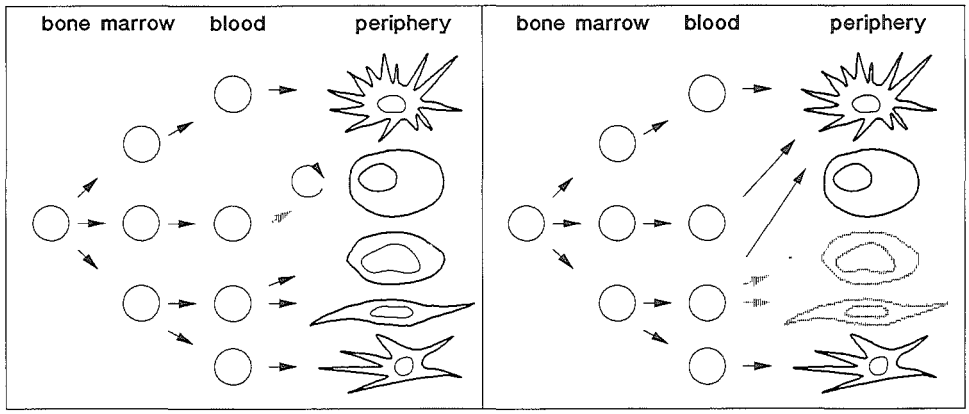

Figure 11.3.

Schematic representation of the "flexible lineage hypothesis" explaining the generation of mature macrophage heterogeneity.

(LEFT). Macrophage differentiation in the steady state. Different lineages originate from the bone marrow, which exhibit preferential development into particular mature macrophage populations. Some peripheral populations might be self-maintaining by local proliferation.

(RIGHT). Macrophage differentiation under immunological stress conditions. The regular differentiation pathways of several lineages are overruled by extreme conditions, such as increased cytokine concentrations. This leads to the enhanced production of particular mature subpopulations.

mononuclear phagocytes that are best suited to challenge the threatening conditions.

The mechanism underlying both the generation of multiple differentiation lineages in the steady state and the flexible modulation of differentiation pathways under stress conditions might be the following. Various cytokines are known to induce differentiation of macrophage precursors. However, each of these factors might induce different patterns of gene expression and therefore instruct the precursor cells to differentiate into different lineages. Initial experimental evidence for this notion has been provided recently. M-CSF and GM-CSF both stimulate precursor cells to proliferate and to differentiate into macrophages (Metcalf, 1984; 1988). However, macrophages obtained from GM-CSF stimulated cultures display a different phenotype and different functional capacities compared to those obtained with M-CSF (Falk and Vogel, 1988; Falk et al., 1988). In this way, many distinct lineages can be generated, given the multitude of factors that influence macrophage differentiation (see chapter 2). Hypothetically, the predominance of a particular cytokine early in the differentiation of macrophages induces the predominant expression of other cytokine receptors and/or homing receptors for various organs (fig. 11.4). The resulting receptor phenotype then determines the

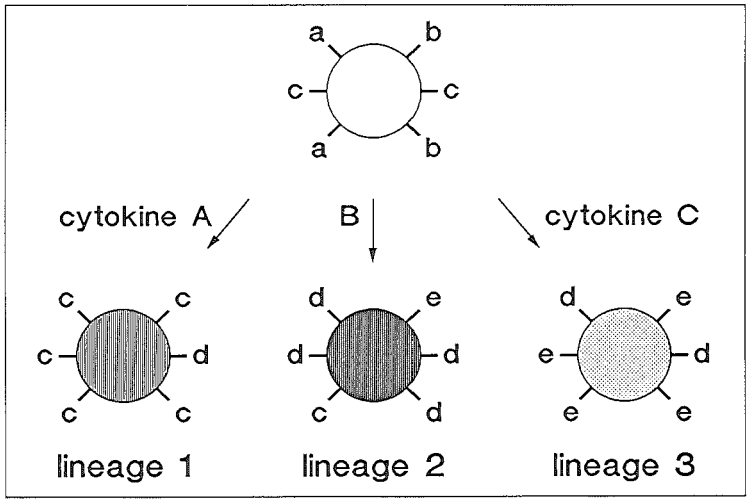

Figure 11.4.

Putative mechanism explaining the generation of different macrophage differentiation lineages. Early macrophage precursor cells display receptors (a, b, and c) for various cytokines (A, B, and C) that can induce maturation of the cells. However, different cytokines induce a differential expression of other cytokine and/or homing receptors (c, d, and e). Hence, different lineages emerge with different sensitivity for particular cytokines or different tendency to home into particular organs. Diversification of macrophages into different lineages thus occurs at the precursor level in the bone marrow.

sensitivity for the cognate cytokines and/or the tendency to home into specific organs.

A consequence of the mechanism outlined above is that the different lineages can be discerned on the basis of their relative sensitivity for the various cytokines or the expression of - hypothetical - macrophage homing receptors. This implies that the distinct lineages are not separated in an absolute sense. Low concentrations of, for example, cytokine 'C' present under steady state conditions, will only stimulate cells ofthe 'privileged' lineage, namely those cells with high sensitivity for cytokine 'C'. However, high levels of 'C' might be present under immunological stress conditions in vivo, or in an experimental situation. Such extreme conditions will also affect the differentiation programs of other, 'non-privileged' lineages, since cells of most of these lineages express low numbers of 'C' receptors. The normal differentiation programs of the 'non-privileged' lineages will therefore be overruled by the presence of high cytokine 'C' concentrations. Consequently, more end-stage cells of the 'C' lineage will be produced (fig. 11.3 - right). The number of lineages involved in such a shift in differentiation programs will depend on the local concentration of cytokine 'C' as well as on the sensitivity for 'C' of the various 'non-privileged' lineages.

In the light of the foregoing, the discussion whether the mononuclear phagocyte system is a continuum of maturation- and activation stages, or a pedigree of separate lineages, is more or less semantic. The various lineages designated as lineage 1, 2, and 3 in figure 11.4 exhibit gradual differences. As such, they can be regarded as part of a continuum. However, comparison of lineages 1 and 3 reveals absolute differences in the potential to respond to different cytokines and/or to home to different organs. These lineages should therefore be regarded as separate pathways of macrophage differentiation.

In conclusion, the "flexible lineage hypothesis" postulated here provides a means to understand the generation of macrophage diversity in terms of distinct differentiation lineages. Simultaneously, however, this hypothesis explains how immunological stress conditions drastically change the regular differentiation pathways. In this way, the mononuclear phagocyte system may respond highly flexible to divergent environmental challenges.

CHAPTER 12

SUMMARY - SAMENVATTING

SUMMARY

Mononuclear phagocytes - macrophages and their precursors - constitute a very heterogeneous cell population. This heterogeneity has two aspects: on the one hand, mononuclear phagocytes perform many different functions, in particular in the defense against threatening agents, such as harmful micro-organisms and tumor cells (outlined in **chapter 2**). On the other hand, concomitant with functional heterogeneity, mononuclear phagocytes display a variety of phenotypes. At present, it is not clear how the diversity of the mononuclear phagocyte system should be described in terms of (i) maturation stages, (ii) activation stages, (iii) distinct differentiation lineages, and (iv) locally maintained peripheral macrophage populations.

The aim of the present study was the establishment of a correlation between macrophage differentiation stage and the phenotype of these cells. To that end, novel monoclonal antibodies were required, directed against stage- or lineage specific cell surface antigens.

Basic prerequisites for the generation of monoclonal antibodies are adequate immunogens and a reliable screening system. For the latter, a macrophage differentiation model was elaborated, based on macrophage tumor cell lines arrested in different maturation stages (described in **chapter 4**). Phenotypical and functional analysis indicated that these cell lines could be ordered in a linear differentiation sequence. Thus, a screening system for monoclonal antibodies against differentiation antigens was created which proved very useful in the following studies.

Especially the phenotypes of cells in the first steps of macrophage differentiation are obscure. More specifically, it is unclear whether different lineages of macrophage differentiation can be discerned already at the precursor level. The main hindrance for studying these early stages is the fact that macrophage precursor cells are hidden within the heterogeneous cell population in the bone marrow. To enable analysis at the *clonal level*, macrophage precursors were immortalized by somatic cell hybridization (described in **chapter 5**). Using this technique, 22 hybrid cell lines were obtained that all exhibited characteristics of macrophage precursor cells. As such, the different hybrids expressed similar phenotypes. However, a striking heterogeneity was observed after induction of differentiation in these hybrids. It appeared that different hybrids expressed different features of mature macrophages upon induction. This observation indicates that different macrophage precursor hybrids carry divergent differentiation programs. Hence, these results argue in favour of the existence of different macrophage differentiation lineages that already occur at the precursor cell level.

Using macrophage precursors hybrids as immunogen, a panel of monoclonal

antibodies was raised (described in chapters 6, 7, and 8). Four antibodies, ER-MP12, -20, -54, and -58, were selected in in vitro models of macrophage differentiation that specifically bind to precursor stages (described in **chapter 6**). Notably, ER-MP12, -20, and -58 were successfully used to isolate normal macrophage precursor cells from a bone marrow cell suspension. ER-MP58 appeared to recognize virtually all bone marrow macrophage precursors, whereas ER-MP12 and -20 recognized precursor subpopulations.

In **chapter 7** another phenomenon changing upon differentiation of macrophages was investigated, i.e. the regulation of cellular iron uptake through transferrin. To this end, antibody ER-MP21 was used, which detects the murine transferrin receptor. In functional studies, this antibody was found to inhibit the proliferation of macrophage precursor cells strongly by reducing the level of cellular iron uptake. However, ER-MP21 hardly affected the proliferation of more mature stages of macrophage differentiation, although the iron uptake by these cells was similarly reduced. In search of the mechanism for this phenomenon, we observed that mature macrophages took up 2 to 3 times as much transferrin-bound iron compared to macrophage precursors. This difference was maintained in the presence of ER-MP21. Hence, mature macrophages most likely escape from ER-MP21- mediated growth inhibition because these cells take up more iron than they actually need for proliferation. Possibly, the increased iron uptake upon macrophage differentiation is related to the important iron storage function of mature macrophages.

A characteristic mature macrophage population associated with connective tissue is specifically detected by monoclonal antibody ER-MP23 (described in **chapter 8**). In reticular connective tissues as well as in the dermis, ER-MP23 positive cells occur which co-express unequivocal macrophage markers, such as F4/80, BM8 and ER-HR3 antigens. Likely, ER-MP23 recognizes a marker expressed by a separate differentiation lineage. This conclusion is based on the heterogeneous expression of ER-MP23 Ag by macrophages in situ, as well as among a panel of mature macrophage cell lines.

Another marker for mature macrophages is described in **chapter 9**: i.e. the antigen recognized by antibody ER-BMDM1. Biochemical analysis revealed that the ER-BMDM1 antigen exhibits aminopeptidase activity, and is most likely homologous to the human myeloid CD13 antigen. ER-BMDM1 Ag was found to be present on a subpopulation of macrophages, dendrocytes - interdigitating or dendritic cells, Langerhans cells and veiled cells - as well as on cells containing microvilli, such as intestinal epithelial cells and the lining cells of bile capillaries in the liver. In all in vitro differentiation models, we observed an increased expression of ER-BMDM1 aminopeptidase with maturation of the macrophages. In vivo, ER-BMDM1 Ag appeared to be restricted to post-monocytic stages. This distribution contrasts markedly to the CD13 expression found in man, where also precursor cells as well as mature granulo-

cytes display this antigen.

Taking advantage of the availability of a number of new monoclonal antibodies directed against mature macrophage populations, we decided to investigate the mononuclear phagocyte content of the spleen in more depth (described in **chapter 10**). The spleen is known to contain a number of macrophage and dendrocyte subpopulations with divergent morphological and phenotypical features. Using these new antibodies, more subpopulations could be discerned than previously known. Especially the macrophages in the red pulp compartment were found to be more heterogeneous than previously thought: at least four phenotypically distinct red pulp macrophage subpopulations were detected. This heterogeneity of splenic macrophages is suggestive of a variety of functions in host defense and hemopoiesis. Furthermore, the observed diversity of mononuclear phagocytes in the spleen clearly illustrates the complexity of the mononuclear phagocyte system.

Finally, in **chapter 11** the results presented in this thesis are discussed with emphasis on the origins of mononuclear phagocyte heterogeneity. A new model, termed the "flexible lineage hypothesis", is postulated which explains the generation of mononuclear phagocyte heterogeneity in terms of different lineages. However, as explained by this model, the regular differentiation pathway of the macrophage lineages can be changed under the influence of extreme environmental stimuli. Thus, the model provides a mechanism to understand the fine-tuned, yet flexible generation of diversity of the complex mononuclear phagocyte system.

SAMENVATTING

Het mononucleaire fagocytensysteem - bestaande uit macrofagen en hun voorlopercellen - is zeer heterogeen. Deze heterogeniteit kent twee aspecten: enerzijds hebben mononucleaire fagocyten vele verschillende functies in met name de afweer tegen bedreigende agentia, zoals schadelijke micro-organismen en tumorcellen (samengevat in **hoofdstuk 2**). Anderzijds kennen mononucleaire fagocyten een veelheid aan fenotypes die samenhangt met de genoemde functionele heterogeniteit. Op dit moment is het niet duidelijk hoe de diversiteit van het mononucleaire fagocyten systeem beschreven kan worden in termen van (i) rijpingsstadia, (ii) activatiestadia, (iii) verschillende lijnen van differentiatie, en (iv) populaties die locaal, in de verschillende weefsels in stand gehouden worden.

Het doel van dit onderzoek was het inzicht te vergroten in de relatie tussen het differentiatiestadium van mononucleaire fagocyten en het fenotype van deze cellen. Om dat doel te bereiken was het noodzakelijk nieuwe monoclonale antistoffen op te wekken, gericht tegen stadium- of differentiatielijn-specifieke antigenen.

Essentiële voorwaarden voor het produceren van monoclonale antistoffen zijn adequate immunogenen en een betrouwbaar testsysteem. Voor dit laatste werd een macrofaag-differentiatiemodel uitgewerkt, gebaseerd op macrofaag-tumorcellijnen die gearresteerd zijn in verschillende rijpingsstadia (beschreven in **hoofdstuk 4**). Uit fenotypische en functionele analyse bleek dat deze cellijnen in een lineaire differentiatiesequentie geplaatst konden worden. Hiermee was een testsysteem voor monoclonale antistoffen gecreëerd dat zeer waardevol bleek in het vervolg van dit onderzoek.

Met name over de fenotypes van de vroegste stadia van macrofaag-differentiatie is weinig bekend. Daarbij is het onduidelijk of verschillende lijnen van macrofaag-differentiatie reeds op voorloper niveau onderscheiden kunnen worden. De belangrijkste belemmering om deze vroege stadia te kunnen bestuderen, is het feit dat macrofaag-voorlopers deel zijn van de heterogene celpopulatie in het beenmerg. Om analyse van macrofaag-voorlopers op *clonaal niveau* mogelijk te maken, werden deze cellen geïmmortaliseerd door middel van somatische hybridisatie (beschreven in **hoofdstuk 5**). Met behulp van deze techniek werden 22 hybride cellijnen verkregen, die alle kenmerken hadden van macrofaag-voorlopercellen. De verschillende hybriden brachten overeenkomstige fenotypes tot expressie. Na inductie van differentiatie echter, vertoonden deze hybriden grote verschillen. Het bleek dat verschillende hybriden, na inductie, verschillende kenmerken van rijpe macrofagen lieten zien. Dit geeft aan dat de verschillende macrofaag-voorloperhybriden verschillende differentiatieprogramma's hebben. Deze resultaten pleiten dus voor het bestaan van verschillende macrofaag-differentiatielijnen die reeds vastliggen op het niveau van de macrofaag-voorlopers.

De genoemde macrofaag-voorloperhybriden werden gebruikt als immunogenen voor het opwekken van monoclonale antistoffen (beschreven in de hoofdstukken 6, 7 en 8). Vier antisera, ER-MP12, -20, -54 en -58, werden geselecteerd in in vitro modellen van macrofaag-differentiatie op basis van hun specifieke binding aan macrofaag-voorloper-stadia (beschreven in **hoofdstuk 6**). Vervolgens werden ER-MP12, -20 en -58 met succes gebruikt voor de isolatie van normale macrofaag-voorlopercellen uit het beenmerg. ER-MP58 herkende vrijwel alle macrofaag-voorlopers uit het beenmerg; ER-MP12 en -20 herkenden subpopulaties van voorlopercellen.

In **hoofdstuk 7** is een eigenschap van macrofagen onderzocht die verandert met de differentiatie van deze cellen, namelijk de regulatie van cellulaire ijzeropname via transferrine. Daartoe werd monoclonale antistof ER-MP21 gebruikt, dat de transferrine-receptor van de muis herkent. In functionele studies werd gevonden dat dit antilichaam de proliferatie van macrofaag-voorlopercellen sterk remde door een reductie van de cellulaire ijzeropname. Daarentegen had ER-MP21 vrijwel geen effekt op de proliferatie van rijpere macrofaag-differentiatiestadia, hoewel de ijzeropname van deze cellen ook geremd was. Op zoek naar een verklaring voor deze waarneming vonden we dat rijpe macrofagen 2 tot 3 maal zoveel transferrine-gebonden ijzer internaliseerden als macrofaag-voorlopers. Deze verhouding werd ook gevonden in aanwezigheid van ER-MP21. Het meest waarschijnlijk is dus dat rijpe macrofagen ontsnappen aan groeiremming door ER-MP21, omdat deze cellen meer ijzer opnemen dan ze feitelijk nodig hebben voor hun proliferatie. Mogelijk houdt deze toegenomen ijzeropname in de loop van de macrofaag-differentiatie verband met de functie van rijpe macrofagen als belangrijke opslagplaats van ijzer.

Een karakteristieke rijpe macrofaag-populatie, die voorkomt in bindweefsel, wordt specifiek herkend door monoclonale antistof ER-MP23 (beschreven in **hoofdstuk 8**). In reticulair bindweefsel, maar ook in de dermis komen ER-MP23 positieve cellen voor die tegelijkertijd antigenen tot expressie brengen zoals F4/80, BM8 en ER-HR3 die kenmerkend zijn voor macrofagen. Waarschijnlijk herkent ER-MP23 een marker die door een aparte differentiatielijn tot expressie gebracht wordt. Deze conclusie is gebaseerd op de heterogene expressie van het ER-MP23 antigeen door macrofagen in situ, alsook door een panel rijpe macrofaag-cellijnen.

Een andere marker voor rijpe macrofagen is beschreven in **hoofdstuk 9**, namelijk het antigeen dat herkend wordt door het antiserum ER-BMDM1. Biochemische analyse leerde dat het ER-BMDM1 antigeen aminopeptidase-activiteit bezit, en hoogstwaarschijnlijk homoloog is aan het humane myeloïde CD13 antigeen. Het ER-BMDM1 antigeen is aanwezig op een subpopulatie macrofagen, dendrocyten - interdigiterende of dendritische cellen, cellen van Langerhans en sluiercellen - en op cellen die bezet zijn met microvilli, zoals darm-epitheelcellen en de cellen die de galcapillairen in de

lever bekleden. In alle in vitro differentiatiemodellen vonden we een toenemende expressie van ER-BMDM1 aminopeptidase met de rijping van de macrofagen. In vivo was het ER-BMDM1 antigeen beperkt tot post-monocytaire stadia. Deze distributie verschilt sterk van de CD13 expressie bij de mens, waar deze marker ook voorkomt op voorlopercellen en op rijpe granulocyten.

Gebruikmakend van een aantal nieuwe monoclonale antistoffen tegen rijpe macrofaag-populaties, werd het voorkomen van mononucleaire fagocyten in de milt in detail onderzocht (beschreven in **hoofdstuk 10**). Het is bekend dat de milt een aantal macrofaag- en dendrocyt-subpopulaties bevat met uiteenlopende morfologische en fenotypische kenmerken. Met behulp van nieuwe antisera werden meer subpopulaties onderscheiden dan eerder bekend waren. Met name de macrofagen in de rode pulpa bleken heterogener dan eerder gedacht werd: tenminste vier fenotypisch verschillende macrofaag-subpopulaties werden aangetoond in de rode pulpa. Deze heterogeniteit van milt-macrofagen wijst op een verscheidenheid aan functies in afweer en hemopoiese. Bovendien vormt de diversiteit van mononucleaire fagocyten in de milt een duidelijke illustratie van de complexiteit van het mononucleaire fagocytensysteem.

Tenslotte worden in **hoofdstuk 11** de resultaten, die beschreven zijn in dit proefschrift besproken in het licht van het ontstaan van de heterogeniteit van mononucleaire fagocyten. Een nieuw model, de "flexibele differentiatielijn-hypothese", wordt gepostuleerd dat de vorming van macrofaag-heterogeniteit verklaart in termen van verschillende differentiatielijnen. Zoals in dit model wordt uitgelegd, kan de reguliere ontwikkeling van macrofaag-differentiatielijnen echter gewijzigd worden onder extreme omstandigheden. Dit nieuwe model nu voorziet in een verklaring voor de nauwkeurig gereguleerde, maar flexibele vorming van diversiteit in het complexe mononucleaire fagocytensysteem.

ABBREVIATIONS

Ag	-	antigen(s)
BMC	-	bone marrow cell(s)
BSA	-	bovine serum albumin
CD	-	cluster of differentiation
CFC	-	colony-forming cell(s)
CFU-GM	-	colony-forming unit - granulocyte-macrophage
CFU-S	-	colony-forming unit - spleen
CM	-	conditioned medium
CML	-	chronic myeloid leukemia
CR3	-	complement receptor type 3
CSF	-	colony-stimulating factor
DC	-	dendritic cell(s)
DDC	-	dendrocyte(s)
DPBS	-	Dulbecco's PBS
FACS	-	fluorescence activated cell sorter / sorting
G-CFC	-	granulocyte CFC
G-CSF	-	granulocyte CSF
GM-CFC	-	granulocyte/macrophage CFC
GM-CSF	-	granulocyte/macrophage CSF
HPP-CFC	-	high-proliferative-potential CFC
IDC	-	interdigitating cell(s)
IFN-	-	interferon-
IL-	-	interleukin-
LC	-	Langerhans cell(s)
LIF	-	leukemia inhibitory factor
LPS	-	lipopolysaccharide
mAb	-	monoclonal antibod(y)(ies)
M-CFC	-	macrophage CFC
M-CSF	-	macrophage CSF
MHC	-	major histocompatibility complex
multi-CSF	-	= IL-3
MW	-	molecular weight
NT	-	5'-nucleotidase
PBS	-	phosphate buffered saline
PGE_2	-	prostaglandin E_2
PHSC	-	pluripotent hemopoietic stem cell(s)
SDS	-	sodium dodecyl sulfate
TGF	-	transforming growth factor
TNF	-	tumor necrosis factor
VC	-	veiled cell(s)

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