ROLE OF CYTOKINES IN ALVEOLAR MACROPHAGE DIFFERENTIATION

Huixin Yang

Department of Microbiology and Immunology
Faculty of Medicine
University of Ottawa
Ottawa, Ontario, Canada

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ABSTRACT

The effects of macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) on rat alveolar macrophage (AM) differentiation in vitro were investigated in this study. Both M-CSF and GM-CSF triggered AM differentiation and multinucleated giant cell (MGC) formation. Morphological analysis further demonstrated that there were two distinct variants of MGC. Type 1 MGC normally contained 3 to 8 nuclei and appeared as a large round cell and type 2 MGC contained a higher number of nuclei (up to 30) and displayed irregular, elongated shapes. We also observed that a greater proportion of type 2 MGC expressed β_3 integrin, thus bringing additional evidence for differences between type 1 and type 2 MGC.

Assessment of the relative proportion of type 1 and type 2 MGC indicated that M-CSF induced the formation of both types of MGC to a similar extent and GM-CSF induced predominantly the formation of type 2 MGC. Experiments with anti-M-CSF or anti-GM-CSF antibody to neutralize and cross-block the effects of M-CSF and GM-CSF further confirmed that M-CSF is associated with type 1 MGC formation whereas GM-CSF is responsible for type 2 MGC formation. Type 2 MGC seen in M-CSF treated groups may result from endogenous production of GM-CSF induced by M-CSF. This is supported by RT-PCR experiments in which M-CSF was shown to stimulate GM-CSF mRNA expression.

Molecular phenotyping of a set of cytokines known to participate in inflammation and AM regulation was performed using RT-PCR at various times (up to 5 days) of AM differentiation. AM freshly obtained by BAL (0 time) did not show mRNA expression of TNF-α, IL-1α and IL-6, indicating these proinflammatory cytokines are not constitutively expressed by rat AM. Compared to the controls, both M-CSF and GM-CSF increased mRNA expression of TNF-α and IL-1α, suggesting these 2 cytokines are involved in M-CSF or GM-CSF induced AM differentiation. A significant increase of IL-6 mRNA expression was observed only in GM-CSF treated groups and the expression appeared early and persistently at all time points studied. Experiments with exogenous IL-6 and antibody to IL-6 receptor further indicated that IL-6 is involved in type 2 MGC formation. TGF-β mRNA was constitutively expressed by rat AM and further enhanced by M-CSF and GM-CSF. Results from exogenous TGF-β suggested that this cytokine favors formation of type 1 MGC over type 2 MGC.

Cytoplasmic expression of TNF-α, PDGF and TGF-β was investigated using immunocytochemical procedures. MGC were found to be able to express these cytokines, suggesting that MGC are a functional population rather than merely dead-end cells. Interestingly, type 1 MGC always showed higher levels of these cytokines than type 2 MGC, suggesting that type 1 MGC may be functionally more active than type 2 MGC.

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LIST OF ABBREVIATIONS

AM	alveolar macrophage
AMV	avian myeloblastosis virus
BAL	bronchoalveolar lavage
BN	bombesin
bp	base pair
CT	calcitonin
CTMT	complete tissue culture medium
DEPC	diethyl pyrocarbonate
ELAM	endothelial leukocyte adhesion molecule
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
GM-AM	GM-CSF-derived alveolar macrophage
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRP	gastrin-released peptide
GuSCN/ME	guanidine isothiocyanate solution/2-mercaptoethanol
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HN	histogranin
ICAM	intercellular adhesion molecule
IFN	interferon

IGF	insulin-like growth factor
IgG	immunoglobulin gamma
IL	interleukin
IMDM	Iscove's modified Dulbecco medium
KDa	kilodalton
LFA	lymphocyte functional antigen
LPS	lipopolysaccharide
LT	leukotriene
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
2-ME	2-mercaptoethanol
MDGFs	macrophage-derived growth factors
MGC	multinucleated giant cell
MLR	mixed lymphocyte reaction
mRNA	messenger ribonucleic acid
m.w.	molecular weight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PKC	protein kinase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte

RA	retinoic acid
RMA	rat macrophage activator
RT	reverse transcription
SDS	sodium dodecyl sulfate
SEM	standard error of mean
[Ser^1]HN	chemically stable analog of histogranin
TBE	Tris base/Boric acid/EDTA
TCM	tissue culture medium
TGF	transforming growth factor
TNF	tumor necrosis factor
UICC	union internationale centre le cancer

1. INTRODUCTION

Macrophages are a population of ubiquitously distributed mononuclear phagocytes responsible for numerous homeostatic, immunological and inflammatory processes. They are recognized as being pluripotential and their wide tissue distribution makes these cells well suited to provide an immediate defence against foreign elements by participating in both specific immunity via antigen presentation and interleukin-1 (IL-1) production and nonspecific immunity against bacteria, viruses, fungi, neoplastic pathogens, organic and inorganic particles (Seljelid and Eskeland 1993, Stein and Keshav 1992, Rutherford and Schook 1992a). However, it is also becoming apparent that macrophages do not perform all these different functions at the same time. Rather they exhibit selective adaptation to the local environment by acquiring specific groups of coordinated activities analogous to distinct phenotypes. Circulating monocytes provide a source of macrophages by migrating into tissue and undergoing further differentiation. There are a number of distinct pathways for the macrophage differentiation and maturation process that are directed by both exogenous and endogenous regulatory factors (Stewart et al. 1994, Naito 1993, Henson and Riches 1994, Falk and Vogel 1990). Macrophages are designated differently depending on their tissue localization. In the lung alveoli, macrophages are termed alveolar macrophages (AM).

1.1. ALVEOLAR MACROPHAGES

Alveolar macrophages (AM) reside on the alveolar epithelial surface, where they provide phagocytic defence against invasive agents and perform a variety of functional activities (Sibille and Reynolds 1990, Nathan 1987). The AM can be defined as being a distinct population of macrophages because of their common location on the alveolar surface and because they have been shown to possess unique antigens not found on other body macrophages (Rumpold et al. 1982, Godleski et al. 1984). However, many studies have demonstrated marked heterogeneity among AM harvested by bronchoalveolar lavage (BAL) in terms of their morphologic, biophysical, biochemical, and functional characteristics, as well as in terms of their cell surface profile of antigens and other constituents (Shellito and Kaltreider 1984, Nibbering et al. 1987, Chandler et al. 1984 and 1986, Lemaire and Lemay 1985). Normal AM from rodents display smooth or ruffled surface membranes with ruffled cells indicating a high capacity for phagocytic and chemotactic activity (Warheit et al. 1984). Alveolar macrophage morphology is also characterized by a lobulated nucleus and vacuolated cytoplasm containing several mitochondria and electron-dense secondary lysosomes (Sibille and Reynolds 1990).

The basis of macrophage diversity has not been clearly resolved, but AM heterogeneity may be due to the coexistence of cells in various stages of differentiation / maturation that are derived from a common mononuclear cell lineage and/or the presence of subset populations of AM derived from

macrophage precursors of distinctly separate cell lineages (Bursuker and Goldman 1983, Fels and Cohn 1986). Macrophage heterogeneity may result from a transient expression of functions during differentiation. Phagocytic capacity, cytotoxicity, expression of transferrin receptor, chemotactic responses, and the production of various molecules associated with inflammation, such as plasminogen activator, inhibitor of fibrinolysis, complement factor C2, and interferon, have all been shown to be expressed maximally at specific stages of differentiation (Sorg 1982, Alpert et al. 1983, Neumann and Sorg 1980). Migration of peripheral blood monocytes into the alveoli, migration of interstitial macrophages into alveoli, and the local proliferation of resident AM have all been postulated as being major processes by which the stability of the AM population is maintained (Tarling et al. 1987, Shellito et al. 1987, Bowden and Adamson 1980, Sawyer 1986). Additionally, environmentally related factors in the alveoli may also contribute substantially to the physical and functional characteristics and heterogeneity of AM (Takemura et al. 1989, Lee et al. 1989). Whether different subpopulations of AM, however defined, have different roles in the clearance and retention of invading agents, and in the production of cytokines which direct locally an inflammatory or fibrogenic reaction, remains to be determined. Some in vitro studies in which the functions of density-defined AM subpopulations have been examined, however, suggest that the least dense AM are somewhat less capable of phagocytosing of some types of particles, and they migrate less

actively in response to chemotactic stimuli compared to AM with a higher density (Oghiso 1987, Brannen and Chandler 1988).

1.2. AM AND CYTOKINE PRODUCTION

AM are a major source of many cytokines involved in immune responses, inflammation and homeostatic processes. Upon stimulation by micro-organisms, microbial products or endogenous factors including cytokines themselves, AM can de novo synthesize and release a large variety of cytokines including macrophage colony-stimulating factor (M-CSF) (Becker et al. 1989), granulocyte-macrophage colony-stimulating factor (GM-CSF) (Agostini et al. 1992), interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), (Strieter et al. 1989, Becker et al. 1989, Beulter et al. 1987, Thomassen et al. 1991, Ulich et al. 1991), platelet-derived growth factor (PDGF) (Bonner et al. 1991), and transforming growth factor (TGF)-β (Denholm and Rollins 1993). In this respect, AM may be regarded as "cytokine factories". On the other hand, AM are also the "target" cells for the cytokines. Some cytokines produced by AM can in turn affect AM and upregulate the production of cytokines while others can inhibit it. Thus cytokines can modulate AM functions and surface marker expression, and can recruit monocyte-macrophages within tissue. They are involved in the pathogenesis of either acute or chronic pulmonary inflammation, granuloma or fibrosis formation via autocrine regulation, as well as paracrine activation of other cells such as

polymorphonuclear leukocytes (PMN), fibroblasts, epithelial cells, or endothelial cells (Howarth et al. 1994, Eller et al. 1994).

1.2.1. OVERVIEW OF CYTOKINE BIOLOGY

The cytokines are extracellular signalling proteins secreted by specific effector cells. These molecules have the ability to modify the behaviour of other closely adjacent cells. The short extracellular distances over which cytokines travel before interacting with target cell surface receptors distinguish them in an important way from the circulating endocrine hormones (Balkwill 1993, Dallman et al. 1991). Cell-to-cell interactions mediated by cytokines have been classified as either paracrine or autocrine in nature. The cytokines, although first recognized for their capacity to modulate proliferation, can also affect other cellular functions such as cytoskeletal arrangement and cell shape, contractility (Berk et al. 1986), and the production of extracellular matrix proteins (Ignotz and Massague 1986, Kovacs 1991). Many of the cytokines exhibit potent activity as chemotaxins. This property is a very common feature of the cytokines, having been demonstrated for PDGF, IL-1, TNF-α, TGF-β as well as others (Nielsen et al. 1994).

Interactions among cytokines and the existence of a cytokine network has recently become apparent. The actions of combinations of cytokines are complex and cannot be predicted based on knowledge of the action of individual cytokines. To add to the complexity of cytokine biology, the individual cytokines have overlapping as well as distinct sets of biological

actions (Warren 1992). Moreover, their ultimate effects may vary depending on the pre-existing potential of the target cell. The growing awareness of these complex interactions has led to the concept that tissue homeostatic mechanisms are controlled by cytokine cascades and networks rather than by individual cytokines (Kohase et al. 1987). Each cytokine acts on a particular cell by interacting with specific high affinity receptors. These receptors are usually glycoproteins integrally located within the cell membrane and having extracellular, transmembrane, and intracellular domains. Cytokine receptors are linked to one or more intracellular pathways involved in second messenger signals (Meager 1991).

1.2.2. COLONY STIMULATING FACTORS

There are four well characterized colony-stimulating factors (CSFs) that influence the development of bone marrow precursor cells. These CSFs include multi-CSF (IL-3), macrophage-CSF (M-CSF or CSF-1), granulocyte CSF (G-CSF) and granulocyte-macrophage CSF (GM-CSF). Among these four CSFs, M-CSF and GM-CSF are more related to monocyte/macrophage differentiation and maturation.

1.2.2.1. Macrophage colony-stimulating factor (M-CSF)

M-CSF is produced by monocytes, macrophages, fibroblasts, epithelial cells and endothelial cells (Clark and Kamen 1987). Molecular cloning data for human M-CSF suggest the existence of two mRNA species. The predominant transcript of 4.0 kb yields a 61 kDa precursor of 554 amino acids which is

processed by removing a 32 amino acid signal peptide and a 333 amino acid carboxyl-terminal peptide to yield the mature 189 amino acid form. The mature polypeptide contains two potential N-glycosylation sites and is secreted as a disulphide-linked homodimer (Welte et al. 1985). The molecular weight of this form is 70-90 kDa for the intact dimer. The smaller 1.8-2.0 kb mRNA transcript codes for a disulphide-linked homodimer of 36-52 kDa containing sequences suggestive of a membrane anchor (Lu et al. 1989). A second, higher molecular weight species of M-CSF has been identified. This protein appears to be a heterodimer of the 43 kDa M-CSF monomer and a 150-200 kDa proteoglycan form of M-CSF formed through alternative splicing (Suzu et al. 1992).

Examination of murine and human M-CSF suggests similarities in structure. M-CSF from both species promotes differentiation and proliferation of monocyte-macrophage progenitors in the bone marrow. Furthermore, human M-CSF has been found to be active on murine cells, although murine M-CSF does not seem to act on human cells (Wong et al. 1987). The ability of M-CSF to stimulate bone marrow progenitors is increased by low concentrations of GM-CSF. It has been suggested that GM-CSF enhances the action of M-CSF by stimulating M-CSF receptor expression (Caracciolo et al. 1987, Broxmeyer et al 1987).

In addition to its effect on the commitment of progenitor cells to the monocyte-macrophage line, M-CSF is also required for the survival of circulating monocytes and tissue macrophages. Furthermore, M-CSF stimulates numerous

immune effector functions of mature macrophages. The immune functions enhanced by M-CSF include increased phagocytic activity and microbial killing (Karbassi et al. 1987), improved resistance to viral infection (Lee and Warren 1987), tumoricidal activity (Ralph and Nakoinz 1987) and the enhancement of antibody-dependent cell-mediated cytotoxicity by monocytes and macrophages (Mufson et al. 1989). Other activities of M-CSF include inhibition of bone resorption by osteoclasts, stimulation of microglial cell proliferation and regulation of placental function via action of decidual cells and trophoblasts (Hattersley et al. 1988). While many of these functions are the direct result of M-CSF activity, some of these actions may be secondary to the increased viability of the macrophages which in turn can produce other cytokines. Macrophages produce IL-1 (Moore et al. 1980), interferon and TNF (Warren and Ralph 1986) in response to M-CSF. Macrophages also produce M-CSF and AM in particular constitutively produce M-CSF which is believed to play a role in the maintenance of the AM population (Becker et al. 1989, Ogawa et al. 1994).

M-CSF exerts its effects through a specific receptor found on virtually all mononuclear phagocytes. Binding is followed by rapid internalization, after which the receptor is recycled back to the membrane surface. M-CSF receptor, a c-fms proto-oncogene product, is a tyrosine kinase capable of auto-phosphorylation (Metcalf 1991, Gliniak and Rohrschneider 1990). Cytokines such as IL-1, IL-3 and IL-6 induce M-CSF receptor expression of the bone marrow precursors (Chen and Clark, 1986, Ikebuchi et al. 1987). In addition to

cytokine-induced upregulation, M-CSF receptor levels increase with monocyte maturation, and high levels of receptors are present on mature macrophages.

1.2.2.2. Granulocyte/macrophage colony-stimulating factor (GM-CSF)

GM-CSF stimulates the production of both granulocytes and macrophages in cultures of human and murine bone marrow cells. Murine GM-CSF is a 23 kDa glycoprotein secreted by activated T cells, endothelial cells, fibroblasts, mast cells, B cells, and macrophages (Burgess and Metcalf 1980, Zupo et al. 1992). Molecular cloning data suggest that the unmodified peptide of 118 amino acids has a molecular weight of 13.5 kDa with two potential N-glycosylation sites. Human GM-CSF has a molecular weight of 18-22 kDa. Cloning information indicates that the mature polypeptide is processed from a 144 amino acid precursor containing a 17 amino acid signal peptide. The 127 amino acid cleavage product has two potential N-glycosylation sites, is 60% homologous with the murine protein sequence and contains four highly conserved cysteine residues (Wong et al. 1985). Murine and human GM-CSF are species-specific in their activities but it has been documented that murine GM-CSF acts on rat cells (Chen et al. 1994).

GM-CSF production and secretion occurs in response to both humoral and physical stimuli. IL-1, IL-2, TNFα and LPS have all been shown to induce GM-CSF production (Sieff et al. 1987, Broudy et al. 1986, Rennick et al 1987). In contrast to M-CSF, AM do not appear to produce GM-CSF constitutively (Rose et al. 1991, Agostini et al, 1992). Of interest, fibronectin, which binds

to specific receptors on macrophage membranes and can act as a surface for macrophage attachment and migration, and the process of phagocytosis both induce GM-CSF gene expression in macrophages (Thorens et al. 1987). Gamma interferon and the anti-inflammatory agent dexamethasone, however, have been shown to inhibit LPS-induced increase in GM-CSF mRNA by macrophages (Thorens et al. 1987).

The biological effects of GM-CSF are mediated through binding to a high affinity receptor. Both in humans and in the mouse, these receptors are found on cells of the monocytic lineage (Walker and Burgess 1985, DiPersio et al. 1988, Gasson et al. 1986). Like the other CSFs, GM-CSF is internalized upon binding to its receptor (Nicola 1987). The human and murine GM-CSF receptors have been cloned and are composed of a ligand-binding low-affinity chain (α-subunit) and a non-ligand-binding chain (β-subunit) (Park et al. 1992). The α-subunit chain is a 378 amino acid protein with a molecular weight of 85 kDa. The murine and human α-subunits exhibit 35% amino acid similarity. The GM-CSF β-subunit is homologous to the 130 kDa subunit of the IL-5 receptor (murine and human) and the IL-3 receptor (human only) (Park et al. 1992).

GM-CSF has been shown to be an effective proliferative stimulus in marrow cultures of granulocyte, macrophage, and eosinophil colonies. Furthermore, GM-CSF acts in synergy with erythropoietin to increase erythroid and multipotential colony formation. Experiments also suggest that GM-CSF acts in concert with other cytokines such as G-CSF and M-CSF to exert its full

spectrum of activities (Bot et al. 1989). It is also capable of stimulating antibody-dependent cytolysis of tumour cells. GM-CSF has been shown to induce the formation of neutrophilic granulocyte, mixed granulocyte/macrophage, pure macrophage and pure eosinophil colonies (Tomonaga 1986). Other activities of GM-CSF include neutrophil migration inhibition activity (Gasson et al. 1984), potentiation of neutrophil responses to physiologic stimuli (Naccache et al. 1988), burst-promoting activity for BFU-E (Sieff et al. 1985), enhancement of antibody-dependent killing by eosinophils (Silberstein and David 1987), promotion of human endothelial cell migration and proliferation (Bussolino et al. 1989), and induction of proliferation of marrow fibroblast precursors (Dedhar et al. 1988).

1.2.3. CYTOKINES AND LUNG INFLAMMATION

During lung inflammation in response to mineral dusts, one of the most consistent features is the presence of accumulating AM (Lemaire 1995). AM are major cells in the inflammatory locus and they act as primary effector cells in the inflammatory reaction with the other cell populations, including lymphocytes and neutrophils. They are key players in the cytokine network, regulating host defence immunological and inflammatory reactions by providing various cytokines, including IL-1, IL-6, TNF-α, PDGF and TGF-β, etc. (Thivierge and Rola-Pleszczynski 1994). Asbestosis and silicosis are diseases caused by chronic exposure to asbestos and silica and are associated with chronic inflammatory reactions characterized by accumulation of macrophages,

including epithelioid and giant cells (Lemaire 1991a, 1995). Several laboratories have explored the role of cytokine production in asbestos and silica-induced lung injury. In response to these inorganic dust particles, AM are recruited to the sites of deposition and are known to secrete reactive oxygen radicals, which are thought to play a role in asbestos- and silica-induced injury (Vallyathan et al. 1988, Thomas et al. 1994). Moreover, it has been shown that silica is able to trigger the secretion of IL-1, IL-6, TNF-α at higher levels than those observed after inert mineral exposure to titanium oxide (Gosset et al, 1991, Schmidt et al. 1984). In addition, it has been shown that AM incubated in the presence of asbestos or silica, produced both leukotriene B4 (LTB4) and TNF-α, and LTB4 could act in vitro to amplify TNF-α production (Dubois et al. 1989). Lemaire et al. (1985a) have previously investigated the kinetics of TNF-α production by AM during the development of asbestos-induced fibrosis. Spontaneous TNF-α production by AM was enhanced only 6 weeks after asbestos exposure subsequent to the appearance of fibrotic lesions. Surprisingly, suppression of LPS-induced TNF release was observed 1 and 3 weeks after asbestos exposure. However, the reduced TNF production did reverse during the course of disease and was significantly increased by 6 weeks (Ouellet et al. 1993). This suggests bidirectional modulation of TNF characterized by an inability to prime AM for TNF production at some stages during the development of asbestos-induced fibrosis. Interestingly these changes were correlated with changes in AM-populations (Lemaire 1995). The

biological relevance of such bidirectional regulation is unknown. Whether TNF should be considered a mediator of pathogenesis or part of a protective mechanism remains an unresolved issue. TNF has been shown to induce lung granulomas and neutrophil infiltration (Kasahara et al. 1989) and to inhibit wound healing (Rapala et al. 1991). Inhibition of TNF in the first stage may represent an anti-inflammatory action and may result in increased effects of fibrogenic cytokines (Steenfos et al. 1989) in an attempt to promote repair of damaged tissue. Up-regulation of TNF at later stages of the reaction may contribute to maintain the inflammatory response. Such repeated episodes of up- and down-regulation of TNF may set a profile for abnormal repair.

Using the same experimental protocol, the kinetics of IL-1 and IL-6 production were also investigated over a 6-week period (Lemaire and Ouellet 1996). IL-1 and IL-6 levels increased concomitantly during the first stages of disease (1 and 3 weeks) and decreased thereafter (6 weeks) although the levels remained higher than those of controls. Changes in IL-1 and IL-6 were inversely correlated with TNF response, suggesting an in vivo interaction among these cytokines. There is a strong evidence in support of a network of cytokine interaction. IL-1 has been shown to induce IL-6 (Navarro et al. 1989), and TNF triggers production of IL-1 and IL-6 (Sheron et al. 1990). IL-6 in turn was reported to inhibit TNF production (Aderka et al. 1989). Therefore, it is likely that during inflammatory responses, IL-1, TNF, and IL-6 act in coordination by regulating one another's production, and thereby direct the outcome of

inflammatory reactions.

During inflammation, migration and adhesion of AM and other cells are controlled by cytokines secreted by AM as well. They are attracted by TGF-β, PDGF, IL-1, TNF-α (Ming et al. 1987, Dinarello 1989). Both TNF-α and IL-1 induce slower changes in expression of adhesive molecules such as endothelial leukocyte adhesion molecule (ELAM)-1 and intercellular adhesion molecule (ICAM) on endothelial cells, which makes them more adhesive to macrophages and neutrophils, as well as to lymphocytes (Beutler and Cerami 1988; Bevilacqua et al. 1989). IL-1 induces production of stromelysin in fibroblasts and macrophages, modulating the ability of these cells to move through the vascular membrane and interstitial extracellular matrix (Frisch and Ruley 1987). TNF-α causes edema, which may be commensurate with increased transmigration of the macrophage vessel wall (Bavilacqua et al. 1989; Brett et al. 1989). In addition, M-CSF and IL-4 increase the expression of two types of plasminogen activator synthesized by macrophages (Hart et al. 1989). Other cytokines such as GM-CSF and G-CSF can induce endothelial cells to produce CSFs and alter their procoagulant ratios to become more adhesive to leukocytes and lymphocytes and to further wall-off wounds (Bussolino et al. 1989).

1.2.4. CYTOKINES AND LUNG FIBROSIS

The investigation of the interaction between immune and mesenchymal cells expanded from wound-healing to fibrotic disorders of the lung, and focused on macrophage-derived growth factors (MDGFs) which are released by

AM and induce the proliferation of fibroblasts, and the production of collagen. Using an ex vivo approach that consisted of collecting AM exposed in vivo to asbestos and culturing them in vitro in the presence of lung fibroblasts, Lemaire et al. have demonstrated that asbestos exposure enhanced the release of fibroblast growth factor (FGF) by sheep and rat AM (Lemaire et al.1983, 1985b). A similar finding was reported in experimental silicosis (Lugano et al.1984), thus supporting a role for MDGFs in lung fibrosis. In addition, stimulation of FGF release from AM was a prolonged phenomenon and was correlated with the presence of fibrotic lesions (Lemaire et al. 1985b, 1986a). Based on these observations, Lemaire et al. (1986b) suggested that production of FGF under rate-limiting conditions during normal repair process is beneficial, whereas chronic stimulation of FGF release may contribute to the uncontrolled proliferation of fibroblasts in asbestotic lesions. Enhanced production of FGF from AM has also been observed in a model of silicosis (Gritter et al. 1986). Subsequent biochemical analysis further determined that MDGFs are cytokines with fibrogenic effects. While the literature suggests that many cytokines, such as IL-1 (Oppenheim et al. 1986), IL-6 (Piguet et al. 1990), TNF (Ouellet et al. 1993), fibroblast growth factor (FGF) (Baird et al. 1985), and insulin-like growth factor I (IGF-I) (Noble et al. 1991, Rom et al. 1988), play a role in fibroblast growth and collagen synthesis, PDGF and TGF-β have been reported to be highly fibrogenic (Sporn and Roberts 1988), and trigger directly the proliferation of fibroblasts and/or stimulate the production of connective tissue.

Characterization of cytokines elaborated by activated macrophages based on their interaction with target cells allowed the distinction to be made between cytokines that directly induce the proliferation of fibroblasts and those that require additional mediators, such as another cytokine or prostaglandins, to stimulate cell growth. Assays used to assess the proliferative response of fibroblasts to cytokines have demonstrated that PDGF and TGF-β directly stimulate in vitro DNA synthesis and replication of fibroblasts. Recent in vitro experiments have indicated that asbestos induces PDGF production by AM (Bauman et al. 1990, Schapira et al. 1991). AM-derived PDGF is of particular interest with respect to lung fibrosis because it has both chemotactic (Osornio-Vargas et al.1990) and mitogenic (Kumar et al. 1988) effects on fibroblasts and smooth muscle cells, two cell types known to accumulate at sites of lesions. Furthermore, AM-derived PDGF has been found to be bound to α_2-macroglobulin, which enhances its mitogenic capacity for fibroblasts (Bonner et al. 1990).

TGF-β is another cytokine which is released by AM and may play an important role in lung fibrosis (Khalil et al. 1989). In addition to chemotactic and proliferative effects on fibroblasts, it also stimulates the synthesis of collagen. Analysis of TGF-β staining in pulmonary macrophages from rats given intratracheal bleomycin reveals that peak TGF-β levels precede maximal collagen synthesis (Hoyt and Lazo 1989). These data suggest not only that the mediators released in culture are present in situ, but also that there is temporal

relationship between the production of TGF-β and the accumulation of connective tissue.

Other growth factors of interest with regard to pulmonary fibrosis are insulin-like growth factor (IGF)-1 and bombesin (BN) [gastrin-released peptide (GRP)]. AM exposed to asbestos have been shown to express IGF-1 mRNA transcripts and release IGF-1-like molecules (Rom et al. 1988). Previous work done in this laboratory revealed that asbestos-exposed rats exhibited increased levels of bombesin in lungs. Isolated lung cell fractions which were greatly enriched for macrophages from asbestos-exposed rats contained high amounts of immunoreactive BN (Day et al. 1985). In addition, Lemaire and co-workers observed that AM from rats exposed to fibrogenic or nonfibrogenic preparations of asbestos released higher levels of BN-like peptide (Lemaire et al. 1991). These changes were correlated with an increase in the proportion of low-density AM, indicating that production of BN may be related to the state of AM differentiation. BN has been shown to enhance IL-1 release by activated AM (Lemaire 1991b) and to synergize with insulin-like growth factors in stimulating fibroblast proliferation (Corps et al, 1985). In that context, BN may act in concert with IGF-1 and/or other growth factors to amplify and maintain the fibroblastic response.

Other studies have suggested that TNF-α plays a role in the pathogenesis of pulmonary fibrosis. The levels of TNF-α mRNA in the lungs of mice after intratracheal administration of bleomycin were increased (Hoyt and Lazo 1989).

Further experiments showed that the diffuse alveolar damage and the increase in total lung hydroxyproline content observed after bleomycin administration could be diminished by administration of anti-TNF-α antibody. Similar observations were made with silica-induced lung injury (Piguet et al. 1990). These studies, however, do not prove that TNF-α is a direct mediator of fibrosis, rather that the role(s) of other mediators may be dependent on TNF-α. Moreover, TNF-α has been reported to act both as a growth inducer and inhibitor (Thornton et al. 1990). At low concentrations, it stimulates fibroblast proliferation and at high concentrations it blocks growth triggered by other cytokines.

In addition, IL-6 has been detected in silicotic nodules of mice (Piguet et al. 1990). In agreement with this, Lemaire et al. (1994) observed that spontaneous as well as LPS-induced IL-6 release from AM were greatly enhanced up to 6 weeks after a single exposure to asbestos.

1.3. MULTINUCLEATED GIANT CELLS

1.3.1. OVERVIEW

As mentioned above, blood monocytes undergo a process of differentiation to macrophages when they migrate into the tissues. Macrophages are not terminal cells. They may undergo further maturation and activation after exposure to certain stimuli, particularly during pathogenesis. Thus in granulomatous reactions, some macrophages differentiate to form

multinucleated giant cells (MGC) (Sone et al, 1981, van der Rhee et al. 1979). The formation of MGC is thought to represent a specialized form of cells of monocyte-macrophage lineage that undergo morphologic changes to form epithelioid cells which subsequently fuse and differentiate into MGC (Kreipe et al. 1988, Murch et al. 1981). Ample evidence exists that MGC are associated with chronic inflammatory diseases such as sarcoidosis, rheumatoid arthritis, tuberculosis, leprosy, schistosomiasis, syphilis, and various fungal and parasitic infections (Mariano and Spector 1974). In addition, MGC are observed in response to nonbiological stimuli including asbestos, silica, beryllium, carbon, and iron particles (Lemaire 1985, Postlethwaite et al. 1982). Lemarie (1991a) has shown that MGC were involved in resolving granulomatous lesions, while these cells were not found in a fibrogenic response, suggesting that MGC may play a role in directing inflammatory reactions.

1.3.2. MECHANISM(S) AND CAUSATIVE AGENTS FOR MGC FORMATION

Considerable evidence exists to suggest that MGC are formed by the fusion of cells rather than abnormal cell division, i.e. nuclear division without cytoplasmic division (Murch et al. 1981, Abe et al. 1983). However, the mechanism(s) of their formation and the involvement of cell surface proteins in that process are not understood as yet (Ejiri et al. 1987, Baskar et al. 1994). MGC formation in human monocytes can be inhibited by protein kinase C (PKC) inhibitors, staurosporine and H7, and the calcium channel blocker verapamil,

implying that MGC are formed by a signal-driven differentiation of cells (Orentas et al. 1992). Evidence is presented that MGC derived from rat AM express a high number of functional receptors for calcitonin (CT), a peptide hormone, which indicates that CT may be involved in MGC formation through the regulation of calcium concentration (Vignery et al. 1991). Monoclonal antibody (mAb) to the α or β chain of LFA-1 and mAb to ICAM-1, one of the ligands of LFA-1, inhibit MGC formation of human monocytes, suggesting a role for adhesion molecules in cell fusion (Most et al. 1990). Recent findings with a murine anti-rat macrophage activator (RMA) antibody which was produced by injection of female Lewis rat AM into male CB6F/J mice, suggests that the binding of this antibody to a 120 kDa surface membrane antigen expressed by rat AM may activate DNA synthesis, and promote clustering and fusion of AM, leading to MGC formation (Lazarus et al. 1990).

A few studies have explored the causative agents involved in MGC formation. Human peripheral blood monocytes cultured in the presence of IFN-gamma (IFN-γ) have been shown to form MGC (Weinberg et al. 1984, Takashima et al. 1993). Phorbol myristate acetate (PMA), Simian immunodeficiency virus SIVsmmPBj 1.9, antibody to class II MHC molecules and conditioned medium generated through concanavalin A (Con A) stimulation of lymphocytes or a mixed lymphocyte reaction (MLR) have all been reported to increase human monocyte fusion and subsequently MGC formation (Hassan et al. 1989, Baskar et al. 1994, Orentas et al. 1992). 1,25-

dihydroxycholecalciferol (1,25-$(OH)_2D_3$) and retinoic acid (RA) have been shown to moderately enhance fusion of murine AM (Abe et al. 1984) and, to a lesser extent, human monocytes (Ohta et al. 1986). Similarly, interleukin-4 (IL-4) appears to induce fusion of murine bone marrow cells (McInnes and Rennick 1988) and has been shown to enhance the differentiation of human monocyte/macrophages and MGC formation (Essner et al. 1989, Te Velde et al. 1988). IL-3 has been demonstrated to cause formation of osteoclasts, a cell closely related to the MGC, from murine bone marrow cells (Barton and Mayer 1989), and its role as an activator of human monocyte/macrophages has been the subject of recent interest (Frendle and Beller 1990, Frendle et al. 1990). A combination of IL-3 and IFN-γ has been shown to increase human monocyte fusion and MGC formation (Enelow 1992). IL-6 has been suggested to enhance MGC formation from human monocytes (Hassan et al. 1990). TNF-α has been implicated in the formation of granuloma in response to mycobacterial infection, and its enhanced effects on MGC formation of human blood monocytes have been reported (Takashima et al 1993). Granulocyte-macrophage-colony stimulating factor (GM-CSF) has been reported to be a major macrophage fusion factor present in conditioned medium of concanavalin A-stimulated spleen cell culture (Abe et al. 1991). To our knowledge, however, the direct effects of M-CSF and GM-CSF in MGC formation from rat AM have not been investigated.

1.3.3. FUNCTION AND CHARACTERISTICS OF MGC

The function of MGC is still unclear. MGC may function as antigen-presenting cells (Papadimitriou and Van Bruggen 1986). Whether they represent "activated" macrophages (Schelesinger et al. 1984) or are merely a disposal device for metabolically exhausted macrophages (Mariano and Spector 1974) is unknown. In comparison with unfused macrophages, MGC have been found to exhibit enhanced activity for α-naphthyl acetate esterase, acid phosphatase, and acid phosphatase tartrate-resistant (Kreipe et al. 1988). A 20-30 fold increase in the production of oxygen-free radicals in response to zymosan has also been shown in MGC. Compared to monocyte-macrophages, however, transcription of the proto-oncogene c-fms is down-regulated in MGC after fusion has occurred, suggesting that MGC represent highly stimulated cells of monocyte-macrophage lineage at a terminal stage of differentiation. Experiments with immunocytochemistry indicate that most of the MGC derived from rat AM are found to accumulate p53, a 53kDa nuclear phosphoprotein encoded by p53 tumour suppressor gene, in cytoplasm, while only few nuclei are stained (Wiethege et al. 1994). The plasma membrane of MGC was found to be enriched in Na-K-adenosinetriphosphatases (ATPases) and the localization of their Na-K-ATPases was restricted to the nonadherent domain of the plasma membrane of cells both in vivo and in vitro, thus imposing a functional polarity on their organization (Vignery et al. 1989). Ultrastructural observations suggested that MGC are single cells but not clustered cells, and their

characteristics are very similar to the basic features of macrophages, except that MGC are poor in lysosomes and phagosomes (Saeki et al. 1994).

1.4. BACKGROUND AND OBJECTIVES

The interactions between AM, cytokine production and modulation are likely to play an important role during the development of chronic lung inflammation. One aspect of AM biology that has not been investigated in detail is the role of AM heterogeneity and/or differentiation in granuloma and fibrosis formation. In previous studies, this laboratory has shown that experimental lung granuloma and fibrosis are associated with selective changes in AM populations and/or state of differentiation. In animals with lung fibrosis there was an increase in proportion of large, more mature AM, whereas resolving granuloma was associated with the presence of MGC (Lemaire 1991a). The presence of AM displaying morphological changes and the occurrence of MGC was observed both in histological sections of lung (Lemaire et al. 1989) and in bronchoalveolar lavage (Lemaire 1985). However, the mechanisms responsible for the regulation of these modified forms of AM as well as the physiological implication of AM derivates are unknown. the precise role of these phenotypically different AM remains to be elucidated.

The formation of MGC may represent an attempt by macrophages to eliminate the dust burden more effectively (Miller, 1978). In this respect, MGC would play a protective role as opposed to a damaging one. MGC may

represent highly stimulated cells of macrophage lineage at a terminal stage of differentiation (Kreipe et al. 1988). However, many questions concerning MGC are left unanswered. What are the biological effects of MGC? What are the conditions that favour formation of MGC? Whether MGC are able to produce certain cytokines and actively participate in inflammatory reactions, or merely represent the end-point of macrophage differentiation, remains unclear. To answer these questions, a simplified and reproducible model for MGC formation is needed.

During the development of lung granuloma and fibrosis, Lemaire et al. have shown the selective changes in AM populations and/or state of differentiation and investigated the production of cytokines that have some relevance to inflammation. Animals with lung fibrosis produced high levels of IL-1 (Lemaire and Beaudoin 1984, Lemaire 1991a), MDGF (Lemaire 1986a, 1986b) and bombesin (BN) (Day et al 1987, Lemaire et al. 1991). However, the profiles of changes found for these cytokines were different. These findings raise important questions concerning the role of AM differentiation, MGC formation and the interplay of cytokines in the maintenance and progression of inflammatory response. In order to elucidate these, I investigated AM differentiation and MGC formation in relation to specific cytokine production using an in vitro model of AM differentiation. The hypothesis was that during the chronic inflammation, AM always appeared in site of lesions. AM activation, differentiation and cytokine production may direct the outcome of the

inflammation. since AM are heterogenous, they may display differences in responses to M-CSF or GM-CSF-induced differentiation and display differences in cytokines' production during the differentiation. More specifically, my objectives were:

(1) to determine the effects of differentiation cytokines (M-CSF and GM-CSF) as well as other cytokines (TGF-β, TNF-α, IL-6) on AM differentiation in vitro and compare the biological differences between M-CSF and GM-CSF in inducing AM differentiation and MGC formation.

(2) to characterize MGC morphologically and explore the possible mechanisms for MGC formation induced by M-CSF and GM-CSF in an in vitro model.

(3) to investigate the relationship between AM differentiation and cytokine production. This should give information on the profile of inflammatory cytokines, such as TNF-α, IL-1 and IL-6, and fibrogenic cytokines such as TGF-β produced during AM differentiation.

(4) to characterize the functional status of MGC, notably their ability to produce cytokines, such as TNF-α, PDGF and TGF-β.

2. MATERIALS AND METHODS

2.1. ANIMALS

Male Wistar rats weighing between 250 and 275 g were purchased from Charles River Canada, Inc. (St-Constant, Québec). These rats were derived from a pathogen-free colony and shipped behind filter barriers. All animals were treated in strict accordance with procedures outlined in the **Guide for the Care and Use of Experimental Animals** endorsed by the Medical Research Council of Canada. Animals were housed 2 per cage in isolated, temperature-controlled quarters in an animal isolator unit (Johns Scientific Inc., Toronto, Ontario) with a 12 hr light/12 hr dark cycle. They were fed with Purina laboratory Chow (Ottawa, Ontario) and water *ad libitum*. The animals were used within 2 weeks of arrival.

2.2. ALVEOLAR MACROPHAGE ISOLATION

Alveolar macrophages (AM) were recovered from rats by bronchoalveolar lavage (BAL) as described previously (Lemaire and Lemay, 1985). The animals received a lethal dose of sodium pentobarbital (100 mg/kg), (M.T.C. Pharmaceuticals Canada Packers Inc., Cambridge, Ontario). The abdominal aorta was severed to exsanguinate the animals, the diaphragm was punctured, and the trachea was cannulated. A total volume of 48 ml of sterile phosphate-buffered saline (PBS, pH 7.4, without calcium or magnesium) in 8 ml aliquots

was infused into the lungs of each animal. The lung surface was gently massaged and the lavage fluid was subsequently withdrawn. The first three aliquots were kept in the lung for 4 min and the final three aliquots for 2 min each. Lavage recovery was at least 95% of the volume infused. The total lavage fluid (BAL) was centrifuged at 200 x g for 10 minutes at 4°C. The cell pellet was resuspended in Iscove's modified Dulbecco medium (IMDM) (Gibco/BRL, Burlington, Ontario) supplemented with 10% dialysed fetal bovine serum (FBS) (Gibco, Grand Island Biological Co. New York, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.8% Hepes (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]), which will henceforth be referred to as complete tissue culture medium (CTCM) unless stated otherwise. Cells were counted using a hemacytometer chamber and the viability was determined by trypan blue dye exclusion. With this procedure, 8-10 x 10^6 cells/animal were consistently recovered and cell viability was more than 97%. In most experiments, cells from each rat were used separately (not pooled with cells from other rats).

2.3. DIFFERENTIAL CELLULAR ANALYSIS

Differential counts of AM population were made from cytocentrifuged smears (Shandon, Johns Scientific Inc., Toronto) prepared with 4 x 10^4 cells in 200 μl tissue culture media containing 20% FBS. The smears were stained with Wright-Giemsa and examined under a light microscope. Differential cellular

analysis has shown that greater than 99% of cells obtained from BAL are of macrophage morphology.

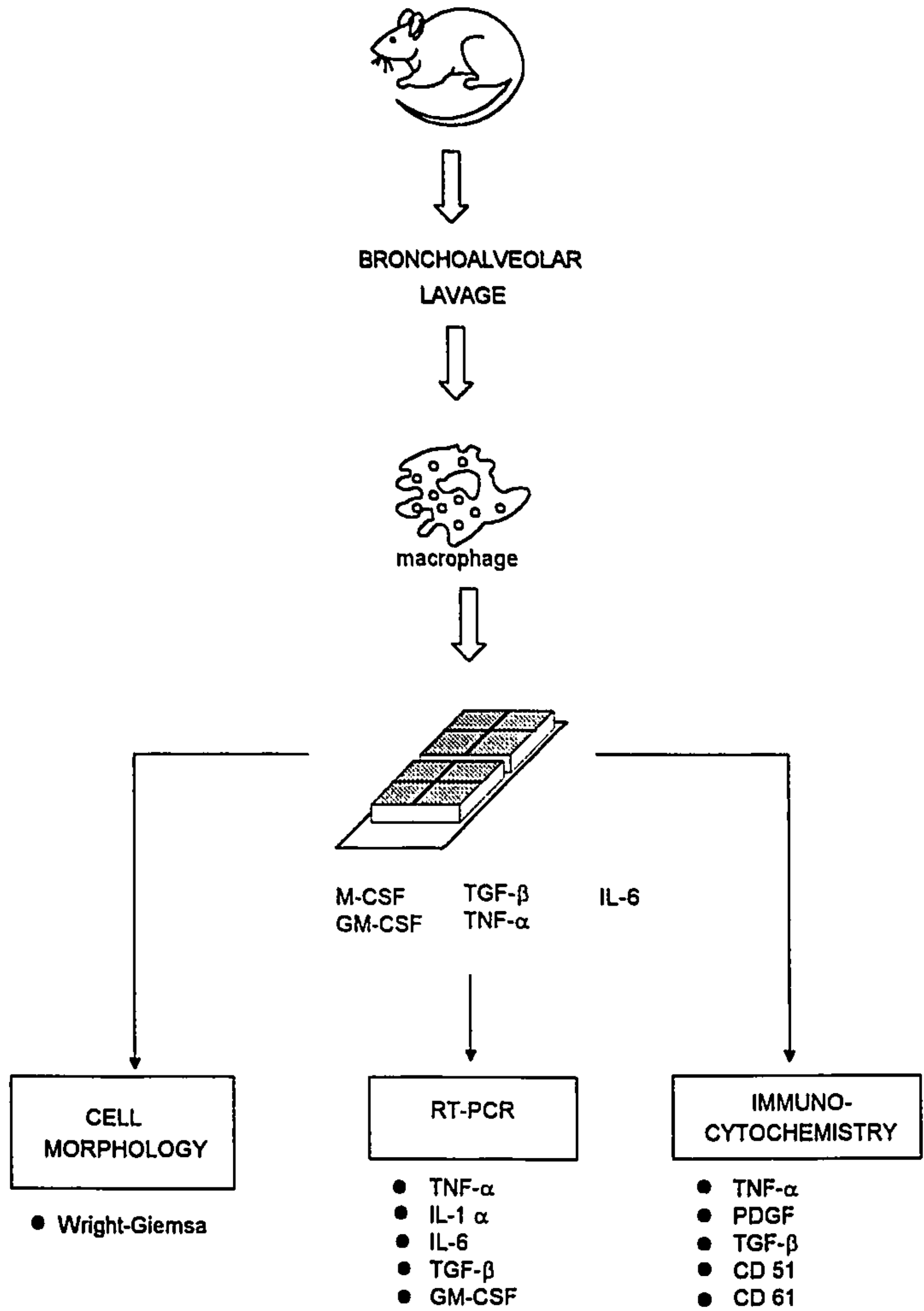
2.4. CELL CULTURE SYSTEM AND AM DIFFERENTIATION IN VITRO

The experimental protocols are schematically shown in Figure 1. The following reagents were used to investigate AM differentiation in vitro and MGC formation: human recombinant macrophage colony-stimulating factor (M-CSF) which has been found to be active on rat cells (Wong et al. 1987) (Genzyme, Cambridge, MA, USA), murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) which can act on rat cells (Chen et al. 1994) (Gibco/BRL, Burlington, Ontario), human recombinant TGF-β (Genzyme), murine recombinant TNF-α (Gibco/BRL), mouse recombinant IL-6 (Genzyme), polyclonal rabbit anti-human M-CSF antibody (Genzyme), polyclonal goat anti-murine GM-CSF antibody (R&D Systems, Minneapolis, MN, USA), polyclonal rabbit anti-mouse TNF-α (Genzyme), and polyclonal rat anti-mouse IL-6 receptor (Genzyme). All these reagents have been found to act on rat AM.

Following BAL, AM were adjusted to 1×10^6 /ml and incubated in a Lab Tek slides (Nunc, Naperville, IL, USA) containing 8 culture chambers. AM (2×10^5 AM in 200 μl medium) were incubated at 37°C under 5% CO_2 in air for various times as indicated in the presence or absence of M-CSF (25-75 U/ml) or GM-CSF (25-75 U/ml). Other cytokines, including TGF-β, TNF-α and IL-6, were added to Lab Tek chambers either alone or in combination with M-CSF or

Figure 1. Experimental protocol. Alveolar macrophages (AM) were recovered from normal rats by bronchoalveolar lavage (BAL) and incubated in Lab Tek chamber slides with different cytokines for various times. Cytokine expression by AM was determined by RT-PCR or immunocytochemistry. Morphological changes were examined by microscope after Wright-Giemsa staining.

EXPERIMENTAL PROTOCOL



GM-CSF. Control groups with no addition of cytokines were carried out in parallel. After incubation, cells were gently washed three times with warm PBS and chambers were disassembled. The slides were stained with Wright-Giemsa and mounted with coverslips and Permount (Fisher Scientific). The presence of MGC with more than 3 nuclei was monitored by light microscopy (magnification x 330) after counting the whole culture well.

2.5. NEUTRALIZATION AND CROSS-BLOCKING WITH ANTIBODY TO M-CSF OR GM-CSF

To examine the respective roles of M-CSF and GM-CSF on AM differentiation in vitro, polyclonal rabbit anti-human M-CSF antibody was used to neutralize M-CSF in the M-CSF (50 U/ml) treated group and polyclonal goat anti-murine GM-CSF antibody was used to neutralize GM-CSF in the GM-CSF (50 U/ml) treated group, respectively and non-specific rabbit or goat IgG was used as control. According to manufacturer's instructions and the calculation of neutralization $dose_{50}$ (ND_{50}), polyclonal rabbit anti-human M-CSF antibody was used at 6.6 μg/ml and polyclonal goat anti-murine GM-CSF antibody was used at 2 μg/ml. The antibodies were incubated with M-CSF or GM-CSF at 4°C for 60 minutes, added to the samples. In some experiments, antibody was added every other day (0, 2, 4 days), and no difference was noticed between these two regimens. To further investigate the effects of M-CSF or GM-CSF on MGC formation and its relationship with the two morphological types of MGC,

cross-blocking experiments were carried out. Polyclonal goat anti-murine GM-CSF IgG was added to M-CSF (50 U/ml) treated groups. Conversely, polyclonal rabbit anti-human M-CSF IgG was added to the groups treated with GM-CSF (50 U/ml). After 5 day incubation in Lab Tek chambers, slides were rinsed with pre-warmed PBS and stained with Wright-Giemsa. The numbers of MGC and their morphology were evaluated under a light microscope (magnification x 330).

2.6 INDUCTION OF LUNG INFLAMMATION IN VIVO

A Union Internationale Contre le Cancer (UICC) standard sample of chrysotile B (21% of fibres longer than 10 μm) was obtained from the National Research Institute for Occupational Diseases, Johannesburg, South Africa. The material was autoclaved for 45 min and suspended in sterile PBS with a Dounce glass homogenizer (Fisher Scientific, Ottawa) before instillation into the animals. Rats received a single transtracheal injection of either saline or UICC chrysotile B (5 mg), as described by Lemaire et al. (1986a). At least four animals in each group were sacrificed 3 and 6 weeks after treatment and were evaluated by bronchoalveolar analyses.

2.7. NORTHERN HYBRIDIZATION ANALYSIS

Measurement of messenger RNA for TNF-α and IL-1α was performed. Total cellular RNA was obtained using the acid guanidinium thiocyanate-

phenolchloroform extraction procedure of Chomczynski and Sacchi (1987). RNA samples (10 μg) were electrophoresed through 1% agarose-formaldehyde/MOPS gels and transferred by capillary action to Pall Biodyne A nylon membranes (Pall Canada Ltd, Mississauga, Ontario, Canada) as described by Fourney et al. (1988). The cDNA probe for human TNF-α (585 bp) was prepared by Hind III and Ava I digestions from plasmid pc DV (ATCC, No 39894) and the cDNA probe for rat IL-1α (920 bp) was prepared by Bam HI and Hind III digestion from plasmid pUC19. The human β-actin probe (850 bp) was obtained from Dr. Izaguirre (AIDS Federal Centre, Ottawa, Ontario). Probe DNA fragments (25 ng) were radiolabeled with α ^{32}P dCTP (3000 Ci/mmol; Dupont/NEN Canada Inc., Mississauga, Ontario) to specific activities 5 x 10^8 dpm/μg using commercial kits (Promega, Madison, WI) for random hexadeoxyribonucleotide synthesis (Feinberg and Vogelstein, 1983). Unincorporated dNTPs were removed by chromatography through sephadex G50 syringe columns (Stratagene cloning Systems, LaJolla, CA). Hybridizations were carried out at 65°C in a mechanical incubator (Robins Scientific Corp., Sunnyvale, CA) in the presence of 1.5 x SSPE (0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA)/7% SDS (sodium dodecyl sulfate)/10% PEG (polyethylene glycol)(m.w. 8000) supplemented with 200 μg/ml sonicated and denatured herring sperm DNA, 500 μg/ml heparin and 2 ng/ml ^{32}P-labelled probe in a procedure adapted from Budowle and Baechtel (1990). Blots were washed at high stringency in 0.1 x SSC (0.15 M NaCl, 0.015 M Na_3 Citrate)/0.1% SDS

(65°C) and subjected to autoradiography for 3 to 5 days at -76°C under Kodak XAR film sandwiched between Dupont Cronex Lightning Plus intensifying screens. Following hybridization with TNF-α or IL-1α probe, membranes were washed 3 x with 1 mM EDTA pH 8.0/0.5% SDS for 20 min at 95°C, then rinsed with 0.1 x SSC/0.1% SDS at room temperature and re-hybridized with ^{32}P-labelled β-actin cDNA probe. Quantitative assessment of mRNA expression levels was accomplished by scanning autoradiograms on a molecular Dynamics Model 300A laser densitometer, and by performing image analysis and volume integrations using the image Quant V3.0 software (Molecular Dynamics, Sunnyvale, CA).

2.8. REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION (RT-PCR)

2.8.1. CELL PREPARATION

Rat AM were collected by bronchoalveolar lavage (BAL) and adjusted to 1 x 10^6/ml in CTCM. AM (2 x 10^5 in 200 μl medium) were incubated in Lab Tek culture chambers with M-CSF (50 U/ml) or GM-CSF (50 U/ml) for 1.5h, 3h, 24h, 72h and 120h. AM in medium with no treatment served as a negative control and lipopolysaccharide (LPS, 1 μg/ml) (Sigma Chemical company, St. Louis, MO, USA) as positive control. To investigate whether certain cytokines are constitutively expressed, rat AM without incubation (0h) were prepared for RNA extraction immediately after BAL.

2.8.2. RNA EXTRACTION

Total RNA was extracted by the single-step method using Glassmax™ RNA microisolation spin cartridge system (Gibco/BRL, Burlington, Ontario). Briefly, after removal of supernatant, ice-cold guanidine isothiocyanate solution/2-mercaptoethanol (GuSCN/ME, 400 μl) was added to each culture chamber to disrupt cells and transferred to RNase-free 1.5 ml eppendorf tube. Absolute ethanol (280 μl) was added to the tube and mixed thoroughly. The suspension was centrifuged at 13,000 x g for 5 min at room temperature. After the centrifugation, the supernatant was carefully removed with a sterile pipette. Binding solution (6 M sodium iodide, 450 μl) was then added, followed by addition of 40 μl of 3 M NaOAc, pH 5.5. The pellet was resuspended by vortexing for one minute and transferred to the GlassMAX spin cartridge. After centrifugation at 13,000 x g for 20 seconds the tube was emptied, followed by 3 washes with 0.5 ml 1x cold (4°C) wash buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM NaCl, 50% (v/v) ethanol) each time. Then cold (4°C) RNase-free 80% (v/v) ethanol (0.5 ml) was added to the spin cartridge. The tube was centrifuged at 13,000 x g for 20 seconds and the supernatant discarded. This washing step was repeated one time. After removing the final wash from the tube and centrifugation at 13,000 x g for 1 min, the cartridge was placed into a fresh sample recovery tube. DEPC-treated water (40 μl, preheated to 65°C) was added to the spin cartridge and the tube was centrifuged at 13,000 x g for 20 sec to elute the RNA. RNA integrity was

verified by electrophoresis through 1% (w/v) agarose-formaldehyde/MOPS gel and staining with ethidium bromide. The quantity and purity of total RNA were determined by diluting a 10 μl aliquot with 490 μl of DEPC-treated water and measuring the optical densities at 260 nm and 280 nm with the spectrophotometer (GeneQuant, Pharmacia). The amount of RNA calculated as total RNA (μg) = [A_{260} x 40 μg / (1 A_{260} x 1 ml)] x 50 (dilution factor) x 0.04 ml (volume), was found to be between 1.6 to 2.3 μg/2 x 10^5 AM and the ratio of absorbance at 260 nm and 280 nm between 1.85 to 1.97.

2.8.3. REVERSE TRANSCRIPTION (RT)

All RNA samples within one experiment were reverse transcribed simultaneously using cDNA cycle kit[R] (InVitrogen, San Diego, CA, USA) following procedures as per manufacturer's instructions. The synthesis of cDNA was carried out in a 20 μl reaction volume containing 1 μg of RNA, 200 ng of oligo (dT), 100 mM Tris-HCl (pH 8.3), 40 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM spermidine, RNase inhibitor (10 U), 5 mM dNTP (1.25 mM each), 4 mM sodium pyrophosphate and Avian myeloblastosis virus (AMV) reverse transcriptase (5 U). In order to make it possible to quantitate RT products, 1 μCi ^{32}P dCTP (3000 Ci/mmol, Dupont/NEN, Canada Inc.) was added. The reaction mixture was incubated at 42°C for 60 minutes, then at 95°C for 2 minutes to denature the RNA-cDNA hybrids. At this point, 5 units of AMV reverse transcriptase were added and cDNA synthesis continued for an additional 60 minutes at 42°C. Samples were then heated at 95°C for 3 more minutes and quickly chilled

on ice. Phenol extraction and ethanol precipitation were performed before freezing and storing the cDNA. The RT products were then diluted to 120 μl with DEPC-treated water and 5 μl were used to estimate the amount of ^{32}P dCTP incorporation and the yield of synthesized cDNA following scintillation counting. Routinely, between 10 and 15 ng of cDNA were obtained from each sample. The RT products were aliquoted and stored at -20°C before subsequent amplification.

2.8.4. POLYMERASE CHAIN REACTION (PCR)

PCR was performed to detect gene expression of TNFα, IL-1α, IL-6, TGF-$_\beta$ and GM-CSF. β_2-microglobulin was used as an internal control. Primers used for PCR together with the expected size of amplicons are listed in Table 1. Primer sequences for GM-CSF were designed (Lemaire et al. 1996a) from a partial rat GM-CSF cDNA sequence (Smith et al. 1994) and primer sequences for TNF-α, IL-1α, IL-6 and TGF-β were synthesized based on the information reported by Pousset (1994[a]). RT products were amplified in a DNA thermal cycler (Perkin-Elmer, Model 2400). The PCR reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 2 mM dNTP (0.5 mM each), 15 pM of each sense and antisense oligonucleotides, 1 unit of the thermostable Taq DNA polymerase (Perkin-Elmer) in a total volume of 50 μl. The step-cycle programme was set to denature at 95°C for 1 minute, anneal at 55°C for 1 minute, and extend at 72°C for 1 minute. After 30 cycles, the duration of the

[a] *personal communication*

Table 1. Primers used for PCR together with the expected size of amplicons. Oligonucleotides were used to detect mRNAs for β_2-microglobulin and various cytokines

mRNA	Sense and antisense primer	Expected size
GM-CSF	5'primer-5'-AGGCCGACATGTGTGCAGAC-3'	
	3'primer-5'-TGGATTCAGAGCTGGCCTGG-3'	261 bp
TNF-*α*	5'primer-5'-AAATGGGCTCCCTCTCATCA-3'	
	3'primer-5'-AGCCTTGTCCCTTGAAGAGA-3'	248 bp
IL-1*α*	5'primer-5'-AACTGGGTCAGTCTTTTGCC-3'	
	3'primer-5'-TTGTGACACCCTGGTTTGAG-3'	207 bp
IL-6	5'primer-5'-TAGAGTCACAGAAGGAGTGG-3'	
	3'primer-5'-GCCAGTTCTTCGTAGAGAAC-3'	210 bp
TGF-*β*	5'primer-5'-ACCAACTACTGCTTCAGCTC-3'	
	3'primer-5'-TGTTGGTTGTAGAGGGCAAG-3'	195 bp
β_2-μglob.	5'primer-5'-ATCTTTCTGGTGCTTGTCTC-3'	
	3'primer-5'-AGTGTGAGCCAGGATGTAG-3'	243 bp

final elongation reaction was increased to 5 minutes at 72°C to permit completion of reaction products. Following PCR, the products (both β_2-microglobulin and cytokine) were analyzed in parallel on a single 10% polyacrylamide TBE precast gel (NOVEX, San Diego, CA, USA), stained with ethidium bromide to verify the expected sizes.

In primary experiments, serial dilutions of cDNA were employed for the best analysis of each individual cytokine in the PCR and a precast 10% polyacrylamide TBE gel evaluation system. Based on the information derived from these primary experiments, GM-CSF was amplified from 0.5 nanogram of RT products, TNFα and IL-1α were amplified from 0.1 nanogram of RT products, IL-6 and TGF-β amplified from 0.25 nanogram of RT products and β_2-microglobulin was amplified from 0.025 nanogram of RT products.

2.9. IMMUNOCYTOCHEMISTRY

2.9.1. CYTOPLASMIC DETECTION FOR CYTOKINES

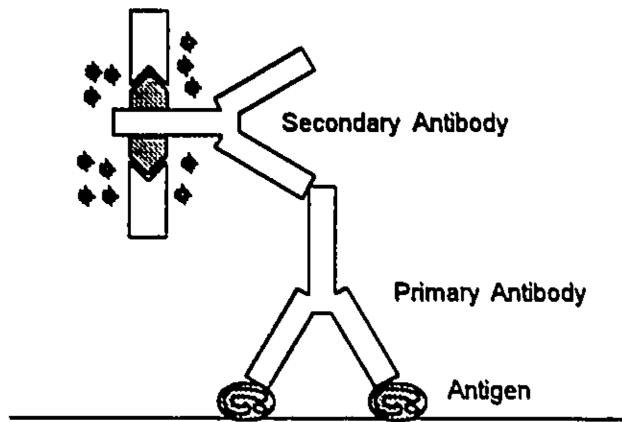
Cytoplasmic expression of TNF-α, PDGF and TGF-β cytokines was examined by immunocytochemical analysis. We used cells grown directly on Lab Tek chamber slides (Nunc, Naperville, IL). AM monolayers were incubated for 5 days with M-CSF (50 U/ml) or GM-CSF (50 U/ml). Control group received no treatment. In some cases, LPS was added as a positive control.

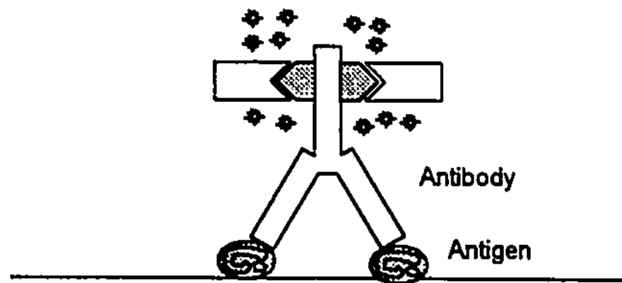
The primary antibodies used to characterize the cytoplasmic expression of TNF-α, PDGF and TGF-β cytokines were: 1) polyclonal rabbit anti-murine

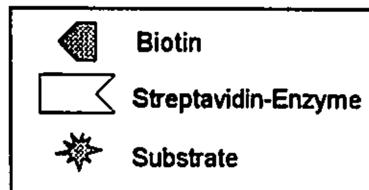
TNF-α (Genzyme, Cambridge, MA, USA) in 1:200 dilution for detection of TNF-α expression by AM, 2) polyclonal goat anti-human PDGF (Collaborative Biomedical Products, Becton Dickinson Labware, Bedford, MA) in 1:200 dilution for detection of PDGF expression by AM, 3) polyclonal goat anti-human TGF-β (TEBU, France) in 1:500 dilution for detection of TGF-β expression by AM. The biotinylated second antibodies used in this set of experiments were goat anti-rabbit IgG (Zymed Laboratory Inc. San Francisco, CA, USA) for rabbit primary antibody against TNFα and rabbit anti-goat IgG (Zymed Laboratory Inc. San Francisco, CA USA) for goat primary antibody against PDGF or TGF-β. Figure 2A illustrated schematically the protocol used for the detection of cytoplasmic expression of cytokines.

The procedures for cytoplasmic detection of TNF-α, PDGF and TGF-β by immunocytochemistry in our experiments are a modification of those reported by Gowen et al. (1990) and Majesky et al. (1990). Briefly, after 5-day incubation in culture medium, Lab Tek chambers were gently rinsed with warm PBS three times, fixed in 4% paraformaldehyde for 10 minutes and rinsed again with PBS 2 times. Absolute methanol was added for 10 minutes to permeablize cells followed by 3 washes in PBS. Prior to staining, the chambers were treated with a 3% hydrogen peroxide solution for 10 minutes in order to inactivate any endogenous peroxidase activity. For TNF-α detection, nonspecific binding of rabbit IgG was blocked by preincubation with a 1:10 dilution of normal goat serum. For PDGF or TGF-β detection, nonspecific binding of goat IgG was

Figure 2. Protocol for immunocytochemistry. A) detection system for cytoplasmic cytokines. B) detection system for membrane integrins.


Detection System for Cytoplasmic Cytokines
A
Secondary Antibody
Primary Antibody
Antigen


Detection System for Membrane Integrins
B
Antibody
Antigen


Biotin
Streptavidin-Enzyme
Substrate

blocked by preincubation with a 1:10 dilution of normal rabbit serum. The serum was removed after 10 minutes at room temperature and the chambers were sequentially incubated with the appropriate primary antibody or a similar dilution of nonimmune serum as negative control. After 60 minutes incubation at room temperature, the wells were rinsed with PBS (3x), overlaid with biotinylated second antibody and incubated for 30 minutes followed by three additional rinses with PBS. The wells were treated with peroxidase-labelled streptavidin for 10 minutes, washed three times with PBS and overlaid with substrate-chromogen of AEC (3-amino-9-ethyl-carbazole) for 5 minutes to allow for colour development. Hematoxylin was used as a counterstain and slides were covered by coverslip with mounting medium. Reagents for peroxidase staining were purchased from Zymed Laboratory Inc. (San Francisco, CA, USA).

2.9.2. MEMBRANE DETECTION FOR INTEGRINS

AM monolayers incubated in Lab Tek chambers for various times in the presence or absence of M-CSF or GM-CSF were directly examined for membrane associated α_V integrin (CD51) and β_3 integrin (CD61). Polyclonal hamster anti-mouse CD51 and anti-CD61 conjugated with biotin (Pharmingen, San Diego, CA, USA) were used at 1:100 dilution, respectively. The procedures were basically the same as those used for cytoplasmic detection of cytokines except that cells were not permeabilized with absolute methanol. In addition, the second antibody was not used since the primary antibody was already

conjugated with biotin. Nonspecific binding was blocked by preincubation with a 1:10 dilution of normal hamster serum. The protocol for immunocytochemical detection of membrane integrins is illustrated in figure 2B.

Immunocytochemically stained slides were examined by light microscope (magnification x 850). Positive AM were determined by counting at least 200 cells. To evaluate the number of positive MGC, at least 200 MGC were counted. Data are presented as percentage of positive cells.

2.10. STATISTICAL ANALYSIS

Data are expressed as mean values ± standard error of the mean (SEM) from at least three experiments. Statistical significance was determined using Student's T test ($p<0.05$). When there are more than three groups to be compared for the statistical analysis, multiple comparisons have been taken into account. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test (Instat, GraphPad, San Diego, CA). Significantly different from control at $p<.05$ (*), $p<.01$ (**), or $p<.001$ (***).

3. RESULTS

3.1. CYTOKINE PRODUCTION BY ACTIVATED ALVEOLAR MACROPHAGES (AM)

3.1.1. DEVELOPMENT OF A SIMPLIFIED HIGH THROUGHPUT PROTOCOL FOR NORTHERN HYBRIDIZATION

Alveolar macrophages (AM) have the ability to produce many cytokines which modulate inflammatory reactions. To investigate this, in the first part of this work, I analyzed the production by activated AM of cytokines that have relevance to inflammation, notably tumour necrosis factor-alpha (TNF-α) and interleukin-1 alpha (IL-1α), at the gene expression level. For this, a simplified high throughput protocol for quantitative determination of cytokine mRNA concentration by Northern blot hybridization analysis was developed.

In the most widely employed protocol, RNA samples are fractionated by denaturing agarose gel electrophoresis, transferred to a solid support matrix and hybridized to radiolabelled complementary probe fragments in the presence of formamide at slightly acidic pH (Davis et al. 1986). The inclusion of formamide permits the use of lower annealing temperature for the formation of probe:target duplexes, thus preserving the integrity of the RNA which would otherwise be compromised at high temperature, especially at neutral and alkaline pH (Williams and Mason 1985). However, formamide retards the rate of duplex formation and limits the sensitivity of target site detection (Budowle

and Baechtel 1990). It is also unstable, volatile and toxic. To overcome these limitations, we developed hybridizations using a simplified version of the tripartite buffer system (henceforth referred to as HYBSOL) described by Budowle and Baechtel for forensic DNA typing (Budowle and Baechtel 1990).

The effect of HYBSOL on the rate of target site detection was determined. To do this, serial dilutions of total RNA from LPS-stimulated AM were applied to duplicate gels, separated by electrophoresis, blotted and probed with TNF-α cDNA. One membrane was hybridized at 65°C using HYBSOL while the other was hybridized at 42°C with conventional formamide cocktail (Davis et al. 1986). The autoradiograms are shown in Figure 3. Quantitative laser densitometry revealed that HYBSOL enhanced (2.5 fold) signal intensity when compared to a conventional formamide-based hybridization cocktail and consistently produced autoradiograms free of background even after prolonged exposures.

This approach was used to monitor the induction of TNF-α and IL-1α gene expression in rat AM. The data are summarized in Figure 4. A representative ethidium bromide stained gel (1% agarose) of serially diluted RNA appears on the left. In agreement with recent reports, low levels of TNF-α mRNA were detected in unstimulated AM (Gosset et al. 1991). As expected, exposure to LPS produced a dramatic induction of steady-state levels of TNF-α and IL-1α mRNA. The increases in TNF-α and IL-1α mRNA levels were respectively 6-fold and 20-fold as determined by quantitative laser densitometry

Figure 3. Effects of HYBSOL and conventional formamide-based hybridization cocktail on the rate of detection of TNF-α transcripts from LPS-stimulated rat AM. Lanes (left to right) correspond to sample loadings of 10 μg, 5 μg and 2.5 μg total RNA, respectively. The blots were exposed to X-ray film for 5 days.

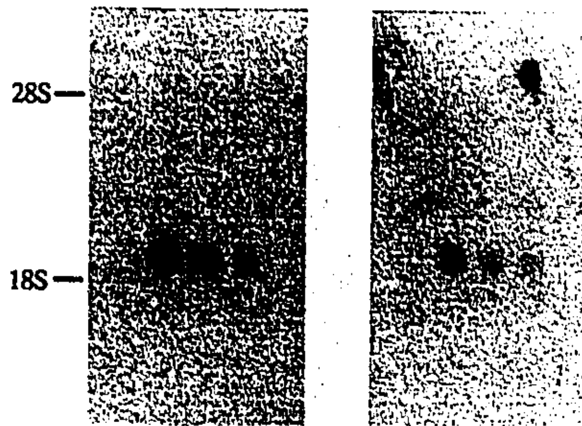
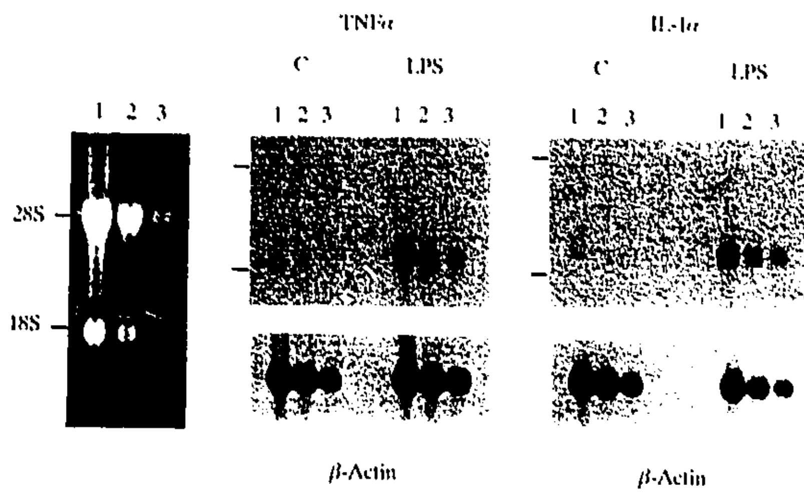

TNFα
28S
18S
HYBSOL
FORMAMIDE

Figure 4. Northern blot hybridization analysis of cytokine mRNA expression in control (C) versus LPS-stimulated rat AM. Lanes 1, 2 and 3 correspond to 10 μg, 5 μg and 2.5 μg total RNA, respectively. Left side is a representative ethidium bromide stained gel (1% agarose) of serially diluted RNA.


TNFα
IL-1α
C
LPS
C
LPS
1 2 3
1 2 3 1 2 3
1 2 3 1 2 3
28S
18S
β-Actin
β-Actin

(Molecular Dynamics, Sunnyvale, CA, USA). Very low levels of IL-1α transcripts were also detected when the highest RNA concentration (10 μg) was used. This may constitute a stress response to in vitro manipulation or adhesion of AM during culture.

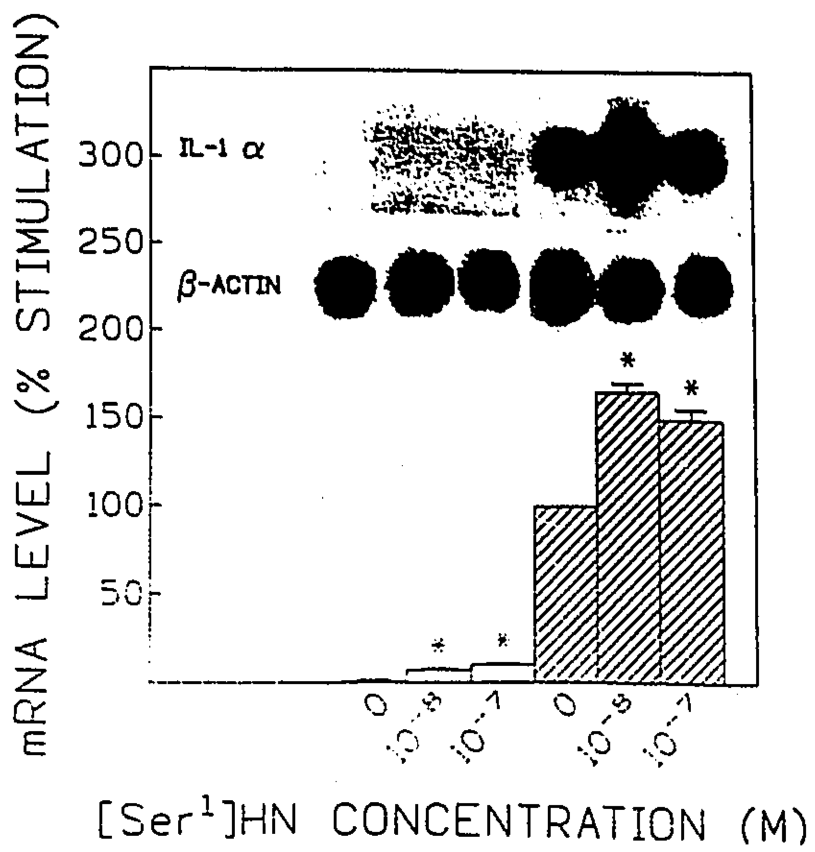
3.1.2. REGULATION OF CYTOKINE PRODUCTION BY AM IN VITRO

The production of a given cytokine by AM can be triggered by more than one factor. Besides LPS, histogranin (HN), a novel endogenous pentadecapeptide, has been shown to stimulate, although slightly, basal release of IL-1α and to significantly enhance the stimulatory effect of LPS on IL-1α release (Ouellet et al. 1993). The effect of HN at various concentrations for 18 h exposure on IL-1 mRNA expression by rat AM was examined. As shown in Figure 5, mRNA expression for IL-1α, a major form of IL-1 produced by rat AM, is also stimulated by HN as measured by laser densitometric scanning following standardization of the mRNA amounts according to the expression of a constant gene, β-actin. Thus LPS and HN stimulate IL-1 production at the gene expression level and this in turn is reflected by increased IL-1 release (Lemaire et al. 1994).

3.1.3. BIPHASIC CHANGES IN TNF-α GENE EXPRESSION BY AM FROM ANIMALS WITH LUNG FIBROSIS

Because it had previously been demonstrated that LPS-induced TNF-α release from AM was suppressed early after asbestos exposure, the pattern of mRNA expression for TNF-α in response to LPS was examined in AM from

Figure 5. Effects of [Ser^1] HN, a chemically stable analog of HN, on spontaneous and LPS-induced IL-1α mRNA expression by AM. AM were incubated for 18 h in the absence (open bars) or presence (hatched bars) of LPS (1μg/ml) with two concentrations (10^{-8}-10^{-7} M) of [Ser^1] HN. Northern blot analysis of IL-1α mRNA in cultured AM was performed as described in **Materials and Methods.** The relative expression of IL-1α mRNA was determined by laser densitometric scanning after standardizing the mRNA amounts according to the expression of a constant gene, β-actin. Results are expressed as % stimulation relative to LPS-induced response (= 100%). Values represent mean $\pm$ SEM of 3 separate preparations.

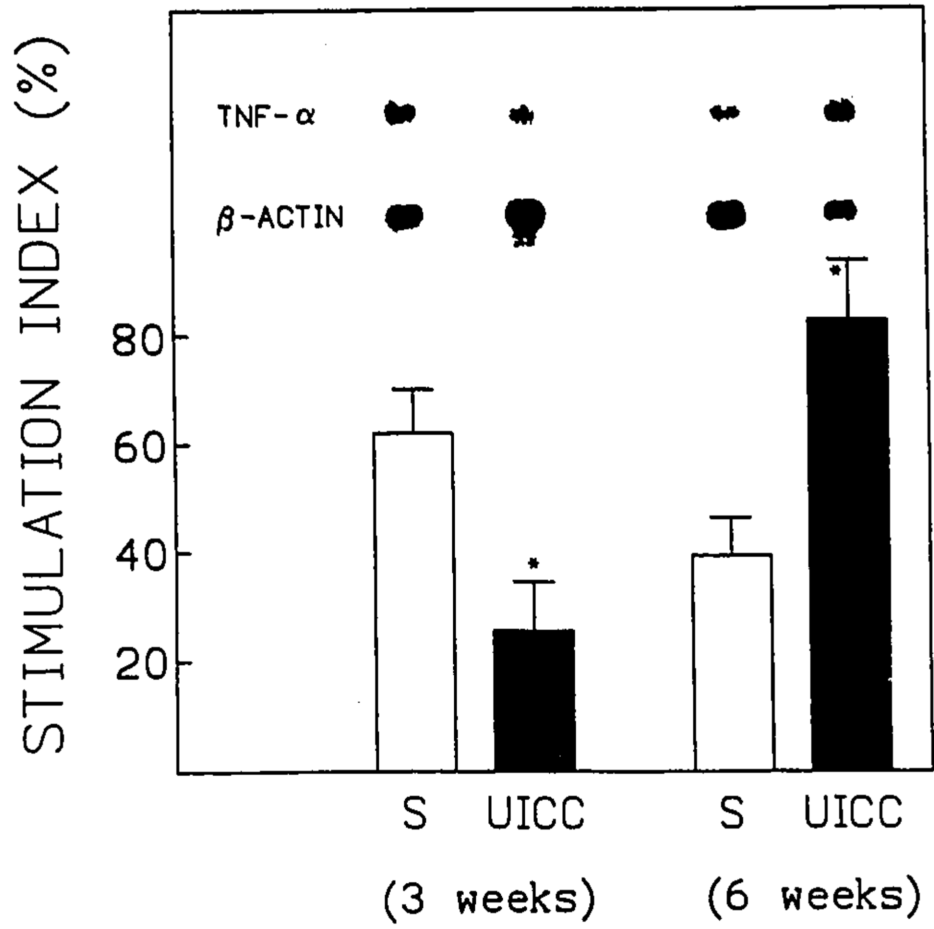

mRNA LEVEL (% STIMULATION)
300
250
200
150
100
50
IL-1 α
β-ACTIN
*
*
*
*
0
10^{-8}
10^{-7}
0
10^{-8}
10^{-7}
[Ser^1]HN CONCENTRATION (M)

animals exposed to fibrogenic asbestos. Three and six weeks after treatment, the BAL cell populations from animals with lung fibrosis were composed exclusively of AM, but significant changes in AM populations were seen, notably an increase in the population of AM with a large diameter (Lemaire 1995). AM isolated from rat lung 3 and 6 weeks post-exposure to chrysotile asbestos or PBS (control) were cultured for 18 h in 1 μg/ml LPS, then RNA was isolated and quantified by Northern hybridization analysis. Northern hybridization analysis showed biphasic changes in TNF-α mRNA expression (Figure 6). Three weeks after treatment, expression of TNF-α mRNA following LPS stimulation was lower in AM from animals exposed to asbestos compared to controls. By 6 weeks, however, TNF-α mRNA levels had increased significantly over controls, indicating that AM were up-regulated for TNF-α production at this stage of the inflammatory response.

Overall, in the first part of this work, an improved protocol for Northern hybridization to analyze cytokine gene expression by AM was used. Biphasic changes in TNF-α production by AM from animals with lung fibrosis were associated with specific changes in AM populations.

It is likely that the regulation of cytokine production, a key element in the resolution of inflammatory responses, may be tightly regulated by differentiation processes and the sequential appearance of distinct AM populations within inflammatory lesions. In connection with this, previous work from this laboratory has demonstrated that experimental granuloma and fibrosis

Figure 6. Northern blot hybridization analysis of TNF-α mRNA in cultured BAL cells. BAL cells obtained from rats 3 and 6 weeks following intratracheal instillation of saline (S) or UICC chrysotile asbestos (UICC) were cultured for 18 h in the presence of LPS (1 μg/ml). (Top) Total cellular RNA was isolated and mRNA expression of TNF-α or β-actin were analyzed; representative of 3 separate experiments. (Bottom) Relative expression of TNF-α in the same groups as determined by laser densitometric scanning after standardizing the mRNA amounts according to the expression of a constant gene, β-actin. Values represent mean $\pm$ SEM of 3 separate experiments. Significantly different at $p < .05$ (*).


TNF-α
β-ACTIN
STIMULATION INDEX (%)
80
60
40
20
S
UICC
S
UICC
(3 weeks)
(6 weeks)

are correlated with selective changes of AM populations and/or state of AM differentiation. In animals with lung fibrosis, there was an increase in the proportion of large, mature AM whereas resolving granuloma was associated with the presence of multinucleated giant macrophages (MGC) (Lemaire, 1991a). To date, however, the mechanisms responsible for the generation of these modified forms of AM as well as the physiological implication of AM derivatives are unknown. To address these issues, in the second and most important part of this work, I investigated the differentiation of AM and the formation of MGC in relation to specific cytokine production.

3.2. AM DIFFERENTIATION AND MGC FORMATION

A cell culture system was developed in an attempt to reproduce some of the cell changes occurring in vivo and to investigate the role of cytokines in this process. The rat is a good model to study AM differentiation since a) adequate numbers of AM are obtained following bronchoalveolar lavage (8.8 $\pm$ 0.76 x 10^6 / rat) (Table 2), b) cell viability is greater than 98%, and c) BAL cell population is composed of a pure population of AM (99%).

3.2.1. INDUCTION OF MGC BY M-CSF AND GM-CSF

We tested the effects of colony stimulating factors (CSFs) known to participate in macrophage differentiation from bone-marrow progenitors. AM were cultured in IMDM supplemented with 10% FBS in the presence or absence of the recombinant human M-CSF or murine GM-CSF. Three different

Table 2. AM recovery and differential analysis

Total cell yield ($x10^6$)*	8.8±0.76
Viability (%)	98.2±0.63
Differential analysis (%)	
AM:	99.6±0.3
PMN:	0.4±0.3
Eosinophil:	0
Lymphocyte:	0

* Data are represented as the mean ± SEM from 10 experiments.

AM: Alveolar macrophage

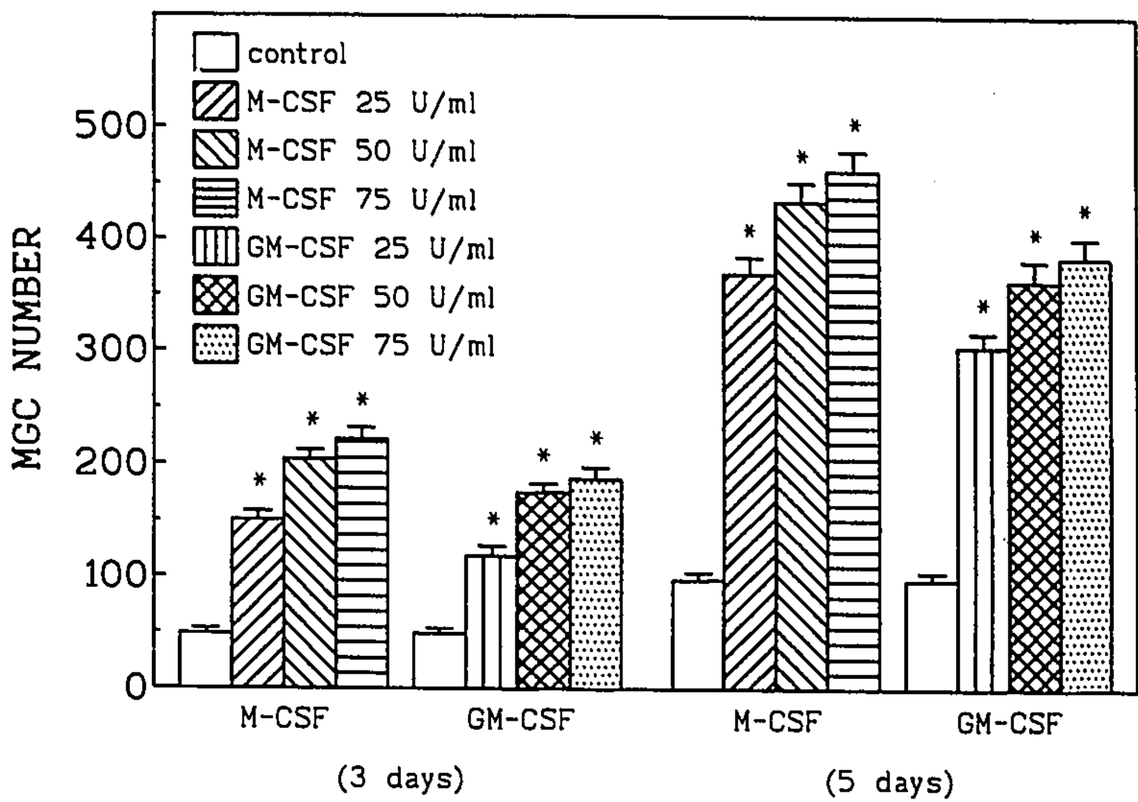
PMN: Polymorphonuclear leukocyte

concentrations of M-CSF or GM-CSF were used, and cells were analyzed after incubation for three and five days. Data are shown in Figure 7. Incubation of AM in tissue culture medium alone resulted in the formation of a low number of MGC. Culture under the same conditions in the presence of either M-CSF or GM-CSF resulted in a significant increase in the number of MGC, indicating a role for CSFs in triggering further differentiation of mature AM within the bronchoalveolar compartment. The stimulatory effects of M-CSF or GM-CSF were dose- and time-dependent. M-CSF or GM-CSF at 25 U/ml concentration increased the number of MGC up to 3-fold whereas 50 U/ml and 75 U/ml M-CSF or GM-CSF resulted in 4 and 4.5-fold increase of MGC formation, respectively. Cultures incubated with M-CSF or GM-CSF for 5 days exhibited higher numbers of MGC than those exposed to M-CSF or GM-CSF for 3 days but the stimulation index was not significantly different because control groups also exhibited a slightly higher number of MGC at 5 days. Assessment of cell number indicated that there was no significant change in total cell number in response to M-CSF or GM-CSF and that increased MGC formation was not related to increased cell proliferation. In addition, the stimulation of MGC formation was also observed in the absence of serum, suggesting that increase in MGC was not due to serum factors other than exogenously added CSFs.

3.2.2. CHARACTERISTICS OF AM-DERIVED MGC

Morphological analysis revealed the presence of two distinct variants of MGC. This was based on many criteria including cell size, shape, number of

Figure 7. Induction of MGC formation in vitro. AM (2×10^5) were incubated in Lab Tek culture chambers in 0.2 ml medium alone or in the presence of M-CSF (25 U/ml-75 U/ml) or GM-CSF (25 U/ml-75 U/ml). After 3 and 5 days, the culture chambers were rinsed with PBS and stained with Wright-Giemsa solution. The number of MGC from each culture were monitored under the microscope (magnification x 330) as described in **Materials and Methods**. Data represent the mean $\pm$ SEM from 3 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.01$ (**) or $p<.001$ (***).


control
M-CSF 25 U/ml
M-CSF 50 U/ml
M-CSF 75 U/ml
GM-CSF 25 U/ml
GM-CSF 50 U/ml
GM-CSF 75 U/ml
MGC NUMBER
500
400
300
200
100
0
M-CSF
GM-CSF
(3 days)
M-CSF
GM-CSF
(5 days)

nuclei and staining with Wright-Giemsa (Table 3). Type 1 MGC normally contained 3 to 8 nuclei and appeared as a large round cell with an average diameter of 18 μm that stained dark blue upon differential staining (Figure 8). In comparison, type 2 MGC contained a higher number of nuclei (up to 30) and was larger than type 1 MGC with an average diameter of 24 μm. Some type 2 MGC exhibited diameters as high as 85 μm. These cells displayed irregular, elongated shapes with a thin and sometimes vacuolated cytoplasm that stained pink with Wright-Giemsa as shown in Figure 9. These characteristics resemble closely those found in BAL fluids of rats treated with non-fibrogenic mineral dusts.

It is generally accepted that the formation of MGC arises from cell fusion (Abe et al. 1983). In agreement with this, AM fusion with MGC was frequently seen in our cell culture system (Figure 10). In some cases, MGC interact through extended projections with AM at a distant location rather than with nearby cells (Figure 11), suggesting that unknown specific factors, possibly adhesion molecules may be involved in this process. To explore this, we studied the expression of adhesion molecules, notably β_3 integrin (CD61) and α_v integrin (CD51) expression by immunocytochemical analysis. This was based on published information that GM-CSF induces selectively β_3 integrin in monocytes whereas M-CSF treated cells ceased to express any pre-existing β_3-mRNA (DeNichillo and Burns 1993). As shown in Figure 12, when type 1 MGC were compared to type 2 MGC for their expression of α_v integrin, no difference

Table 3. Characteristics of MGC induced *in vitro* and *in vivo*

	AM		Type 1 MGC		Type 2 MGC	
	1[a]	2[b]	1	2	1	2
Nuclei	1	1	3-7	3-8	8-20	8-30
Shape	round	round	round	round	irregular	irregular
Diameter (μm)	6-15	6 ± 1.4[c]	21-30	18 ± 3[c]	30-41	24 ± 5[c]
Wright-Giemsa staining	blue	blue	dark blue	dark blue	pink	pink
Density	17%<1.075	-	100%>1.075	-	100%>1.075	-

[a]in vivo. Diameter was evaluated as cytospin preparation which results in a slightly greater diameter.
[b]in vitro. Mean diameter was evaluated in adherent cell preparation cultured in Lab Tek for 5 days.
[c]mean diameter was determined in 200 cells.

Figure 8. Type 1 MGC (magnification x 330). AM (2×10^5) were incubated in Lab Tek culture chambers in 0.2 ml in the presence of M-CSF (50 U/ml) for 5 days and the culture chambers were rinsed with PBS and stained with Wright-Giemsa solution.

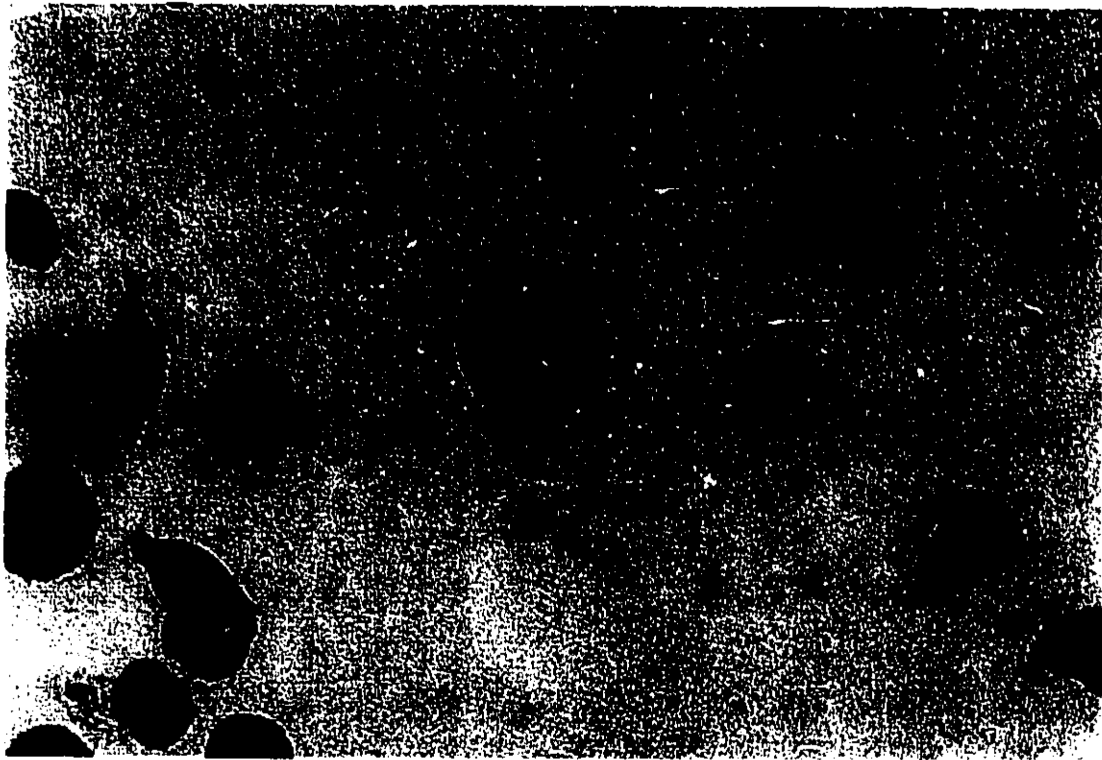


Figure 9. Type 2 MGC (magnification x 330). AM (2 x 10^5) were incubated in Lab Tek culture chambers in 0.2 ml in the presence of GM-CSF (50 U/ml) for 5 days and the culture chambers were rinsed with PBS and stained with Wright-Giemsa solution.

Figure 10. AM fusion with MGC (magnification x 330). AM (2 x 10^5) were incubated in Lab Tek culture chambers in 0.2 ml in the presence of M-CSF (50 U/ml) for 5 days and the culture chambers were rinsed with PBS and stained with Wright-Giemsa solution.

Figure 11. Type 2 MGC interacts selectively with AM at a distant location (magnification x 330). AM (2 x 10^5) were incubated in Lab Tek culture chambers in 0.2 ml in the presence of GM-CSF (50 U/ml) for 5 days and the culture chambers were rinsed with PBS and stained with Wright-Giemsa solution.

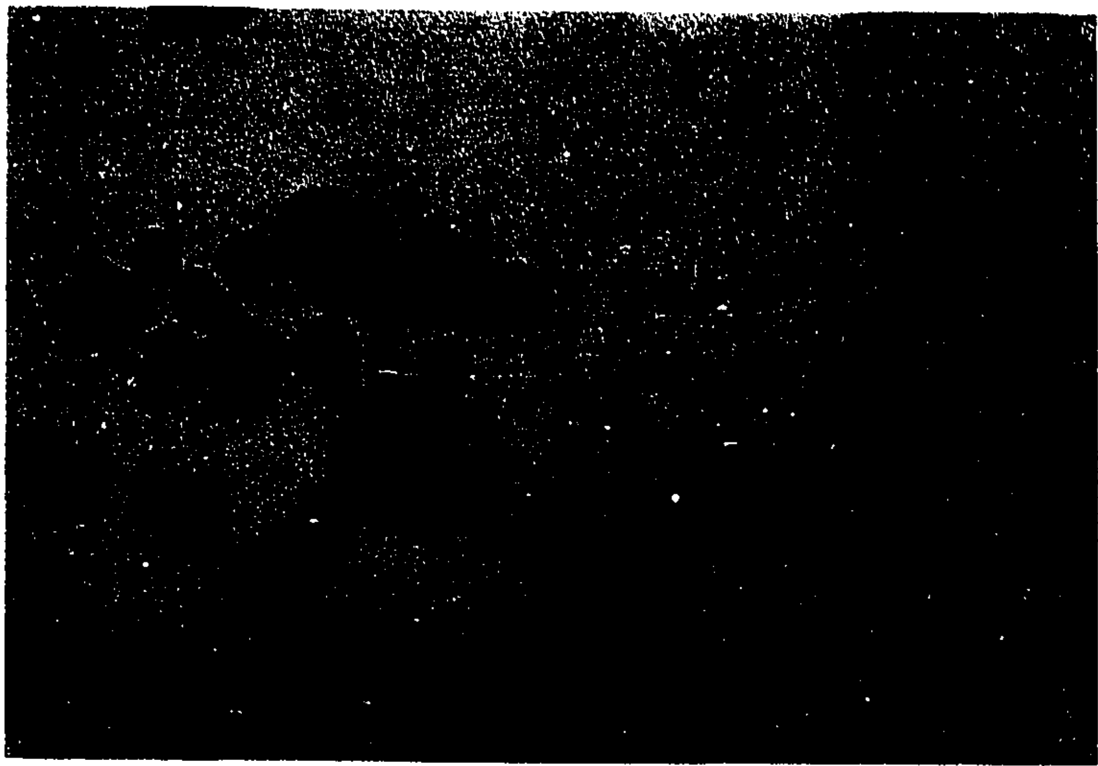
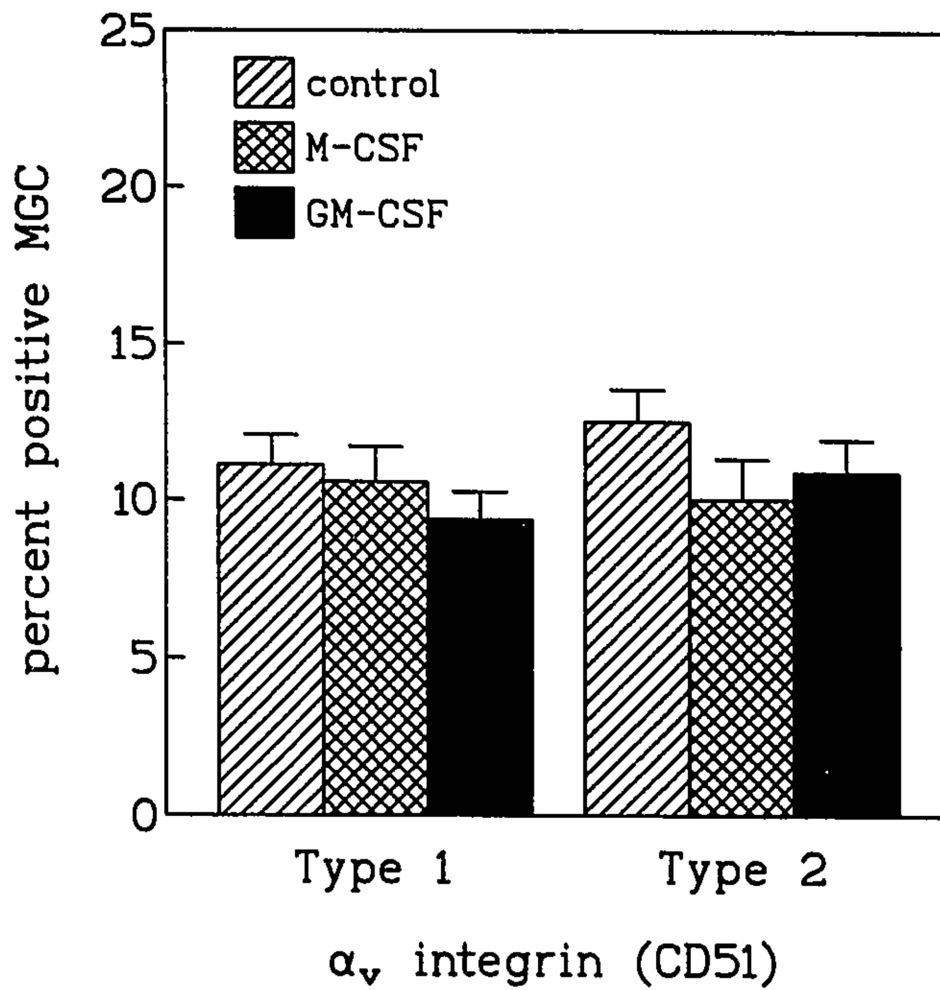


Figure 12. Expression of α_v integrin in type 1 and type 2 MGC. AM (2×10^5) were incubated in Lab Tek culture chambers in 0.2 ml medium alone or in the presence of M-CSF (50 U/ml) or GM-CSF (50 U/ml) for 5 days. The culture chambers were rinsed with PBS and stained by immunocytochemistry as described in **Materials and Methods.** More than 200 MGC were counted under the microscope (magnification x 825) and percent positive cells were calculated.



25
20
15
10
5
0
percent positive MGC
control
M-CSF
GM-CSF
Type 1
Type 2
α_v integrin (CD51)

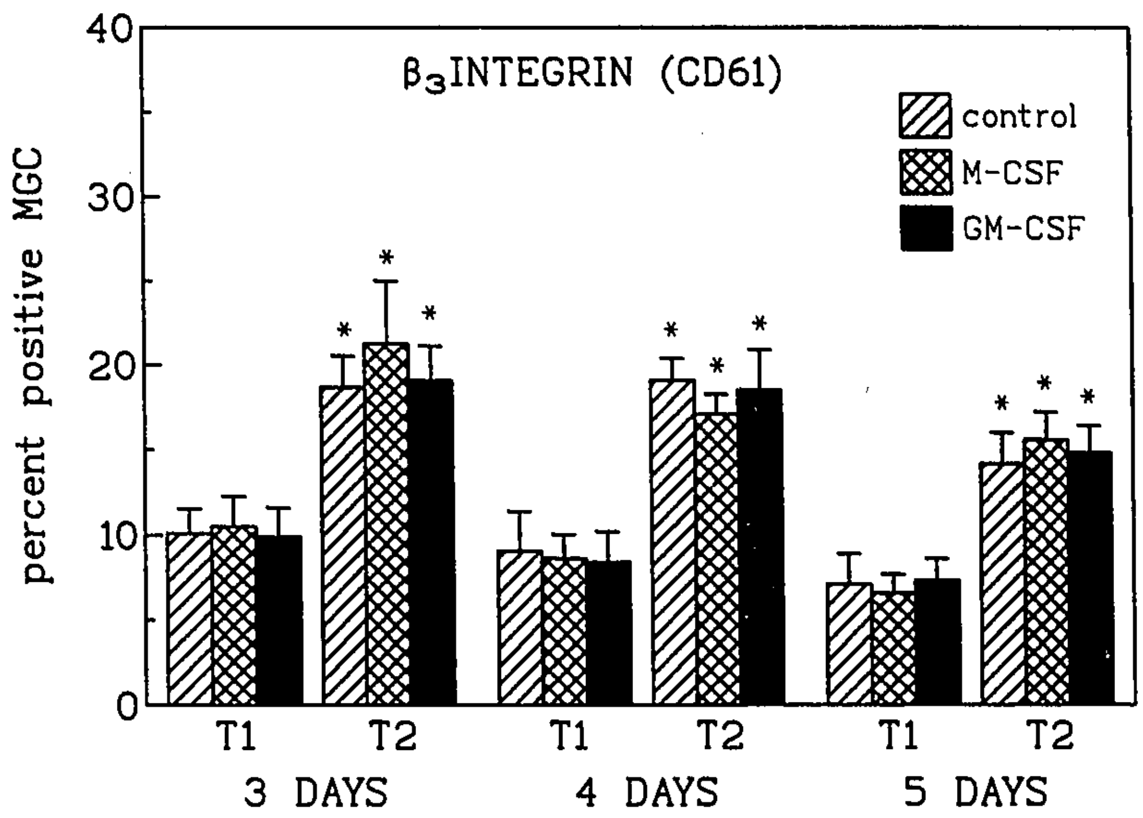
was seen. However, we observed that a greater proportion of type 2 MGC expressed β_3 integrin compared to type 1 MGC. As illustrated in Figure 13, a greater proportion of type 2 MGC expressed β_3 integrin at all time points studied in the presence or absence of M-CSF or GM-CSF, thus bringing additional evidence for differences between type 1 and type 2 MGC.

3.2.3. DIFFERENTIAL EFFECTS OF M-CSF AND GM-CSF ON MGC FORMATION

Morphological analysis also revealed different actions of M-CSF and GM-CSF on AM. In response to M-CSF, the general morphology of AM remains relatively unchanged except for the presence of MGC as shown in Figure 14. In contrast, AM treated with GM-CSF presented an elongated, almost fibroblastoid phenotype (Figure 15). Assessment of the relative proportion of type 1 and type 2 MGC during in vitro differentiation of AM indicated that culture in the presence of 50 U/ml of M-CSF induced the formation of both types of MGC to a similar extent. An equal formation of both types of MGC was also observed during culture of unstimulated AM. In contrast, culture in 50 U/ml of GM-CSF induced predominantly the formation of type 2 MGC (Figure 16).

To investigate further the respective roles of M-CSF and GM-CSF in the formation of distinct types of MGC, neutralization experiments were performed using antibodies against M-CSF and GM-CSF respectively, as described in **Material and Methods.** Addition of anti-M-CSF to control and M-CSF-treated cell

Figure 13. Expression of β_3 integrin in type 1 and type 2 MGC. AM (2×10^5) were incubated in Lab Tek culture chambers in 0.2 ml medium alone or in the presence of M-CSF (50 U/ml) or GM-CSF (50 U/ml) for 3, 4 and 5 days respectively. The culture chambers were rinsed with PBS and stained by immunocytochemistry as described in Materials and Methods. More than 200 MGC were counted under the microscope (magnification x 825) and percent positive cells were calculated. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p < .05$ (*).



β_3INTEGRIN (CD61)
control
M-CSF
GM-CSF
percent positive MGC
40
30
20
10
0
T1
T2
T1
T2
T1
T2
3 DAYS
4 DAYS
5 DAYS

Figure 14. General morphology of AM differentiation in response to M-CSF (50 U/ml) (magnification x 165). AM (2 x 10^5) were incubated in Lab Tek culture chambers in 0.2 ml in the presence of M-CSF (50 U/ml) for 5 days and the culture chambers were rinsed with PBS and stained with Wright-Giemsa solution.

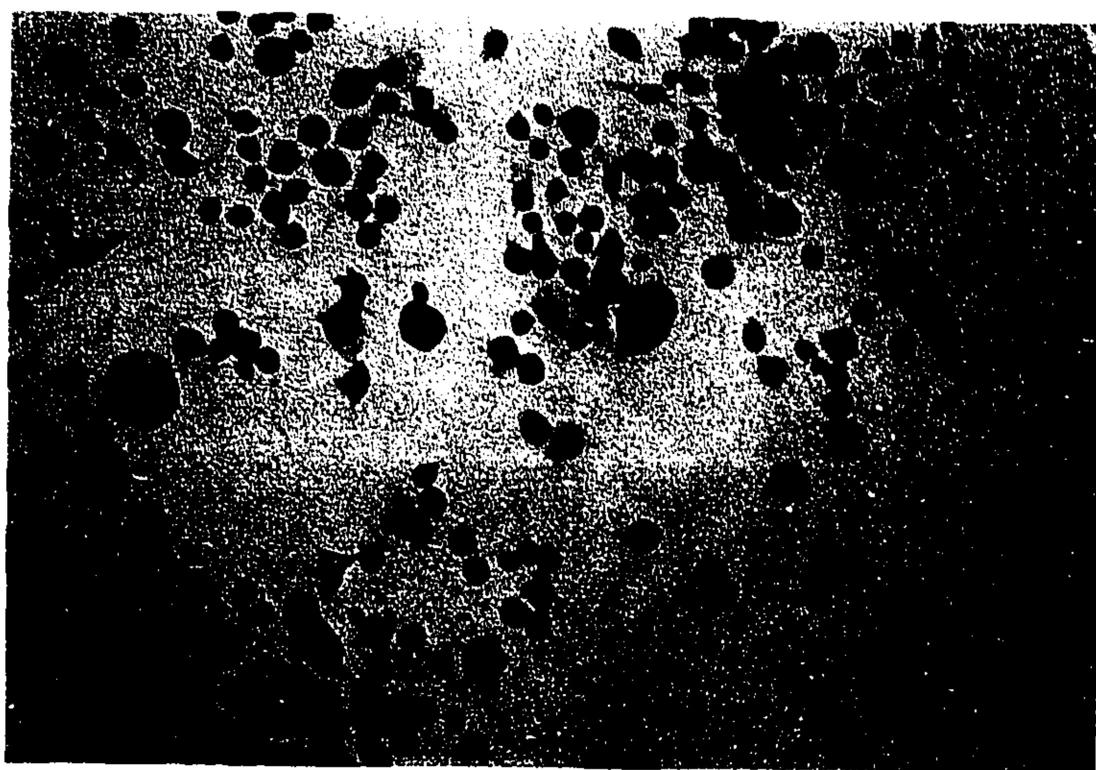


Figure 15. General morphology of AM differentiation in response to GM-CSF (50 U/ml) (magnification x 165). AM (2×10^5) were incubated in Lab Tek culture chambers in 0.2 ml in the presence of GM-CSF (50 U/ml) for 5 days and the culture chambers were rinsed with PBS and stained with Wright-Giemsa solution.

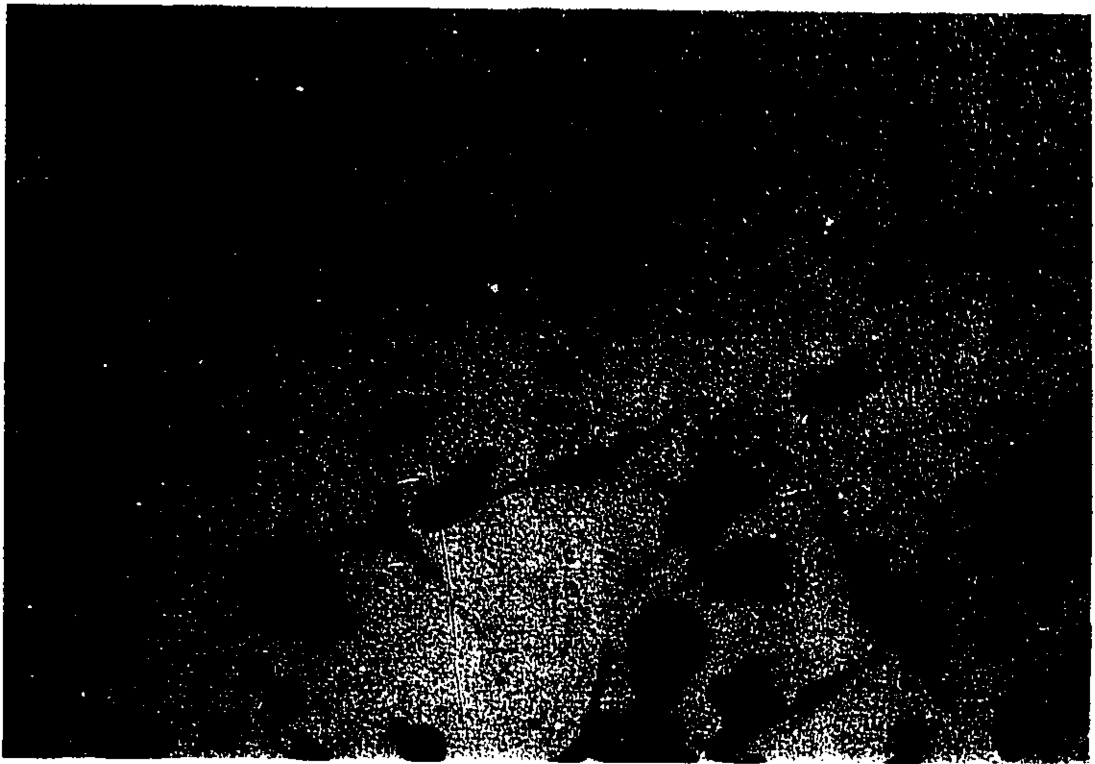
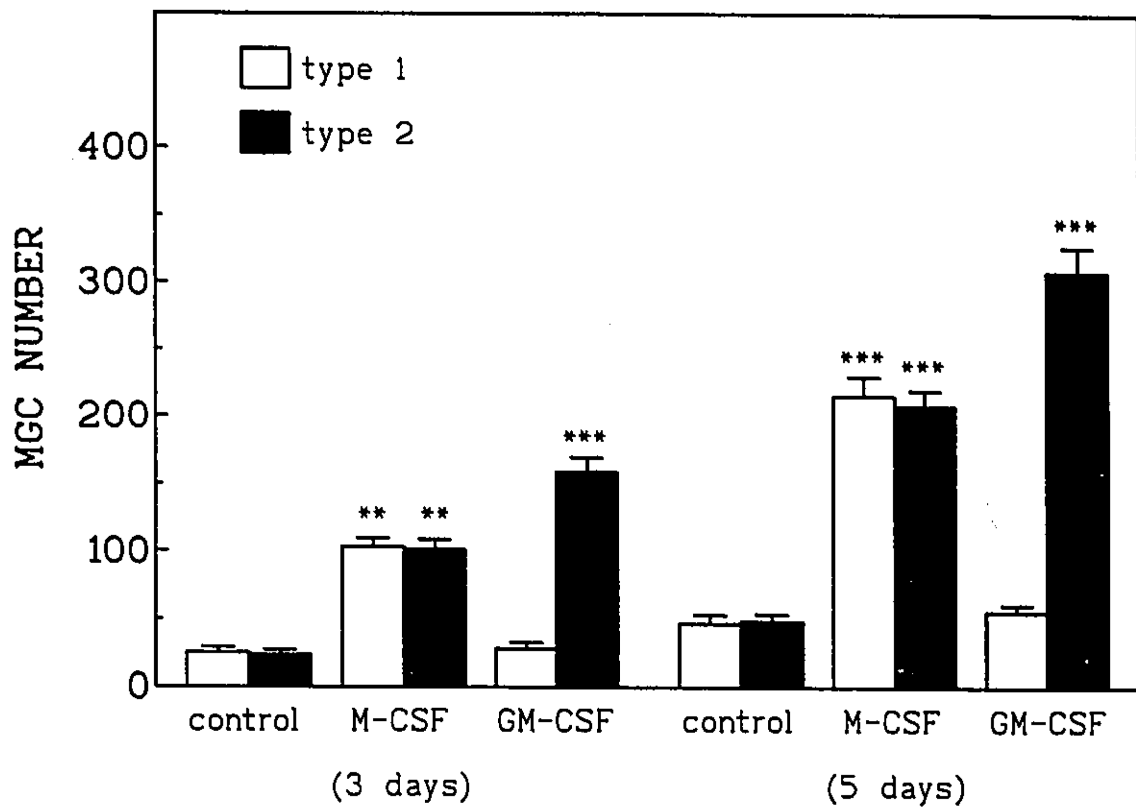


Figure 16. Differential effects of M-CSF and GM-CSF on type 1 and type 2 MGC formation. AM (2 x 10^5) were incubated in Lab Tek culture chambers in 0.2 ml medium alone or in the presence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). After 3 and 5 days, the culture chambers were rinsed by PBS and stained with Wright-Giemsa solution. The number of MGC were monitored under the microscope (magnification x 330) as described in **Materials and Methods.** Data represent the mean $\pm$ SEM from 3 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.01$ (**) or $p<.001$ (***).



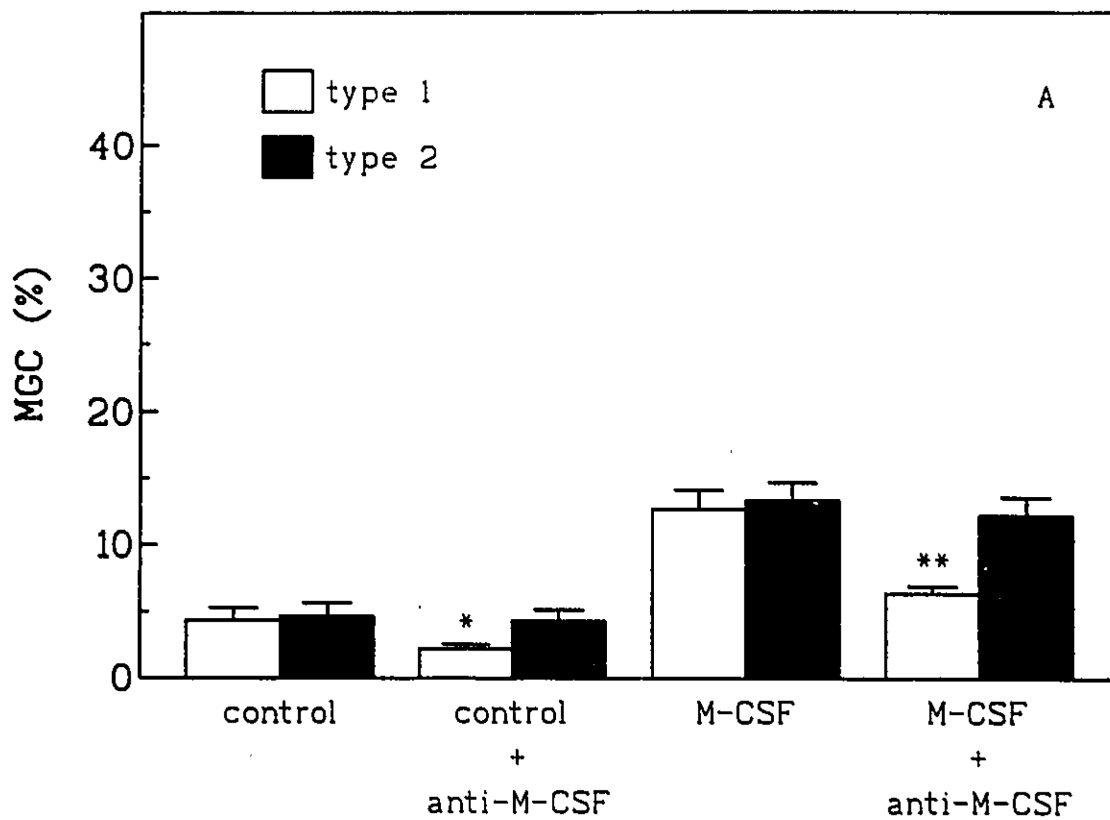
type 1
type 2
MGC NUMBER
400
300
200
100
0
***
***
***
** **
***
control
M-CSF
GM-CSF
control
M-CSF
GM-CSF
(3 days)
(5 days)

cultures caused a decrease of type 1 MGC, but had no effect on type 2 MGC formation (Figure 17A), suggesting that type 2 MGC are not directly derived from type 1 MGC. On the other hand, addition of anti-GM-CSF suppressed the formation of type 2 MGC in control and GM-CSF-treated cultures without affecting the formation of type 1 MGC (Figure 17B). These results suggest that M-CSF induces type 1 MGC formation whereas GM-CSF is responsible for type 2 MGC formation, and suggest that different pathways are likely to be involved in the formation of the two types of MGC.

3.2.4. INVOLVEMENT OF GM-CSF IN TYPE 2 MGC FORMATION INDUCED BY M-CSF

In this in vitro model, a small number of both type 1 and type 2 MGC was observed in control cultures in the absence of serum and without exogenous addition of CSFs. Since it is known that AM produce constitutively low levels of M-CSF (Becker et al. 1989, Ogawa et al. 1994), it is possible that increased type 2 MGC formation in unstimulated cells and in response to M-CSF may result from endogenous production of GM-CSF induced by M-CSF. To investigate this, cross-blocking experiments with antibodies were carried out. As shown in Figure 18A, GM-CSF did not stimulate type 1 MGC over controls and addition of anti-M-CSF to GM-CSF-treated cultures selectively decreased type 1 MGC formation as in controls. More interestingly, addition of anti-GM-CSF to M-CSF-treated AM selectively decreased type 2 MGC formation (Figure 18B), suggesting a role for endogenously produced GM-CSF. Further support

Figure 17. Effects of neutralizing antibodies against M-CSF or GM-CSF on type 1 and type 2 MGC formation. AM (2×10^5) were incubated in Lab Tek culture chambers. Antibody to human M-CSF was added to the culture in the presence or absence of M-CSF (A) and antibody to murine GM-CSF was added to the culture in the presence or absence of GM-CSF (B) as described in the **Materials and Methods.** The culture chambers were rinsed with PBS and stained with Wright-Giemsa solution after 5-day incubation. The number of type 1 and type 2 MGC was monitored under the microscope (magnification x 330) by counting at least 20 fields. Data represent the mean $\pm$ SEM from 4 experiments and values are expressed as percentage. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*), $p<.01$ (**), or $p<.001$ (***).


type 1
type 2
A
MGC (%)
0
10
20
30
40
*
**
control
control
+
anti-M-CSF
M-CSF
M-CSF
+
anti-M-CSF

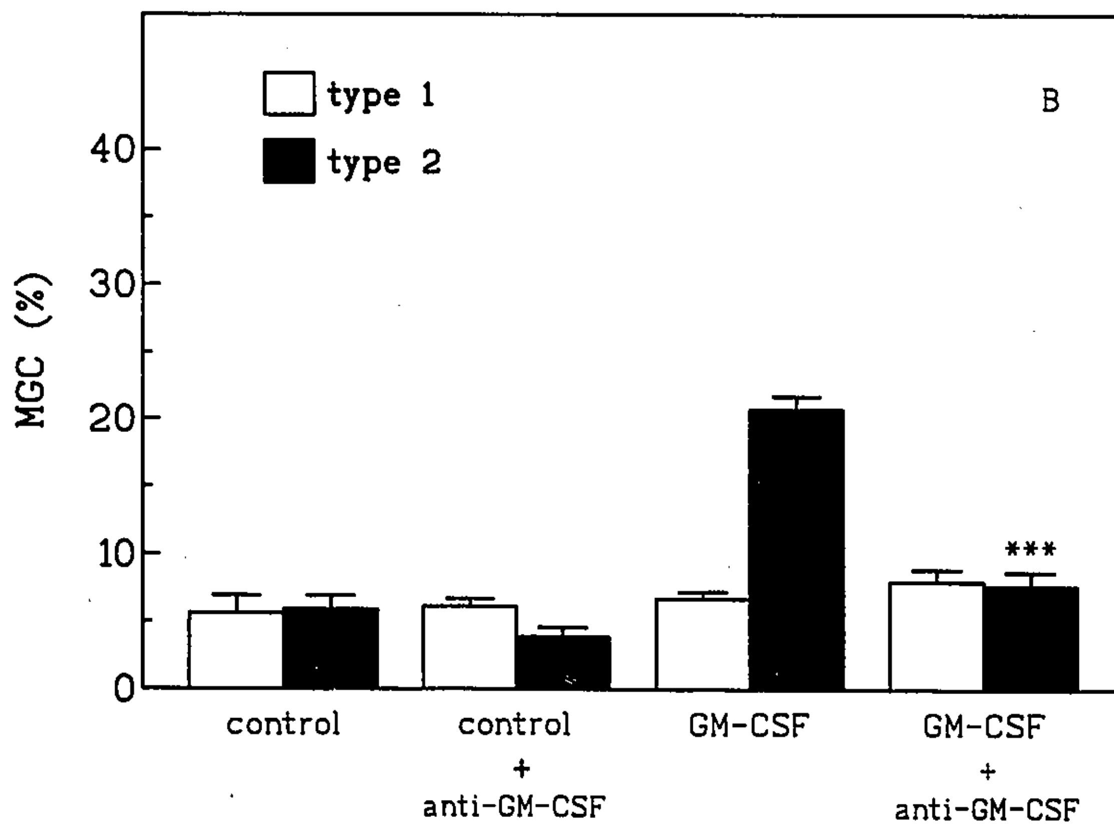
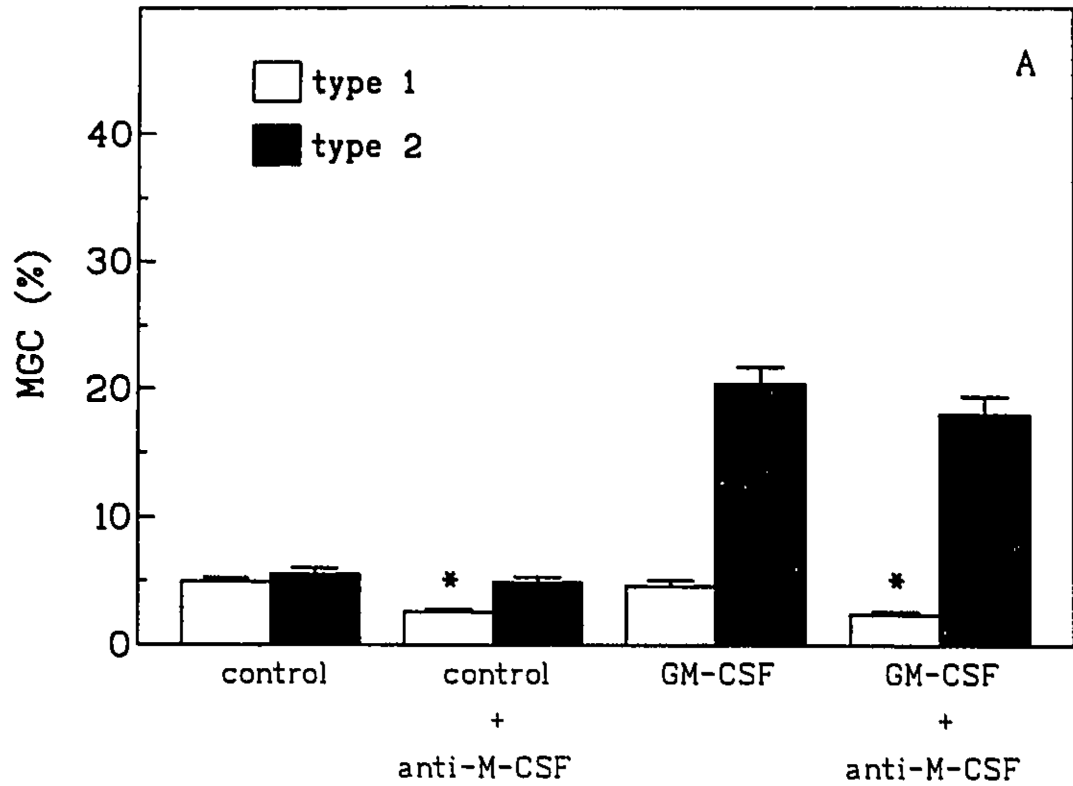
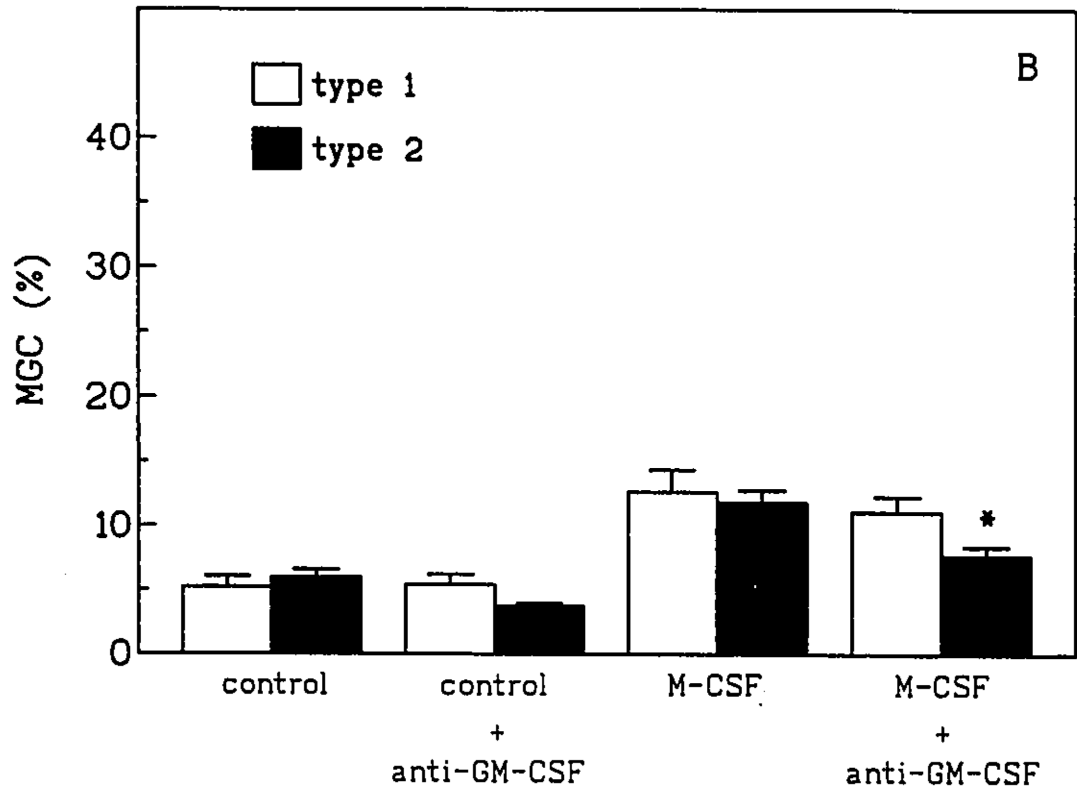

type 1
type 2
B
MGC (%)
0
10
20
30
40
***
control
control
+
anti-GM-CSF
GM-CSF
GM-CSF
+
anti-GM-CSF

Figure 18. Cross-blocking of type 1 and type 2 MGC formation by antibody to M-CSF or GM-CSF. Antibody to human M-CSF was added to the culture in the presence or absence of GM-CSF (A) and antibody to murine GM-CSF was added to the culture in the presence or absence of M-CSF (B) as described in the **Materials and Methods.** The culture chambers were rinsed with PBS and stained with Wright-Giemsa solution after 5-day incubation. The number of type 1 and type 2 MGC was monitored under the microscope (magnification x 330) by counting at least 20 fields. Data represent the mean $\pm$ SEM from 4 experiments and values are expressed as percentage. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p < .05$ (*).



type 1
type 2
A
MGC (%)
40
30
20
10
0
*
*
control
control
+
anti-M-CSF
GM-CSF
GM-CSF
+
anti-M-CSF



type 1
type 2
B
MGC (%)
40
30
20
10
0
*
control
control
+
anti-GM-CSF
M-CSF
M-CSF
+
anti-GM-CSF

for this hypothesis comes from the observations that unstimulated AM incubated under similar conditions expressed low levels of GM-CSF mRNA during culture as measured by RT-PCR (Figure 19). In contrast, freshly obtained AM did not express detectable mRNA for GM-CSF. Moreover, as found for LPS, a positive control, M-CSF stimulated GM-CSF mRNA levels at all time-points studied. Therefore, under this culture conditions, AM have the ability to produce GM-CSF and this could be further stimulated by M-CSF. Thus, type 2 MGC seen in unstimulated and M-CSF treated groups may result from endogenous production of GM-CSF induced by M-CSF.

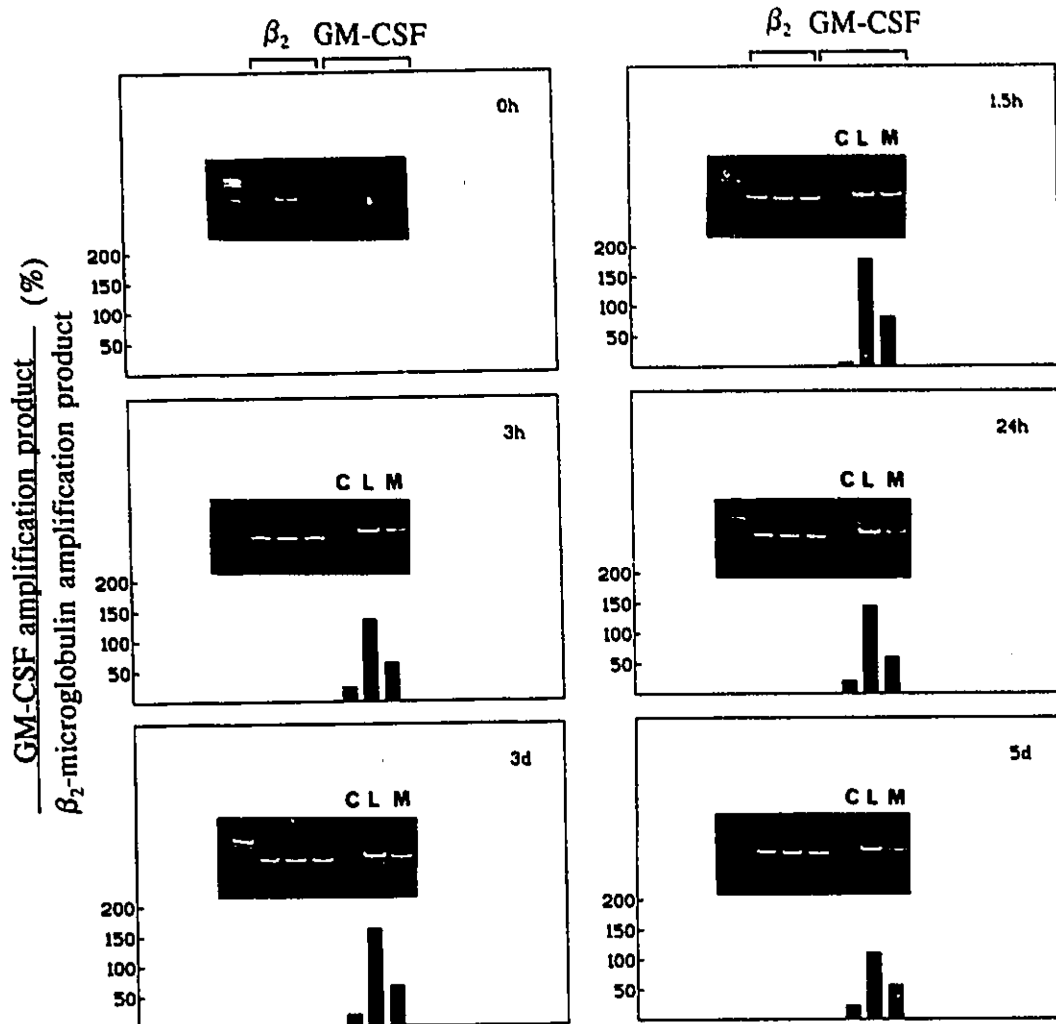
3.3. CYTOKINE MODULATION DURING AM DIFFERENTIATION

3.3.1. CYTOKINE PROFILE DURING AM DIFFERENTIATION

The functional status of the overall cell microenvironment that accompanies the selective changes induced by M-CSF and GM-CSF in culture were examined. The molecular phenotyping of cytokines known to participate in inflammation and AM regulation was performed using RT-PCR. This technique was adapted to the culture conditions and permitted the analysis of individual cultures simultaneously and specifically for the presence of many mRNA species including TNF-α, IL-1α, IL-6 and TGF-β. β_2-microglobulin was used as an internal control in parallel at all time-points studied.

RNA was directly extracted from Lab Tek-cultured AM (2×10^5) which were incubated for 1.5h, 3h, 24h, 72h (3d) and 120h (5d) in the presence or

Figure 19. GM-CSF mRNA expression detected by RT-PCR during AM differentiation from a representative experiment. RNA was directly extracted from Lab Tek cultures of AM which were incubated for 1.5h, 3h, 24h, 72h and 120h in the presence or absence of M-CSF (50 U/ml). In parallel, LPS (1 μg/ml) stimulation was applied as a positive control. AM freshly obtained after BAL were used as a 0 time control. GM-CSF mRNA level was measured by RT-PCR as described in **Materials and Methods.** β_2-microglobulin was used as an internal control. Data are represented as the relative expression of GM-CSF as determined by quantitative densitometric analysis with GPTool program after standardizing the amounts according to the expression of β_2-microglobulin. Values are expressed as percentage of β_2-microglobulin mRNA expression from each group. C, control. L, LPS treated sample. M, M-CSF treated sample.



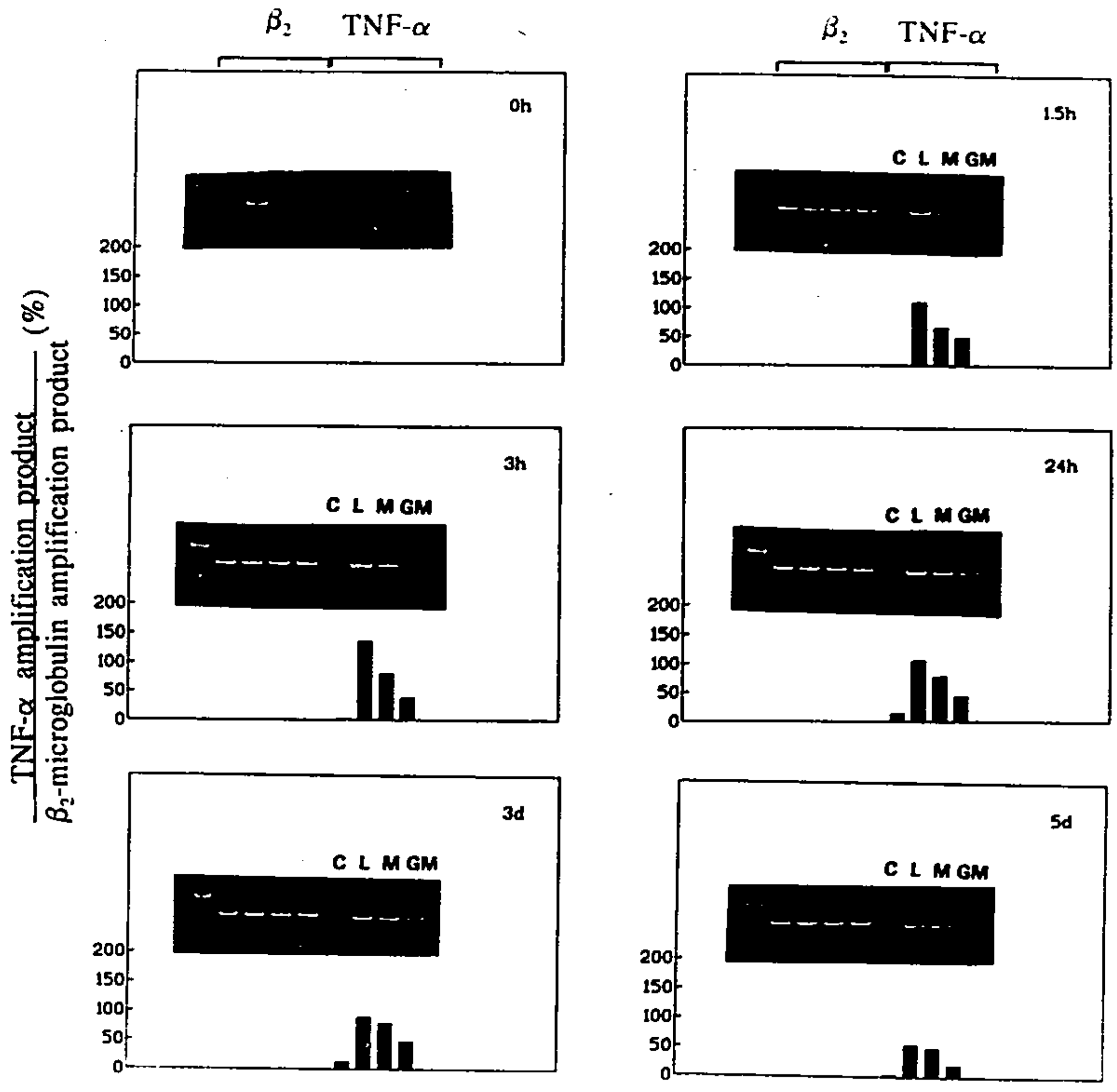
β2 GM-CSF
0h
1.5h
3h
24h
3d
5d
C L M
200
150
100
50
GM-CSF amplification product
β2-microglobulin amplification product
(%)

absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml) as described in **Materials and Methods**. In parallel, LPS (1 μg/ml) stimulation was used as a positive control. AM freshly obtained by BAL were used as a 0 time control to determine whether cytokines investigated in our experiments were constitutively expressed.

3.3.1.1 Tumour necrosis factor-alpha (TNF-α)

TNF-α has been described as an important mediator of inflammatory responses as it influences the growth, differentiation, and functions of several cell types (Weber et al. 1993). TNF-α mRNA expression during AM differentiation 1.5h, 3h, 24h, 72h and 120h post-addition of M-CSF or GM-CSF was determined by RT-PCR and results are presented in Figure 20. TNF-α was not detected in 0 time group, indicating this cytokine is not constitutively expressed in rat AM. As expected, TNF-α transcription was significantly increased upon stimulation with LPS. The highest expression was detected at 3h and 24h post-LPS addition. Incubation of AM with M-CSF also induced the TNF-α mRNA expression at all time-points studied. The kinetics of TNF-α mRNA expression by differentiated AM in the presence of M-CSF was similar to that seen with LPS-treated samples except that the levels were lower in the former. Addition of GM-CSF also increased TNF-α mRNA expression at all time-points compared to controls. In comparison with M-CSF induction, however, AM treated with GM-CSF exhibited lower TNF-α mRNA expression. Interestingly, small but detectable levels of TNF-α mRNA expression were seen in controls

Figure 20. TNF-α mRNA expression detected by RT-PCR during AM differentiation from a representative experiment. RNA was directly extracted from Lab Tek cultures of AM which were incubated for 1.5h, 3h, 24h, 72h and 120h in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). In parallel, LPS (1 μg/ml) stimulation was applied as a positive control. AM freshly obtained after BAL were used as a 0 time control. TNF-α mRNA levels were measured by RT-PCR as described in Materials and Methods. β_2-microglobulin was used as an internal control. Data are represented as the relative expression of TNF-α as determined by quantitative densitometric analysis with GPTool program after standardizing the amounts according to the expression of β_2-microglobulin. Values are expressed as percentage of β_2-microglobulin mRNA expression from each group. C, control. L, LPS treated sample. M, M-CSF treated sample. GM, GM-CSF treated sample.



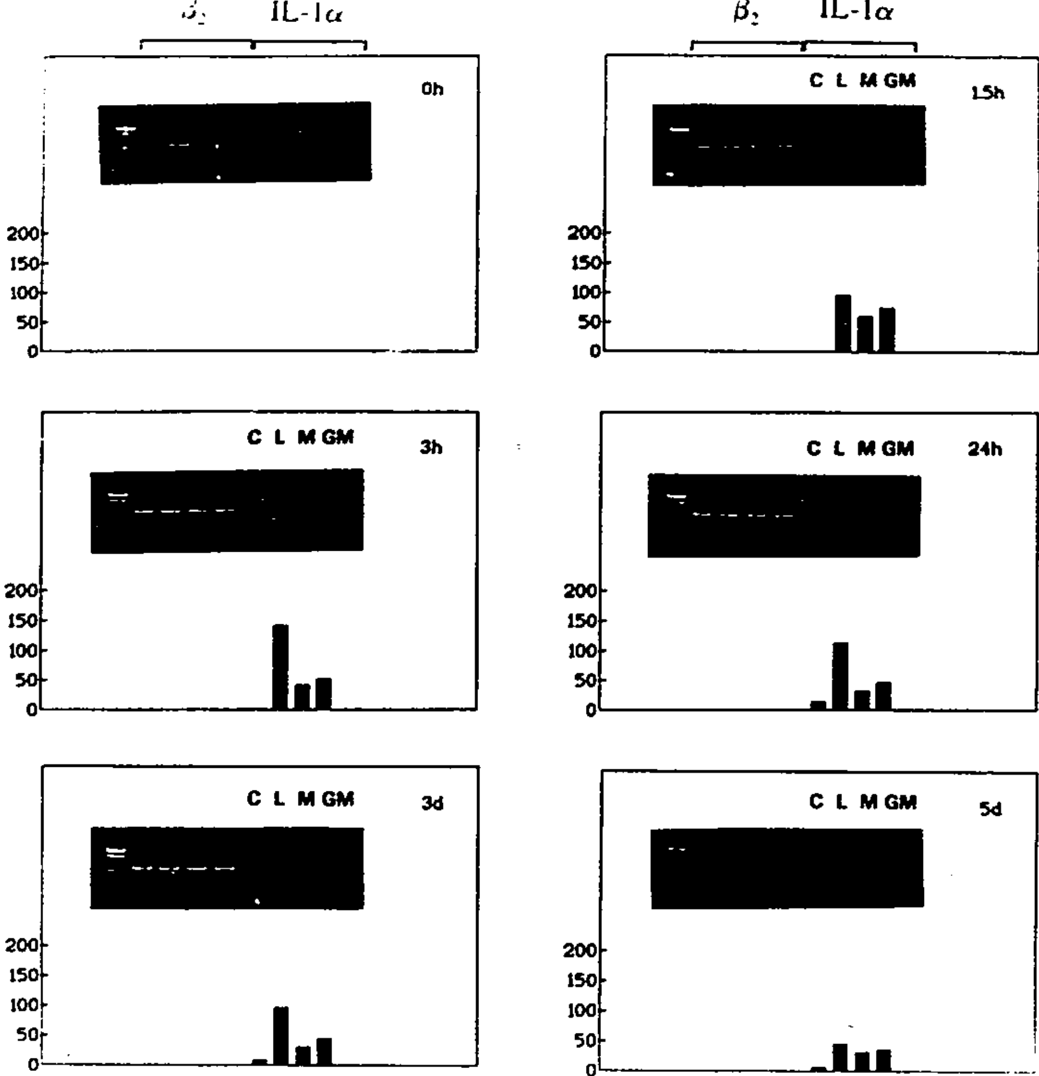
β_2
TNF-α
0h
1.5h
3h
24h
3d
5d
C L M GM
200
150
100
50
0
TNF-α amplification product / β_2-microglobulin amplification product (%)

(unstimulated AM) starting at 3h and thereafter, suggesting that AM differentiation is associated with expression of TNF-α. Parallel experiments done by others in the laboratory (Lemaire et al. 1996b) revealed that these patterns of TNF-α expression were correlated with increased TNF-α protein and activity as measured by enzyme-linked immunosorbent assay (ELISA) and L929 bioassay.

3.3.1.2. Interleukin-1 (IL-1α)

IL-1α is plays an important role in immune responses and inflammatory reactions (Oppenheim et al. 1986). IL-1α transcription during AM differentiation was detected by RT-PCR and results are presented in Figure 21. As for TNF-α, IL-1α mRNA expression was not detected in the 0 time group, suggesting this cytokine is not constitutively expressed in rat AM. As expected, LPS (1 μg/ml) stimulated IL-1α mRNA expression with a significant increase seen as early as 1.5h post-LPS treatment. Controls did not show IL-1α mRNA expression up to 5 days of incubation. M-CSF and GM-CSF induced IL-1α mRNA expression at all time-points studied. The levels of IL-1α mRNA expression, however, were not as high as those seen after LPS stimulation. When the expression of IL-1α mRNA induced by GM-CSF was compared to that induced by M-CSF, GM-CSF had slightly higher inducible effects on IL-1α mRNA expression at 1.5h, 3h, 24h and 3 days. However, levels of IL-1α mRNA expression were generally decreased with time in culture among all groups.

Figure 21. IL-1α mRNA expression detected by RT-PCR during AM differentiation from a representative experiment. RNA was directly extracted from Lab Tek cultures of AM which were incubated for 1.5h, 3h, 24h, 72h and 120h in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). In parallel, LPS (1 μg/ml) stimulation was applied as a positive control. AM freshly obtained after BAL were used as a 0 time control. IL-1α mRNA levels were measured by RT-PCR as described in **Materials and Methods.** β_2-microglobulin was used as an internal control. Data are represented as the relative expression of IL-1α as determined by quantitative densitometric analysis with GPTool program after standardizing the amounts according to the expression of β_2-microglobulin. Values are expressed as percentage of β_2-microglobulin mRNA expression from each group. C, control. L, LPS treated sample. M, M-CSF treated sample. GM, GM-CSF treated sample.



IL-1α amplification product / β2-microglobulin amplification product (%)
β2
IL-1α
C L M GM
0h
1.5h
3h
24h
3d
5d
0
50
100
150
200

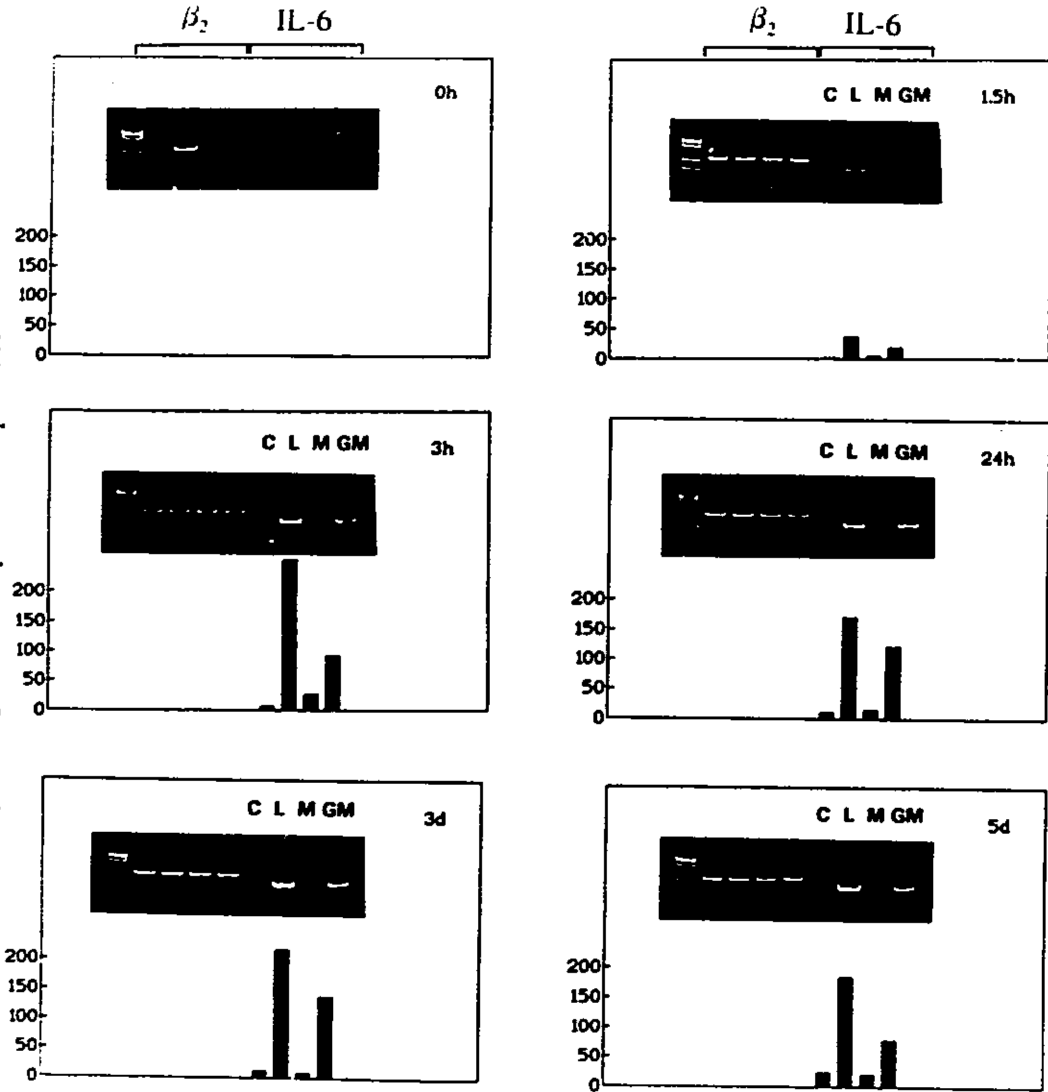
3.3.1.3. Interleukin-6 (IL-6)

IL-6 is produced by macrophages and has been shown to play an important role in cell differentiation (Chiu and Lee 1989, Oritani et al. 1992, Tanigawa et al. 1995). Expression of IL-6 mRNA was determined in our experimental groups by RT-PCR and data are illustrated in Figure 22. IL-6 mRNA is not constitutively expressed since no IL-6 PCR products were detected in the 0 time group while the internal control of β-$_2$ microglobulin was. As for TNF-α and IL-1α, LPS significantly stimulated IL-6 mRNA expression as early as 1.5h post exposure. In contrast to TNF-α and IL-1α, IL-6 mRNA expression in unstimulated and stimulated AM increased with time (up to 5 days). Differentiated AM induced by M-CSF showed levels of IL-6 mRNA comparable to those seen in unstimulated (control) AM at all time-points studied. In contrast, differentiated AM induced by GM-CSF displayed a significant increase of IL-6 mRNA expression at all time-points studied. The effect of GM-CSF on IL-6 mRNA expression appears to be long-lasting (up to 5 days) and the kinetics were similar to those observed for LPS stimulation. Higher levels of IL-6 PCR products could be seen at all time-points studied post-GM-CSF induction and such levels correlated with IL-6 activity as determined by the B9 cell bioassay (Lemaire et al. 1996b).

3.3.1.4. Transforming growth factor-β (TGF-β)

TGF-β appears to play an important role in wound-healing and fibrosis, and may contribute to modulate AM differentiation. The results of TGF-β mRNA

Figure 22. IL-6 mRNA expression detected by RT-PCR during AM differentiation from a representative experiment. RNA was directly extracted from Lab Tek cultures of AM which were incubated for 1.5h, 3h, 24h, 72h and 120h in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). In parallel, LPS (1 μg/ml) stimulation was applied as a positive control. AM freshly obtained after BAL were used as a 0 time control. IL-6 mRNA levels were measured by RT-PCR as described in Materials and Methods. β_2-microglobulin was used as an internal control. Data are represented as the relative expression of IL-6 as determined by quantitative densitometric analysis with GPTool program after standardizing the amounts according to the expression of β_2-microglobulin. Values are expressed as percentage of β_2-microglobulin mRNA expression from each group. C, control. L, LPS treated sample. M, M-CSF treated sample. GM, GM-CSF treated sample.



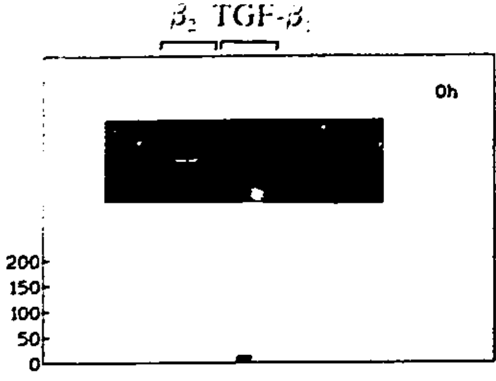
β2
IL-6
0h
1.5h
C L M GM
3h
24h
3d
5d
200
150
100
50
0
IL-6 amplification product / β2-microglobulin amplification product (%)

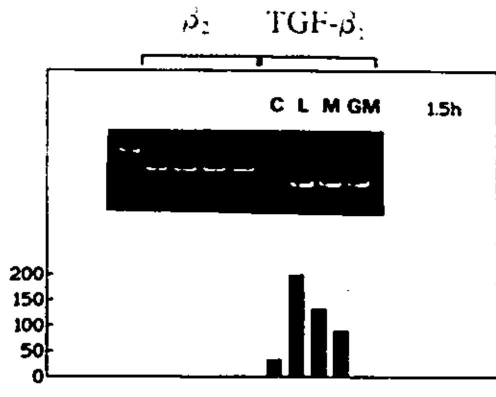
expression determined by RT-PCR in our experiments are presented in Figure 23. Low levels of TGF-β mRNA expression were detected in the 0 time sample, indicating that this cytokine is constitutively expressed by rat AM at the mRNA level. Compared to controls, LPS stimulated TGF-β mRNA expression. Both M-CSF and GM-CSF induced TGF-β mRNA expression as well. AM treated with M-CSF displayed slightly higher levels of TGF-β mRNA expression than AM treated with GM-CSF at 1.5h, 3h, 24h and 3 days. By 5 days, however, the levels of TGF-β mRNA expression were comparable.

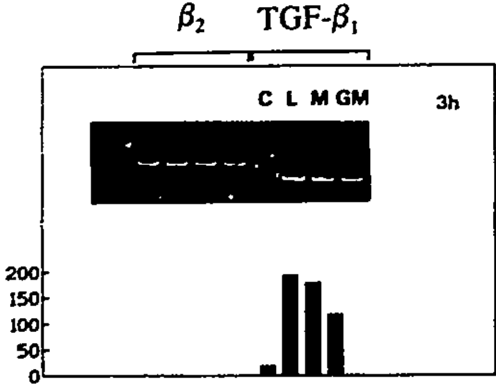
Overall, the respective profile of cytokine mRNA expression in response to M-CSF and GM-CSF is summarized in Table 4 and table 5. In unstimulated AM (control), expression of TNF-α, TGF-β and IL-6 was seen to increase with time in the culture. In response to M-CSF, which is associated with Type 1 MGC formation, TNF-α and TGF-β are expressed early and consistently for up to 5 days whereas IL-6 expression is not modulated by M-CSF. In response to GM-CSF which is associated with Type 2 MGC formation, TNF-α and TGF-β are also expressed early and persistently. In contrast to M-CSF, increased IL-6 expression was observed in GM-CSF treated-AM, suggesting IL-6 is modulated by GM-CSF and possibly during type 2 MGC formation.

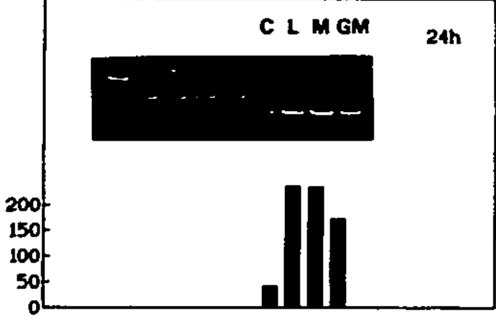
Figure 23. TGF-β mRNA expression detected by RT-PCR during AM differentiation from a representative experiment. RNA was directly extracted from Lab Tek cultures of AM which were incubated for 1.5h, 3h, 24h, 72h and 120h in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). In parallel, LPS (1 μg/ml) stimulation was applied as a positive control. AM freshly obtained after BAL were used as a 0 time control. TGF-β mRNA levels were measured by RT-PCR as described in Materials and Methods. β_2-microglobulin was used as an internal control. Data are represented as the relative expression of TGF-β as determined by quantitative densitometric analysis with GPTool program after standardizing the amounts according to the expression of β_2-microglobulin. Values are expressed as percentage of β_2-microglobulin mRNA expression from each group. C, control. L, LPS treated sample. M, M-CSF treated sample. GM, GM-CSF treated sample.

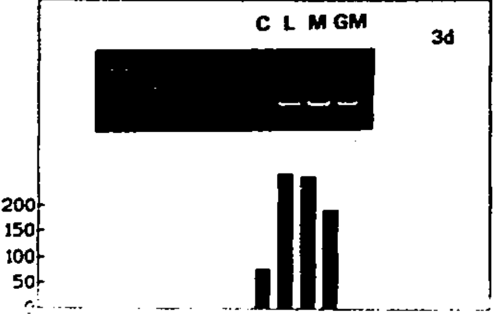
$$\frac{\text{TGF-}\beta_1 \text{ amplification product}}{\beta_2\text{-microglobulin amplification product}} \ (\%)$$











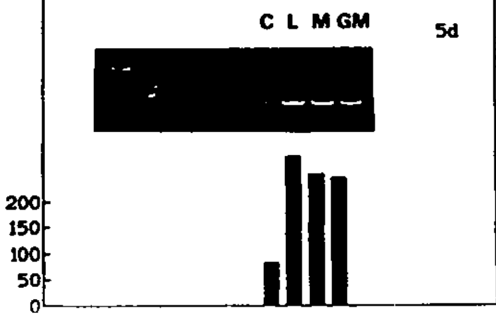
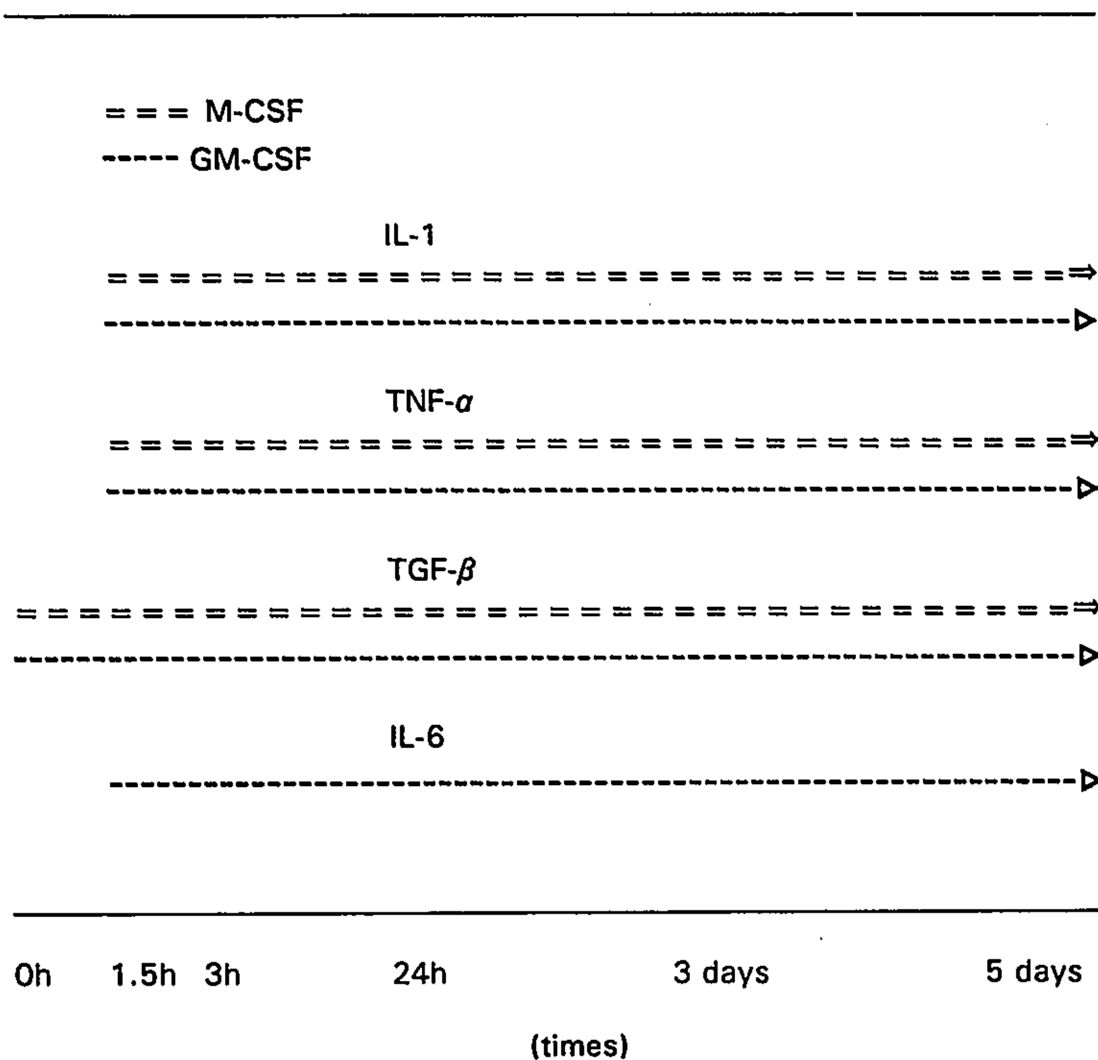


Table 4. cytokine mRNA expression of AM in response to M-CSF or GM-CSF

	TNF-α	IL-1	IL-6	TGF-β
0 time	no	no	no	yes
1.5h				
M-CSF	↑	↑	-	↑↑
GM-CSF	↑	↑	↑	↑
3h				
M-CSF	↑↑	↑	-	↑↑
GM-CSF	↑	↑↑	↑↑	↑↑
24h				
M-CSF	↑↑	↑	-	↑↑
GM-CSF	↑	↑↑	↑↑	↑↑
72h				
M-CSF	↑	↑	-	↑↑
GM-CSF	↑	↑	↑↑	↑↑
120h				
M-CSF	↑	↑	-	↑↑
GM-CSF	↑	↑	↑↑	↑↑

Table 5. Kinetics of cytokine mRNA expression of AM in response to M-CSF or GM-CSF



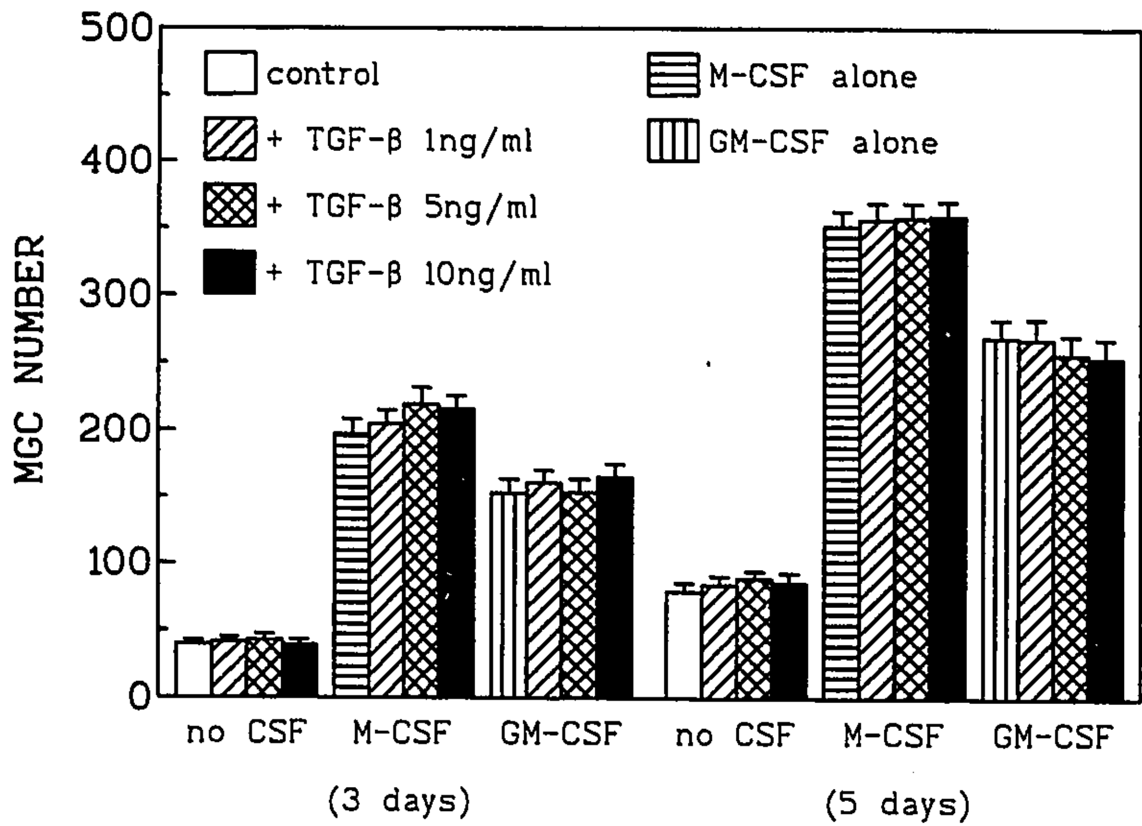
3.3.2. MODULATION OF TYPE 1 AND TYPE 2 MGC FORMATION BY EXOGENOUS CYTOKINES

To delineate further the respective role of TGF-β, TNF-α and IL-6 with respect to Type 1 and Type 2 MGC formation, we studied in a first set of experiments, the effects of exogenously added TGF-β, TNF-α and IL-6 on MGC formation.

3.3.2.1. Effect of TGF-β on MGC formation

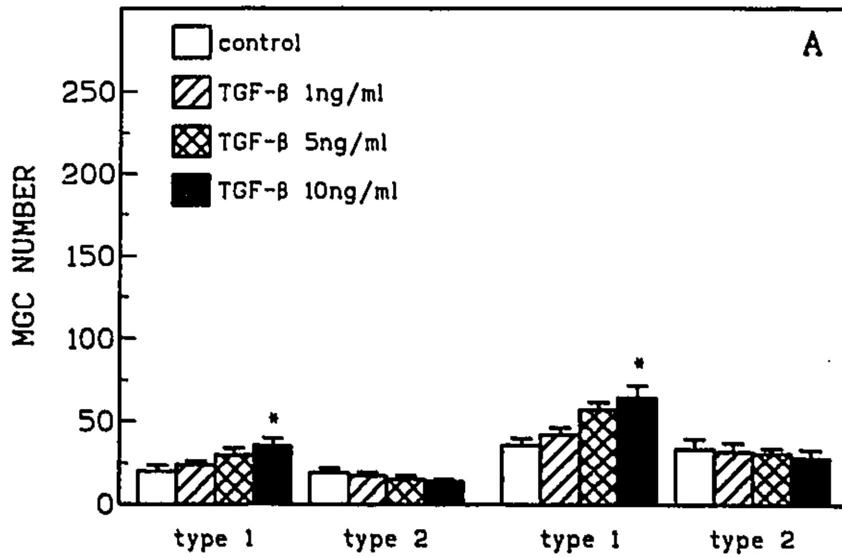
As shown in Figure 24, addition of exogenous TGF-β at concentrations of 1 ng/ml, 5 ng/ml and 10 ng/ml either alone or in combination with M-CSF or GM-CSF did not significantly affect the total number of MGC both at 3 days and 5 days compared to controls. However, when type 1 and type 2 MGC were analyzed, TGF-β alone or in combination with M-CSF or GM-CSF elevated type 1 MGC formation in a dose-dependent fashion, whereas type 2 MGC formation was decreased. Enhancement of type 1 MGC formation in response to TGF-β alone was significant ($P<.05$) at concentrations of 10 ng/ml both at 3 and 5 days incubation (Figure 25A). When AM were treated with a combination of TGF-β with M-CSF (Figure 25B), or TGF-β with GM-CSF (Figure 25C), an increase of type 1 and a decrease of type 2 MGC formation were also observed in a dose-dependent fashion of TGF-β. These data suggest that TGF-β may be involved in the modulation of type 1 and type 2 MGC formation by favouring the formation of type 1 over type 2.

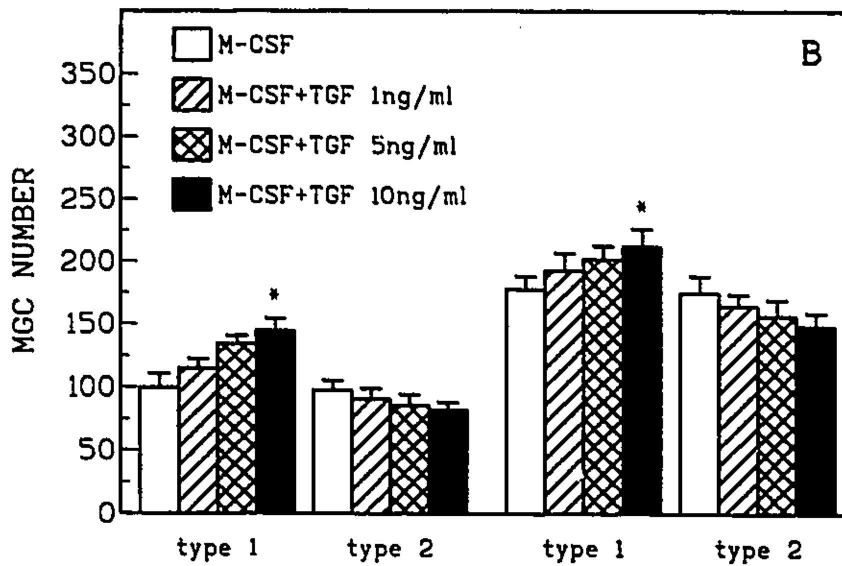
Figure 24. Induction of total MGC formation by exogenous TGF-β. AM (2 x 10^6) were incubated in Lab Tek culture chambers alone, in the presence of TGF-β (1 ng/ml, 5 ng/ml, and 10 ng/ml), M-CSF (50 U/ml), GM-CSF (50 U/ml) or a combination of TGF-β with M-CSF or GM-CSF. After 3 and 5 days, the culture chambers were rinsed by PBS and stained with Wright-Giemsa solution. The number of MGC were monitored under microscope (magnification x 330) as described in **Materials and Methods**. Values represent the mean $\pm$ SEM from 3 experiments.

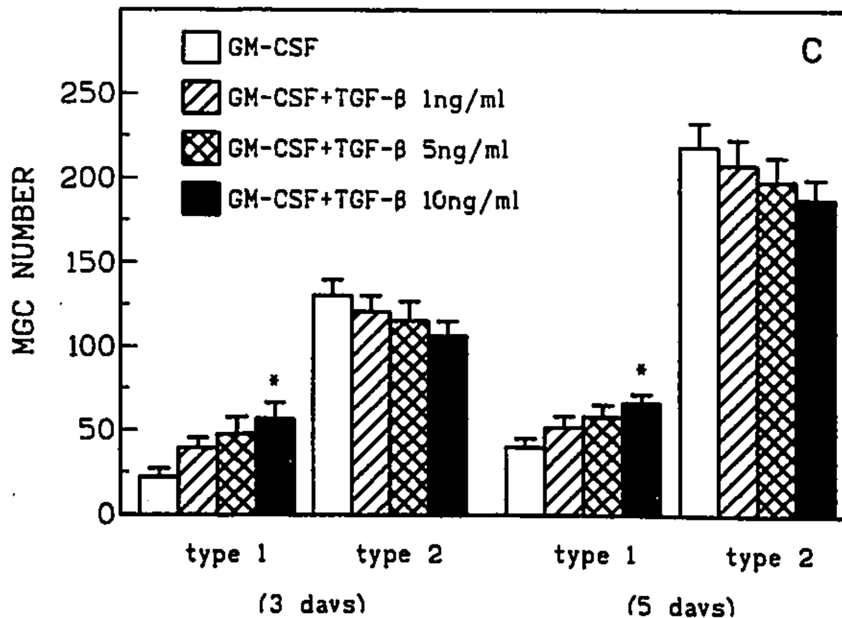


500
400
300
200
100
0
MGC NUMBER
control
+ TGF-β 1ng/ml
+ TGF-β 5ng/ml
+ TGF-β 10ng/ml
M-CSF alone
GM-CSF alone
no CSF
M-CSF
GM-CSF
no CSF
M-CSF
GM-CSF
(3 days)
(5 days)

Figure 25. Induction of type 1 and type 2 MGC formation by exogenous TGF-β and the combination of TGF-β with M-CSF or GM-CSF. (A) AM (2×10^5) were incubated in Lab Tek culture chambers alone or in the presence of TGF-β at various concentrations; (B) M-CSF (50 U/ml) or a combination of M-CSF with various concentrations of TGF-β; (C) GM-CSF (50 U/ml) or a combination of GM-CSF with various concentrations of TGF-β. After 3 and 5 days, the culture chambers were rinsed by PBS and stained with Wright-Giemsa. Type 1 and type 2 MGC were monitored under microscope (magnification x 330) as described in **Materials and Methods**. Values represent the mean $\pm$ SEM from 3 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p < .05$ (*).


control
TGF-β 1ng/ml
TGF-β 5ng/ml
TGF-β 10ng/ml
A
MGC NUMBER
250
200
150
100
50
0
*
*
type 1
type 2
type 1
type 2


M-CSF
M-CSF+TGF 1ng/ml
M-CSF+TGF 5ng/ml
M-CSF+TGF 10ng/ml
B
MGC NUMBER
350
300
250
200
150
100
50
0
*
*
type 1
type 2
type 1
type 2


GM-CSF
GM-CSF+TGF-β 1ng/ml
GM-CSF+TGF-β 5ng/ml
GM-CSF+TGF-β 10ng/ml
C
MGC NUMBER
250
200
150
100
50
0
*
*
type 1
type 2
type 1
type 2
(3 days)
(5 days)

3.3.2.2. Effect of TNF-α on MGC formation

Exogenous TNF-α at concentrations of 1 ng/ml, 20 ng/ml and 100 ng/ml was added to Lab Tek cultures of AM for 5 days to see the effect of TNF-α on AM differentiation and MGC formation. Figure 26 (A, B and C) presents the results of these experiments. Compared to the controls, addition of exogenous TNF-α at concentrations of 1 ng/ml, 20 ng/ml and 100 ng/ml did not display any effect on type 1 and type 2 MGC formation. When TNF-α was added to the culture in combination with either M-CSF (50 U/ml) or GM-CSF (50 U/ml), there was no significant change in type 1 and type 2 MGC formation compared to M-CSF or GM-CSF alone. These data suggest that exogenous TNF-α in this study does not directly affect the formation of type 1 or type 2 MGC.

3.3.2.3. Effect of IL-6 on MGC formation

To investigate the effects of IL-6 on AM differentiation and MGC formation, exogenous IL-6 at concentrations of 1 ng/ml, 20 ng/ml and 100 ng/ml was added to Lab Tek cultures of AM for 3 and 5 days. Data are presented in Figure 27 (A, B and C). Although addition of IL-6 alone or in combination with M-CSF or GM-CSF did not affect type 1 MGC formation, an increase of type 2 MGC formation was observed when IL-6 was added to the cultures. Compared to control, IL-6 alone significantly ($p<0.05$) increased type 2 MGC formation at 100 ng/ml concentration at 3 day-incubation. When IL-6 was added to the cultures in combination with M-CSF or GM-CSF, the significant increase ($p<0.05$) of type 2 MGC formation was observed at 20

Figure 26. Induction of type 1 and type 2 MGC formation by exogenous TNF-α. (A) AM (2 x 10^5) were incubated in Lab Tek culture chambers alone or in the presence of TNF-α at various concentrations; (B) M-CSF (50 U/ml) or a combination of M-CSF with various concentrations of TNF-α; (C) GM-CSF (50 U/ml) or a combination of GM-CSF with various concentrations of TNF-α. After 5 days, the culture chambers were rinsed by PBS and stained with Wright-Giemsa solution. Type 1 and type 2 MGC were monitored under microscope (magnification x 330) as described in Materials and Methods. Values represent the mean $\pm$ SEM from 2 experiments.

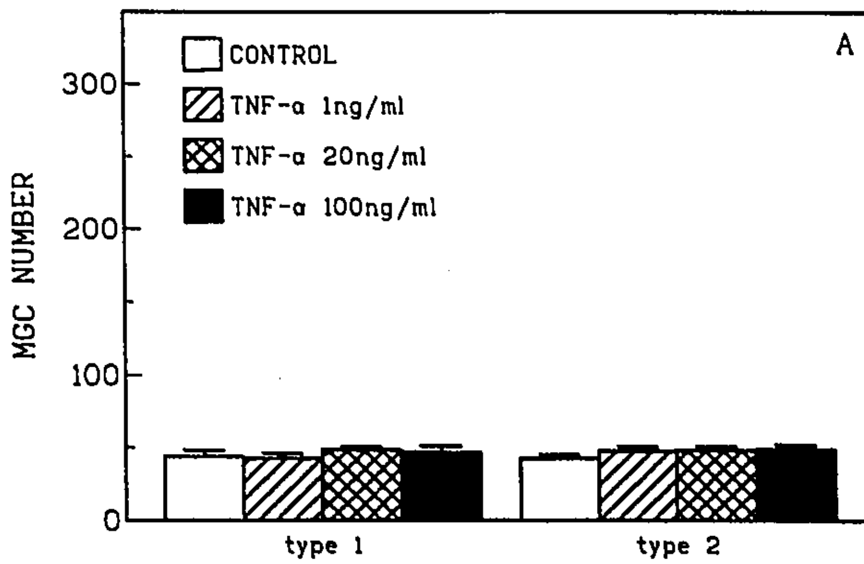
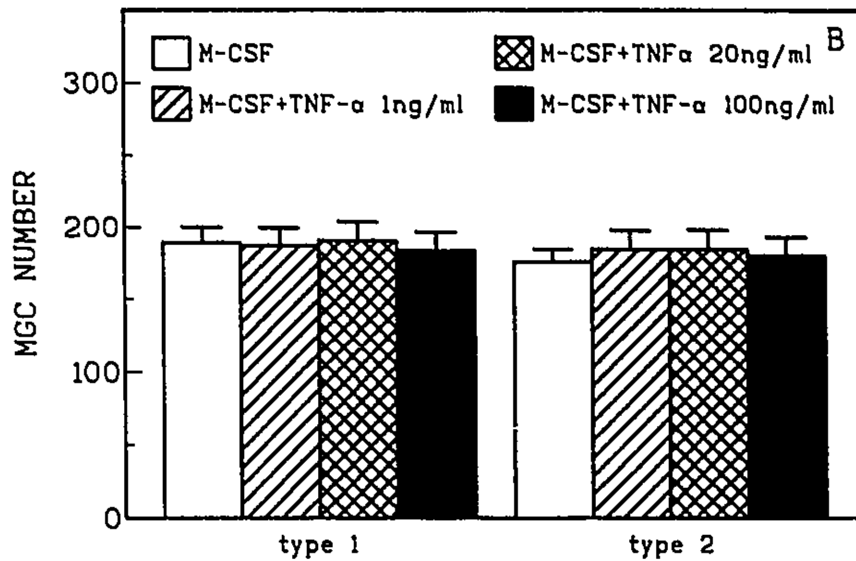
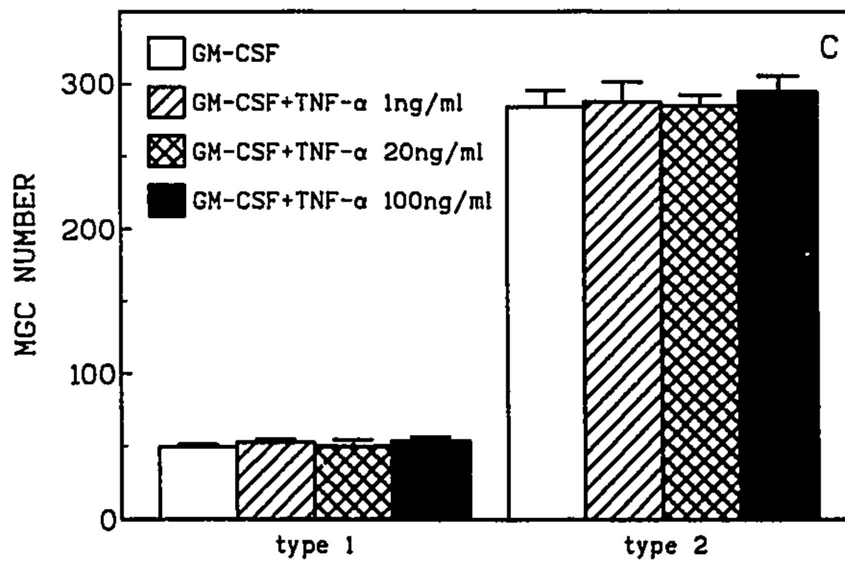
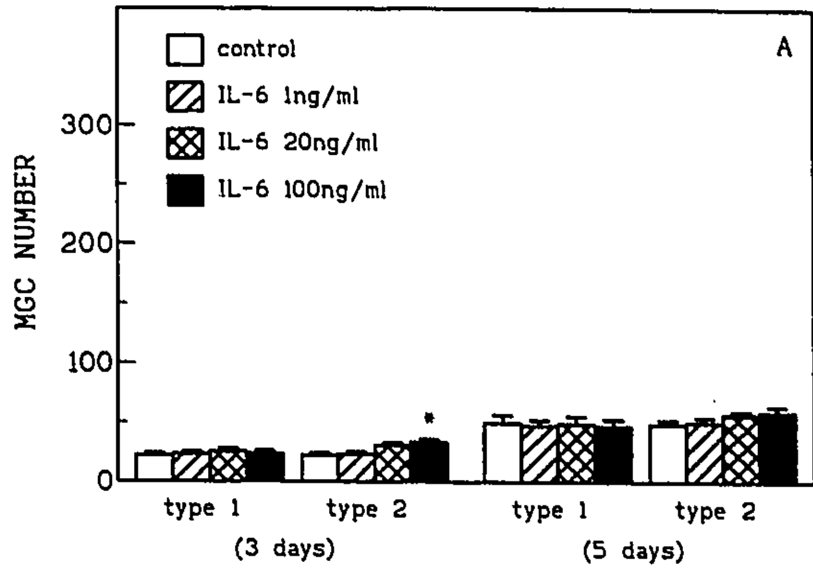
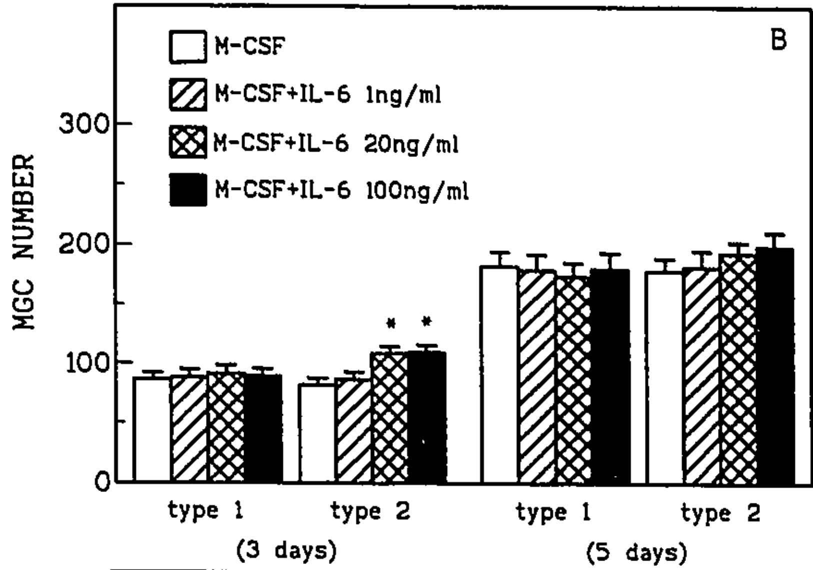
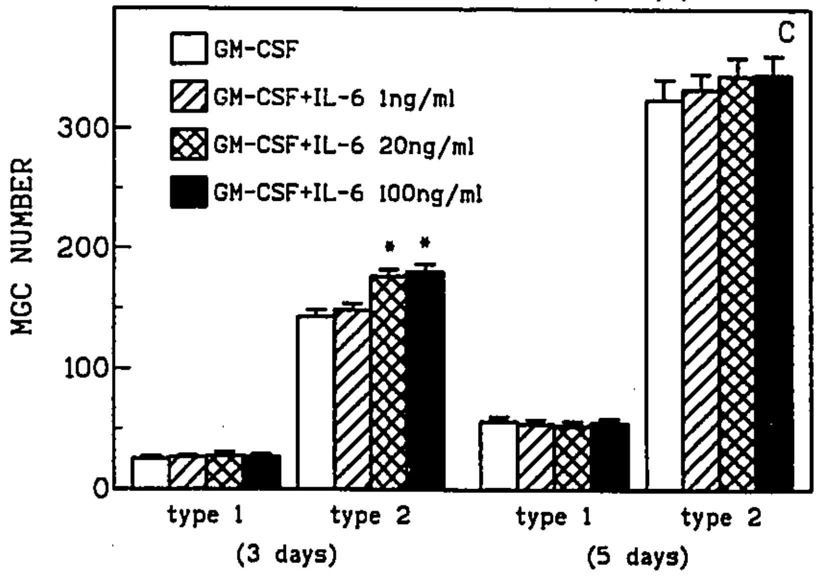

CONTROL
TNF-α 1ng/ml
TNF-α 20ng/ml
TNF-α 100ng/ml
A
MGC NUMBER
300
200
100
0
type 1
type 2

M-CSF
M-CSF+TNF-α 1ng/ml
M-CSF+TNFα 20ng/ml
M-CSF+TNF-α 100ng/ml
B
MGC NUMBER
300
200
100
0
type 1
type 2

GM-CSF
GM-CSF+TNF-α 1ng/ml
GM-CSF+TNF-α 20ng/ml
GM-CSF+TNF-α 100ng/ml
C
MGC NUMBER
300
200
100
0
type 1
type 2

Figure 27. Induction of type 1 and type 2 MGC formation by exogenous IL-6. (A) AM (2×10^5) were incubated in Lab Tek culture chambers alone or in the presence of IL-6 at various concentrations; (B) M-CSF (50 U/ml) or a combination of M-CSF with various concentrations of IL-6; (C) GM-CSF (50 U/ml) or a combination of GM-CSF with various concentrations of IL-6. After 3 and 5 days, the culture chambers were rinsed by PBS and stained with Wright-Giemsa solution. Type 1 and type 2 MGC were monitored under microscope (magnification x 330) as described in **Materials and Methods.** Values represent the mean $\pm$ SEM from 2 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*).


control
IL-6 1ng/ml
IL-6 20ng/ml
IL-6 100ng/ml
A
MGC NUMBER
300
200
100
0
type 1
type 2
type 1
type 2
(3 days)
(5 days)


M-CSF
M-CSF+IL-6 1ng/ml
M-CSF+IL-6 20ng/ml
M-CSF+IL-6 100ng/ml
B
MGC NUMBER
300
200
100
0
type 1
type 2
type 1
type 2
(3 days)
(5 days)


GM-CSF
GM-CSF+IL-6 1ng/ml
GM-CSF+IL-6 20ng/ml
GM-CSF+IL-6 100ng/ml
C
MGC NUMBER
300
200
100
0
type 1
type 2
type 1
type 2
(3 days)
(5 days)

ng/ml and 100 ng/ml concentration. Therefore, IL-6 may be involved in modulating type 2 MGC formation. The lack of effect of IL-6 seen at 5 day-incubation may be due to the degradation of active IL-6 with incubation time in the cultures.

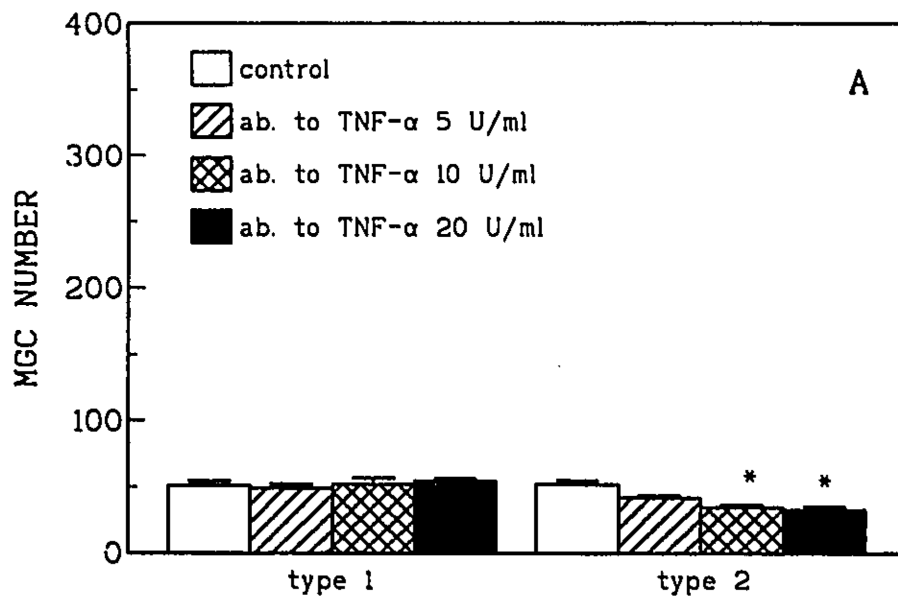
3.3.3. MODULATION OF TYPE 1 AND TYPE 2 MGC FORMATION BY ENDOGENOUS TNF-α and IL-6

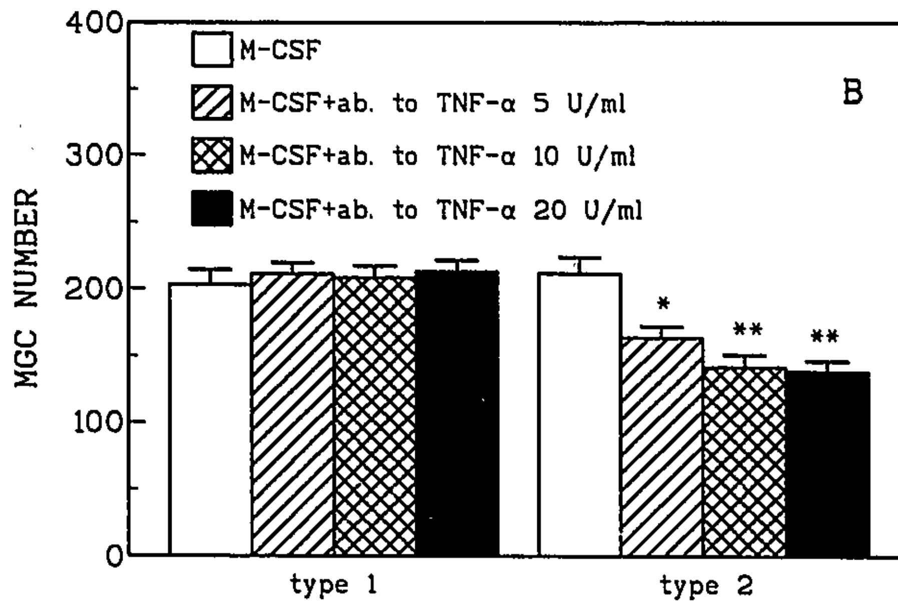
Data from RT-PCR experiments indicate that TNF-α and IL-6 expression is increased in unstimulated AM as a function of time in culture. In addition, TNF-α and IL-6 are differently regulated by M-CSF and GM-CSF, suggesting that endogenous TNF-α and IL-6 may play a role in MGC formation. In the next set of experiments, we used anti-TNF-α antibody and anti-IL-6 receptor antibody to further examine the role of endogenous TNF-α and IL-6 in AM differentiation and MGC formation.

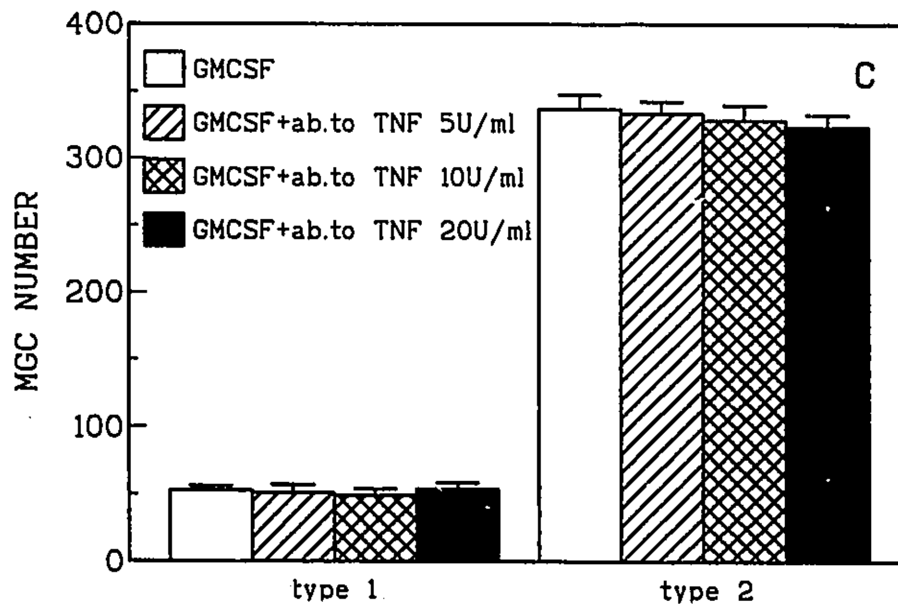
3.3.3.1. Effect of anti-TNF-α on type 1 and type 2 MGC formation.

Antibody to TNF-α at concentrations of 5 U/ml, 10 U/ml and 20 U/ml was added to the Lab Tek cultures in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). Experimental results are presented in Figure 28 (A, B and C). Addition of anti-TNF-α to unstimulated AM resulted in a decrease of type 2 MGC formation in a dose-dependent fashion, whereas type 1 MGC formation was not affected compared to the controls (Figure 28A). Similarly, anti-TNF-α decreased significantly type 2 MGC formation in AM treated with M-CSF, while type 1 MGC formation remain unchanged compared to M-CSF

Figure 28. Effect of antibody to TNF-α on type 1 and type 2 MGC formation. (A) AM (2×10^5) were incubated in Lab Tek culture chambers alone or in the presence of anti-TNF-α at various concentrations; (B) M-CSF (50 U/ml) or a combination of M-CSF with various concentrations of anti-TNF-α; (C) GM-CSF (50 U/ml) or a combination of GM-CSF with various concentrations of anti-TNF-α. After 5 days, the culture chambers were rinsed with PBS and stained with Wright-Giemsa. Type 1 and type 2 MGC were monitored under microscope (magnification x 330) as described in **Materials and Methods.** Values represent the mean $\pm$ SEM from 2 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*), $p<.01$ (**).


400
300
200
100
0
MGC NUMBER
control
ab. to TNF-α 5 U/ml
ab. to TNF-α 10 U/ml
ab. to TNF-α 20 U/ml
A
*
*
type 1
type 2


400
300
200
100
0
MGC NUMBER
M-CSF
M-CSF+ab. to TNF-α 5 U/ml
M-CSF+ab. to TNF-α 10 U/ml
M-CSF+ab. to TNF-α 20 U/ml
B
*
**
**
type 1
type 2


400
300
200
100
0
MGC NUMBER
GMCSF
GMCSF+ab.to TNF 5U/ml
GMCSF+ab.to TNF 10U/ml
GMCSF+ab.to TNF 20U/ml
C
type 1
type 2

treatment alone (Figure 28B). In contrast, anti-TNF-α antibody had no effect on type 2 MGC formation induced by GM-CSF (Figure 28C). These data suggest that endogenous TNF-α may be involved in modulating the effects of M-CSF on type 2 MGC formation.

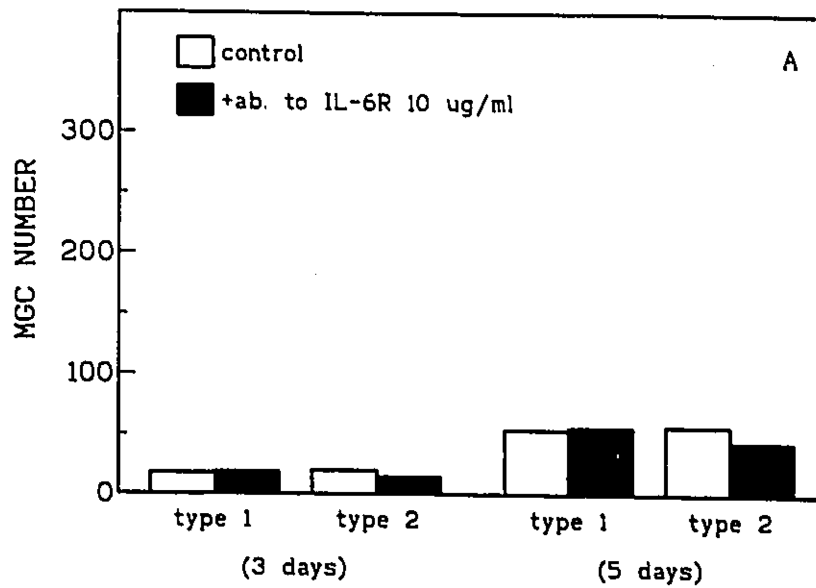
3.3.3.2. Effect of anti-IL-6 receptor on type 1 and type 2 MGC formation.

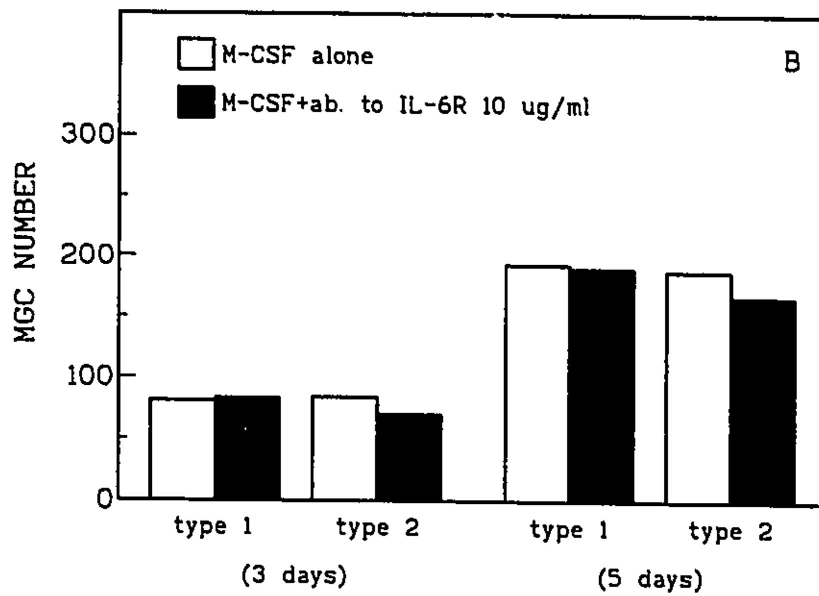
To further investigate the effect of endogenous IL-6 on MGC formation, antibody against IL-6 receptor at a concentration of 10 μg/ml was added to the Lab Tek cultures of AM for 3 and 5 days in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). Results from one experiment are presented in Figure 29. Compared to the control, addition of anti-IL-6 receptor does not affect type 1 but decreased type 2 MGC formation in both 3 and 5 day culture (Figure 29A). Similar results were observed in M-CSF treated AM (Figure 29B) and GM-CSF treated AM (Figure 29C). Compared to M-CSF treatment alone or GM-CSF treatment alone, addition of anti-IL-6 receptor caused a slightly decrease in type 2 MGC whereas type 1 MGC remained unchanged. Although results from bring further support for the involvement of IL-6 in modulating type 2 MGC formation, additional experiments are needed to further confirm it.

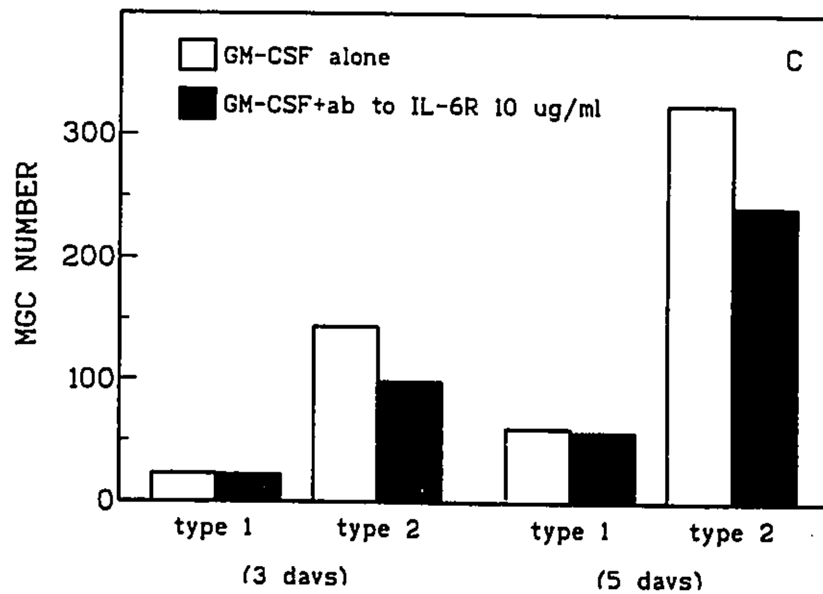
3.4. CYTOKINE PRODUCTION BY MGC

Despite the well documented observation of MGC in a variety of infectious and non-infectious diseases characterized by chronic inflammation,

Figure 29. Effect of anti-IL-6 receptor on type 1 and type 2 MGC formation. (A) AM (2 x 10^5) were incubated in Lab Tek culture chambers alone or in the presence of anti-IL-6 receptor at concentration of 10 μg/ml; (B) M-CSF (50 U/ml) or a combination of M-CSF with anti-IL-6 receptor (10 μg/ml); (C) GM-CSF (50 U/ml) or a combination of GM-CSF with anti-IL-6 receptor (10 μg/ml). After 3 and 5 days, the culture chambers were rinsed with PBS and stained with Wright-Giemsa. Type 1 and type 2 MGC were monitored under microscope (magnification x 330) as described in Materials and Methods. Values are from one experiment.


control
+ab. to IL-6R 10 ug/ml
A
MGC NUMBER
300
200
100
0
type 1
type 2
type 1
type 2
(3 days)
(5 days)


M-CSF alone
M-CSF+ab. to IL-6R 10 ug/ml
B
MGC NUMBER
300
200
100
0
type 1
type 2
type 1
type 2
(3 days)
(5 days)


GM-CSF alone
GM-CSF+ab to IL-6R 10 ug/ml
C
MGC NUMBER
300
200
100
0
type 1
type 2
type 1
type 2
(3 days)
(5 days)

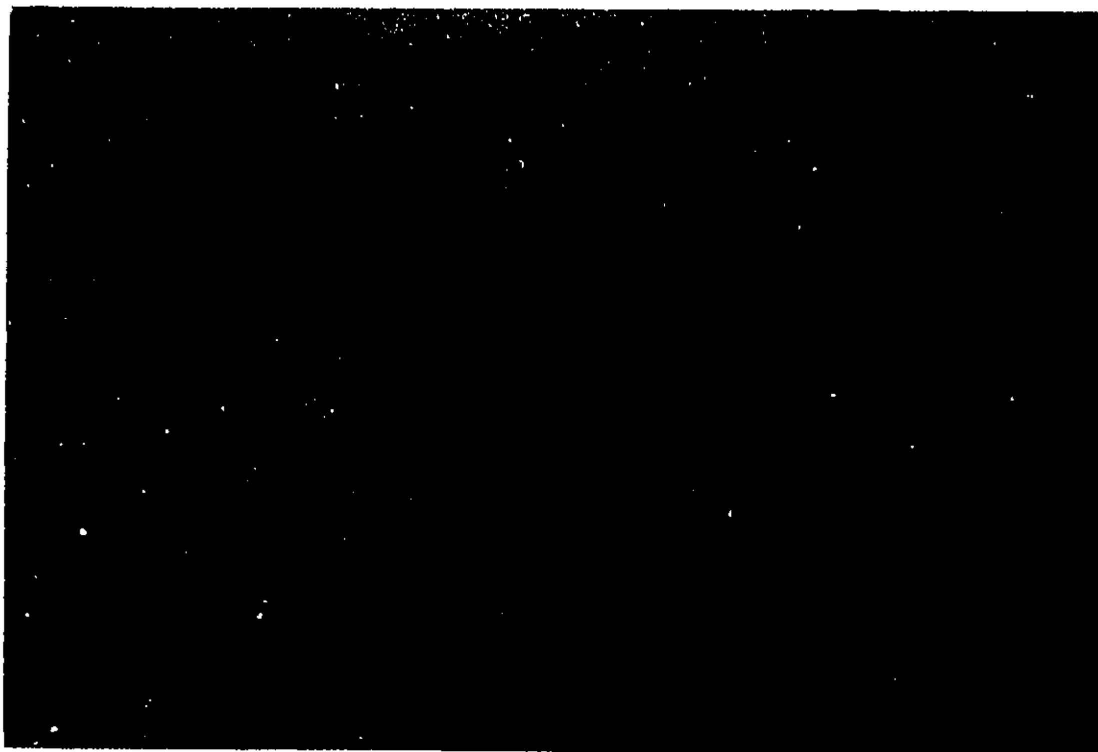
many questions are left unanswered regarding the role of these cells, including their primary biological function and whether they are essential or they merely represent highly stimulated cells at a terminal stage of differentiation. To address these issues, the functional status of MGC was investigated, notably their capacity to produce cytokines/growth factors. Therefore, cytoplasmic expression of TNF-α, PDGF and TGF-β by differentiated AM and AM-derived MGC was evaluated using specific antibodies and immunocytochemistry. TNF-α was chosen because it was associated with the development of lung inflammation and subsequent formation of fibrosis (Ouellet et al. 1993). Similarly, PDGF and TGF-β were chosen to be investigated because they are chemotactic and mitogenic for fibroblasts and play a major role in stimulating fibroblast proliferation (Ross et al. 1986, Kumar et al. 1988; Khalil et al. 1989). For analysis of immunocytochemistry, controls with nonimmune serum rather than specific first antibody to TNF-α, PDGF or TGF-β for all cultures were always used in parallel.

3.4.1. TUMOUR NECROSIS FACTOR-α (TNF-α)

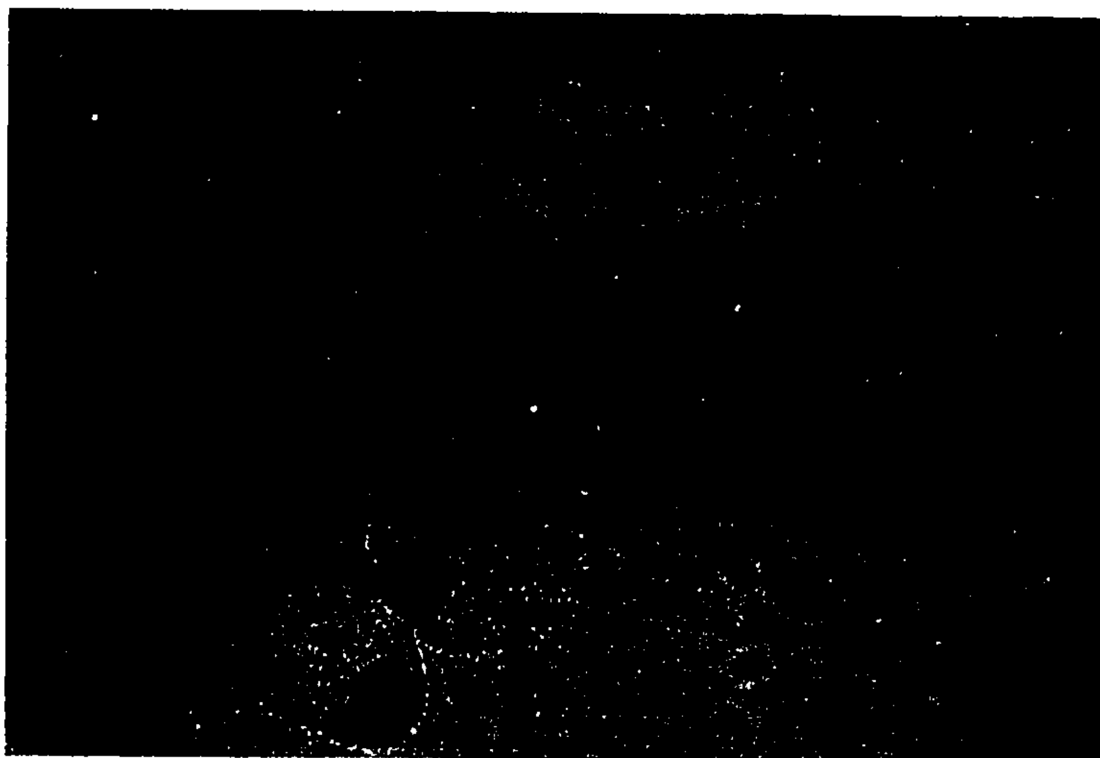
AM incubated in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml) for 5 days were evaluated for cytoplasmic expression of TNF-α. The profiles of TNF-α cytoplasmic expression of total AM are presented in Figure 30. Figure 30A shows the negative control staining from a sample stimulated with LPS and incubated with nonimmune serum rather than specific first antibody to TNF-α. Figures 30B, 30C and 30D show the immunocytochemical

Figure 30. Representative TNF-α immunocytochemistry staining of total AM (magnification x 825). AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. TNF-α was detected by immunocytochemistry as described in **Materials and Methods**. A, negative control with nonimmune serum from the sample stimulated by LPS for 3h; B, control (unstimulated AM); C, M-CSF-treated AM; D, GM-CSF-treated AM; E, LPS stimulation of AM for 3h.

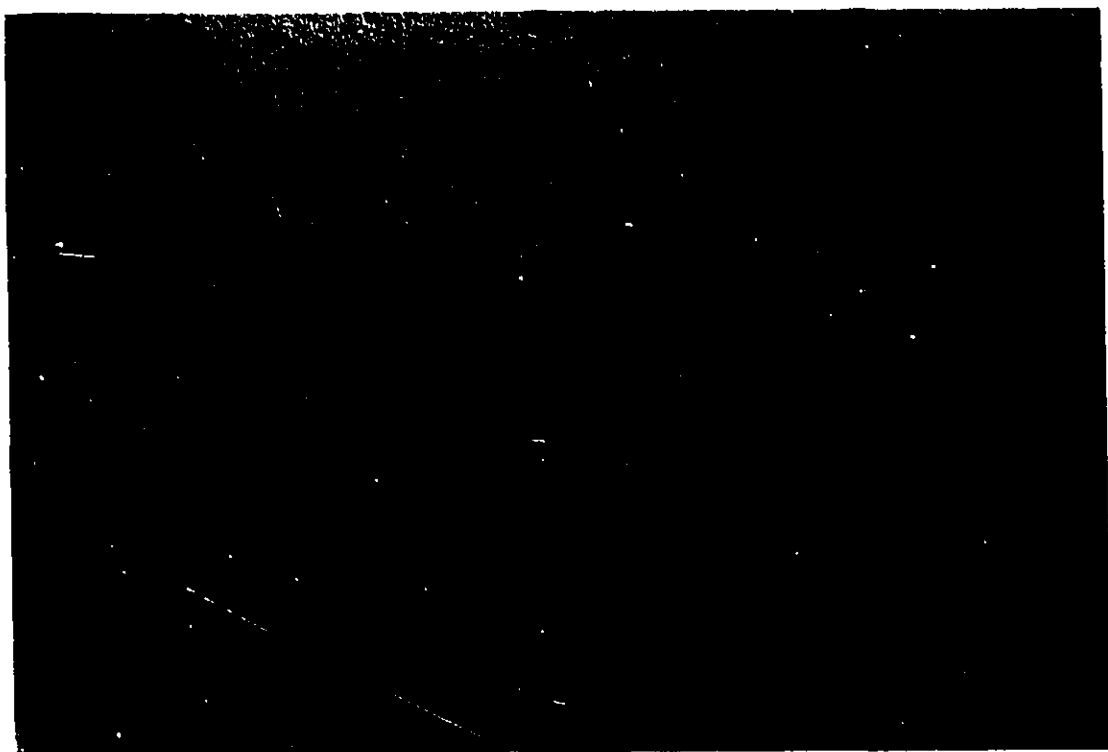
30A



30B

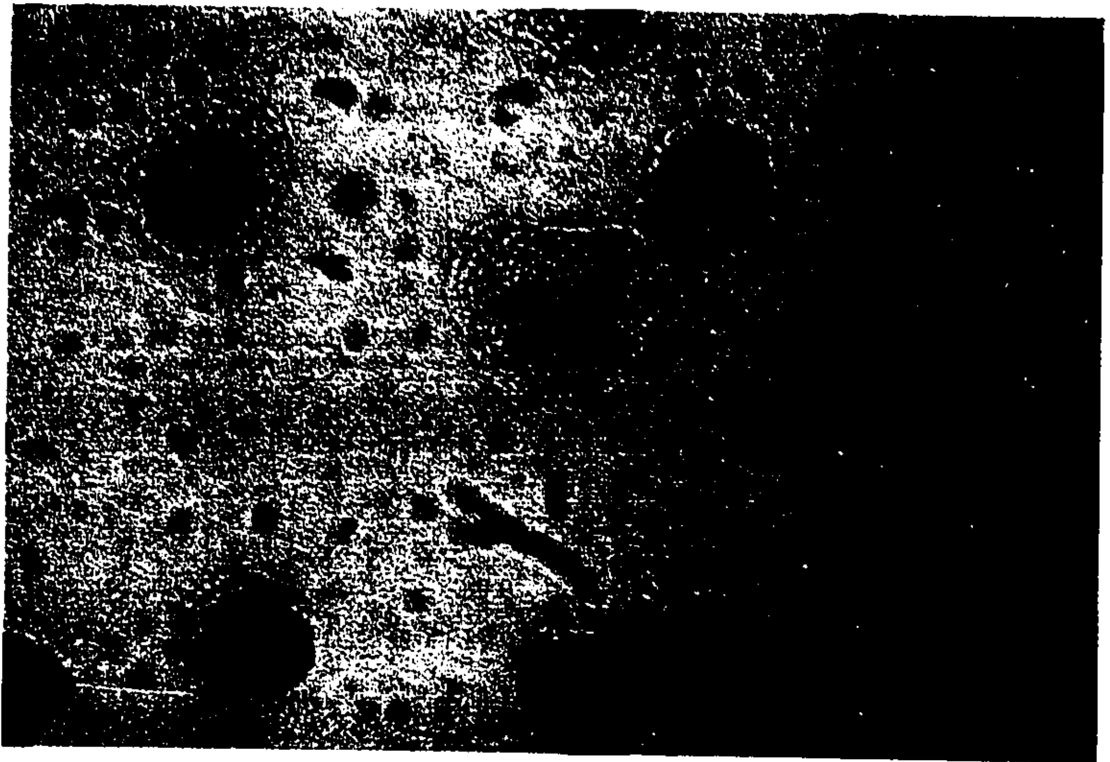


30C



30D

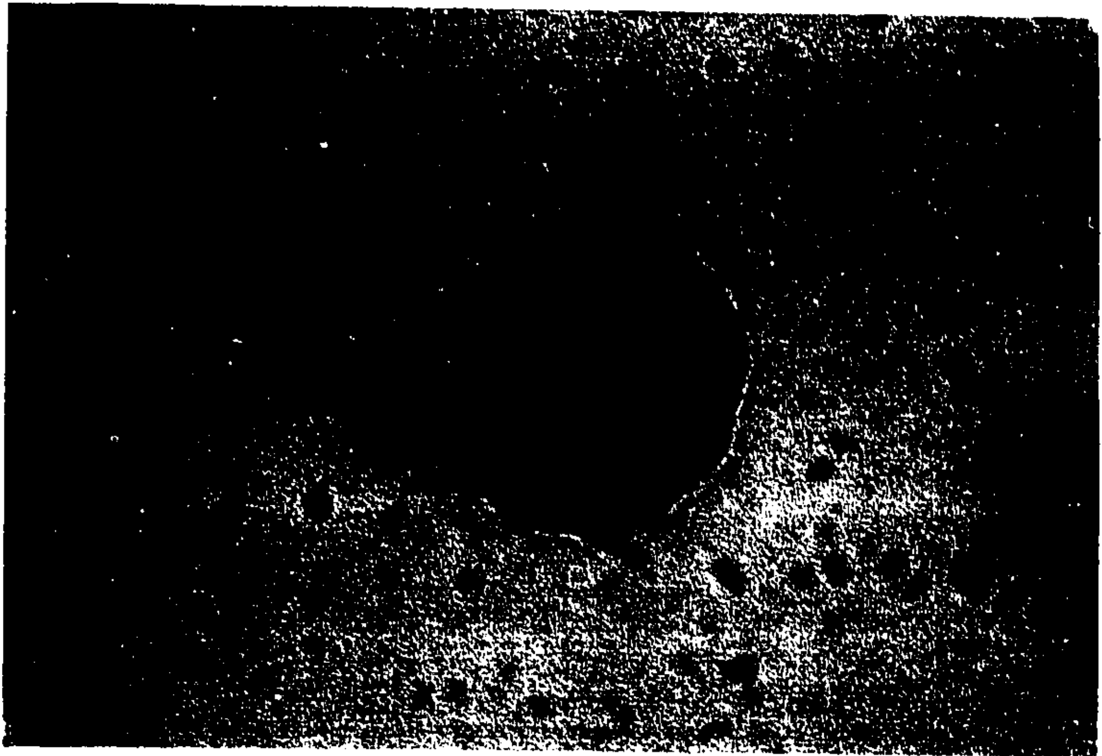
30E



staining for TNF-α in control, M-CSF and GM-CSF treated groups respectively, and Figure 30E shows TNF-α expression following 3h LPS stimulation of total AM following 5-day incubation. Compared to control, M-CSF and GM-CSF increased the proportion of cells expressing TNF-α, which is consistent with the findings of RT-PCR evaluation. We also analyzed whether AM-derived MGC still express this cytokine and found that some MGC did express TNF-α, suggesting that these MGC may be functionally active. However, when attention was paid to staining intensity of TNF-α cytoplasmic expression, it was noticed that generally type 1 MGC showed higher cytoplasmic expression of TNF-α than type 2 MGC (Figure 31A and B). Evaluation of the proportion of MGC expressing TNF-α was performed and data from 3 experiments are presented in Figure 32. Treatment with M-CSF or GM-CSF increased the number of total AM expressing TNF-α compared to controls ($p<.01$). A higher proportion of AM expressed TNF-α in response to M-CSF compared to GM-CSF. Type 1 and type 2 MGC were also monitored for cytoplasmic expression of TNF-α. In unstimulated cells, a similar percentage of type 1 MGC expressed TNF-α compared to total AM, whereas a lower percentage of type 2 MGC expressed TNF-α. M-CSF increased the number of type 1 MGC expressing TNF-α over controls ($p<.01$), whereas GM-CSF did not have a significant effect. Both M-CSF and GM-CSF increased the number of type 2 MGC expressing TNF-α compared to unstimulated cells. However, in all cases, the percentage of type 2 MGC expressing TNF-α was lower than that observed for type 1 MGC.

Figure 31. Representative TNF-α immunocytochemistry staining of MGC (magnification x 825). AM were incubated in the presence of M-CSF or GM-CSF for 5 days. TNF-α was detected by immunocytochemistry as described in **Materials and Methods.** A, type 1 MGC after culture of AM with M-CSF (50 U/ml); B, type 2 MGC after culture of AM with GM-CSF (50 U/ml).

31A



31B

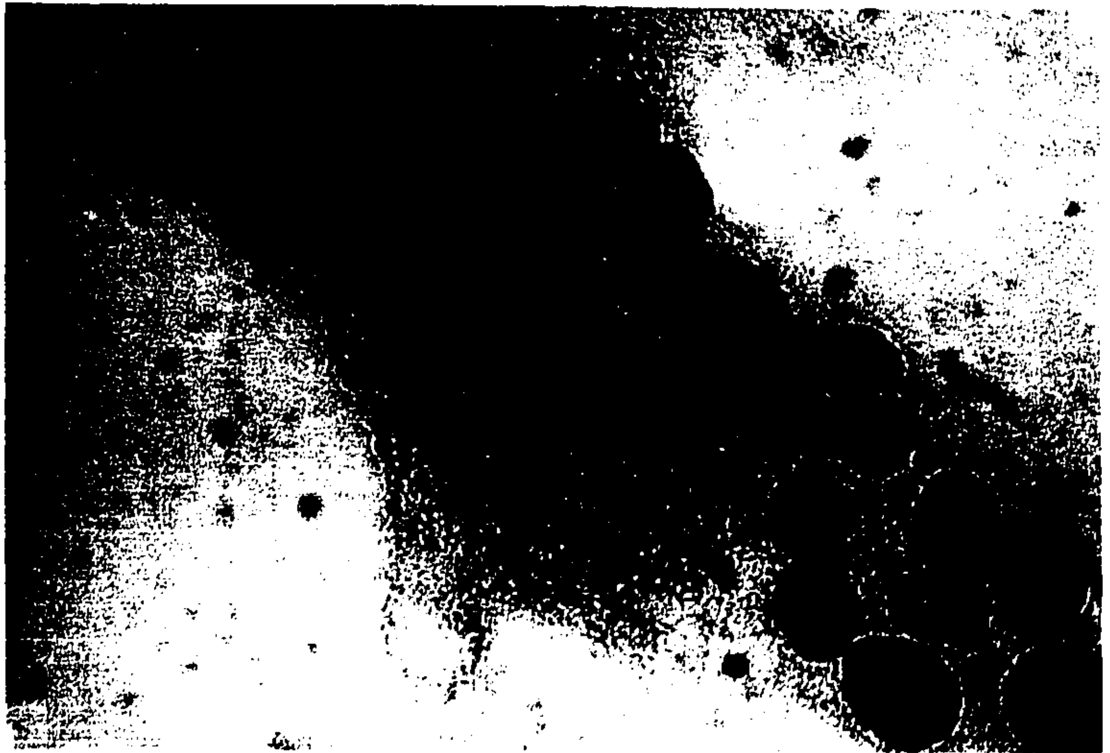
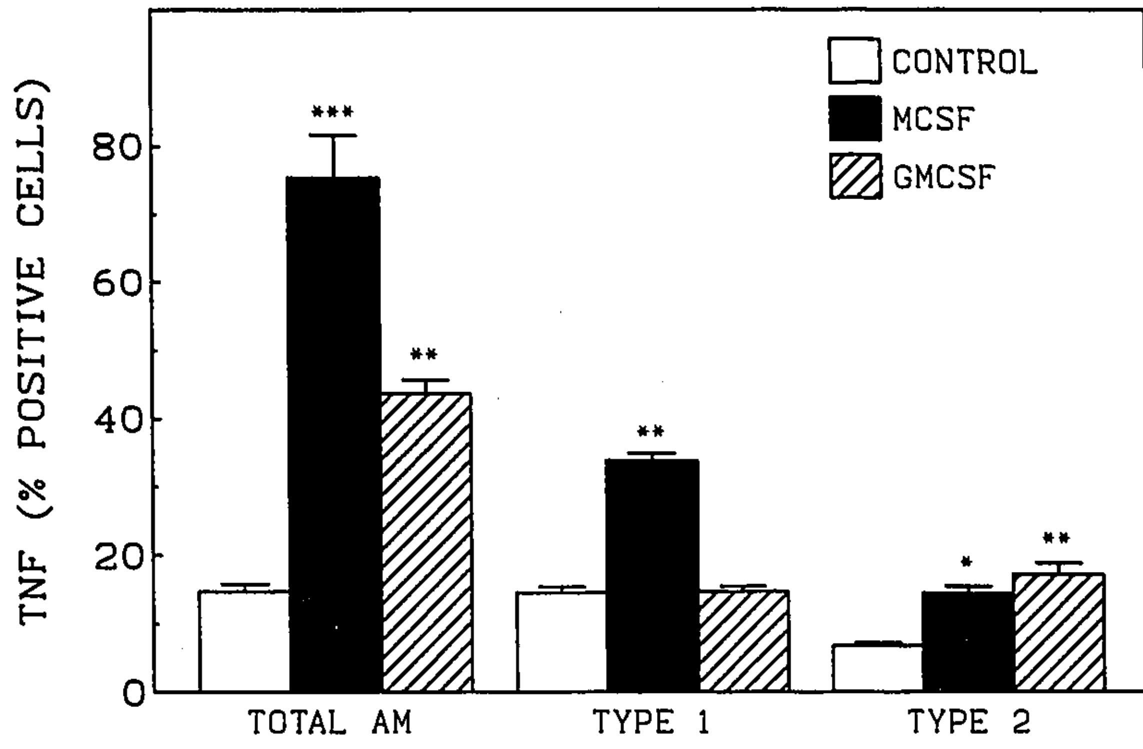
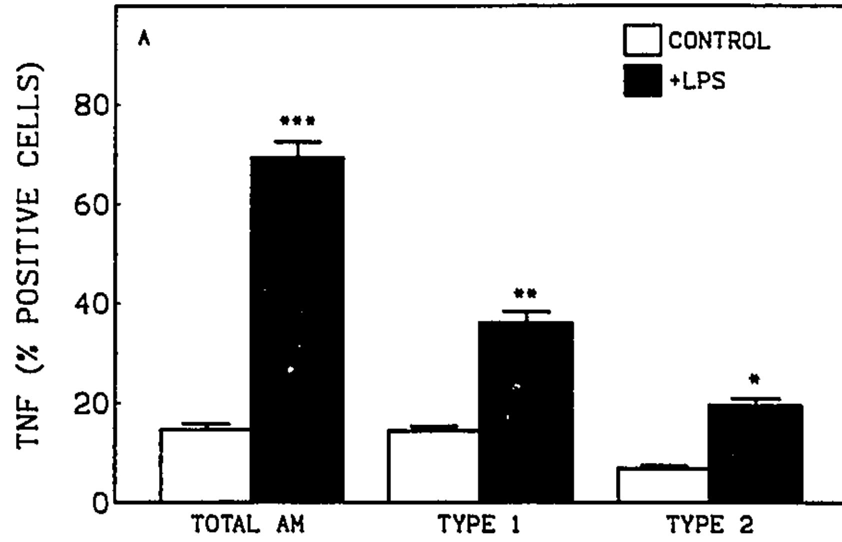


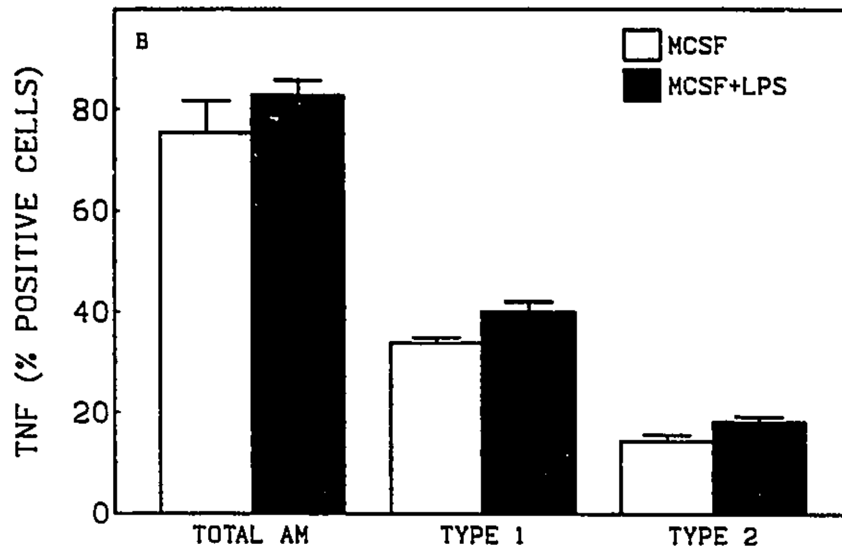
Figure 32. Percentage of total AM, type 1 and type 2 MGC expressing cytoplasmic TNF-*α*. AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. TNF-*α* was detected by immunocytochemistry as described in **Materials and Methods.** More than 200 AM or MGC were counted under the microscope (magnification x 825) and percent positive cells calculated. Values represent the mean $\pm$ SEM from 3 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*), $p<.01$ (**), or $p<.001$ (***).

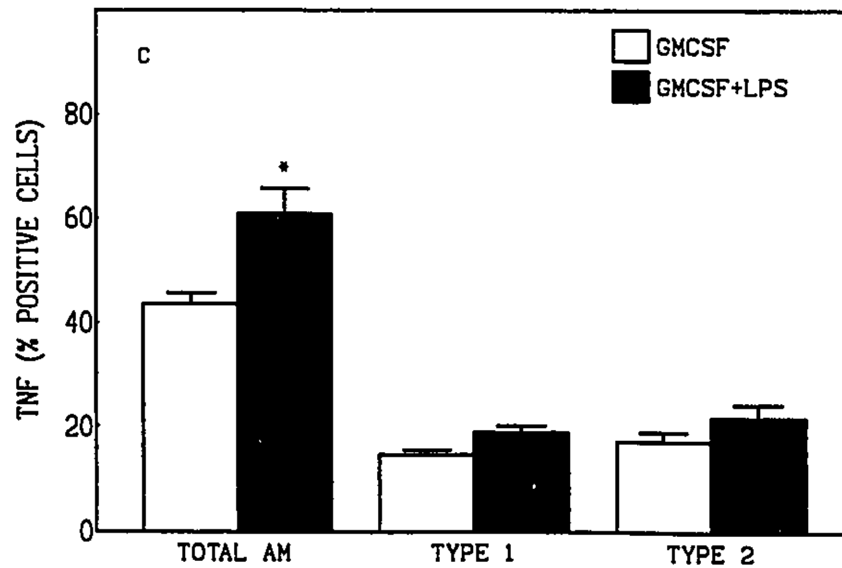

CONTROL
MCSF
GMCSF
TNF (% POSITIVE CELLS)
80
60
40
20
0
***
**
**
*
**
TOTAL AM
TYPE 1
TYPE 2

In order to determine whether the differentiated AM or MGC were still responsive to a classical stimulus such as LPS, AM incubated for 5 days with M-CSF or GM-CSF were stimulated with LPS (1 μg/ml) 3 hours prior to completion of incubation. Figure 30E shows immunocytochemical staining for TNF-α after LPS stimulation of total AM following 5-day culture. LPS still increased the proportion of cells expressing TNF-α under these conditions. As shown in Figure 33 (A, B and C), unstimulated AM incubated for 5 days were still responsive to LPS stimulation. Similarly, both type 1 and type 2 MGC were responsive to LPS stimulation (Figure 33A). Compared to total AM, however, the number of positive cells after LPS stimulation was lower in type 1 and type 2 MGC with type 1 MGC displaying a higher response than type 2 MGC. When AM were incubated in the presence of M-CSF for 5 days, addition of LPS did not significantly increase the percentage of total AM, type 1 and type 2 MGC expressing TNF-α (Figure 33B), suggesting that a maximal proportion of these cells already expressed TNF-α in the presence of M-CSF and did not further respond to LPS. When AM were incubated in the presence of GM-CSF for 5 days, addition of LPS did not significantly increase the number of type 1 and type 2 MGC expressing TNF-α (Figure 33C) but significantly increased the proportion of total AM able to express TNF-α ($p < .05$). Overall, comparison of the intensity and percentage of cells expressing TNF-α after 5-day incubation and LPS stimulation, suggests that type 1 MGC have a greater capacity to express TNF-α compared to type 2 MGC.

Figure 33. Percentage of total AM, type 1 and type 2 MGC expressing cytoplasmic TNF-α after LPS stimulation. AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. LPS was added to the cultures 3 hours prior to completion of incubation. TNF-α was detected by immunocytochemistry as described in Materials and Methods. More than 200 AM or MGC were counted under the microscope (magnification x 825) and percent positive cells calculated. Values represent the mean $\pm$ SEM from 3 experiments. A, AM incubated in the absence of M-CSF or GM-CSF and stimulated with LPS; B, AM incubated in the presence of M-CSF and stimulated with LPS; C, AM incubated in the presence of GM-CSF and stimulated with LPS. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*), $p<.01$ (**) or $p<.001$ (***).


A
CONTROL
+LPS
TNF (% POSITIVE CELLS)
0
20
40
60
80
***
**
*
TOTAL AM
TYPE 1
TYPE 2


B
MCSF
MCSF+LPS
TNF (% POSITIVE CELLS)
0
20
40
60
80
TOTAL AM
TYPE 1
TYPE 2

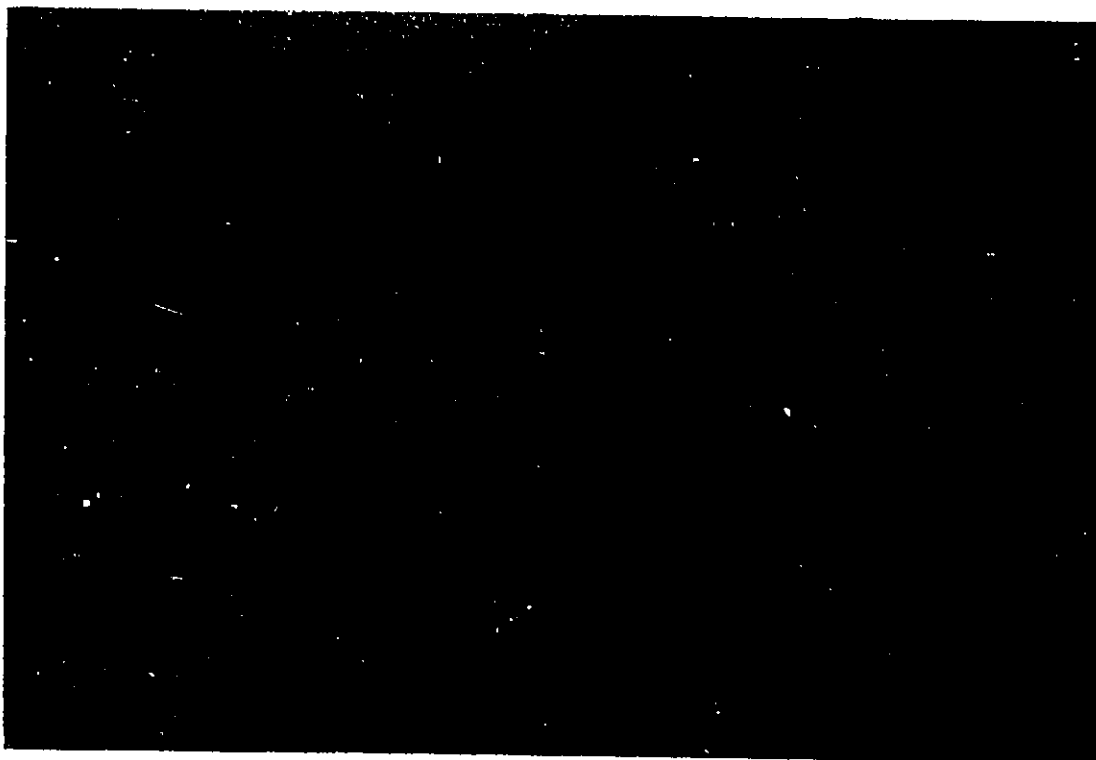

C
GMCSF
GMCSF+LPS
TNF (% POSITIVE CELLS)
0
20
40
60
80
*
TOTAL AM
TYPE 1
TYPE 2

3.4.2. PLATELET-DERIVED GROWTH FACTOR (PDGF)

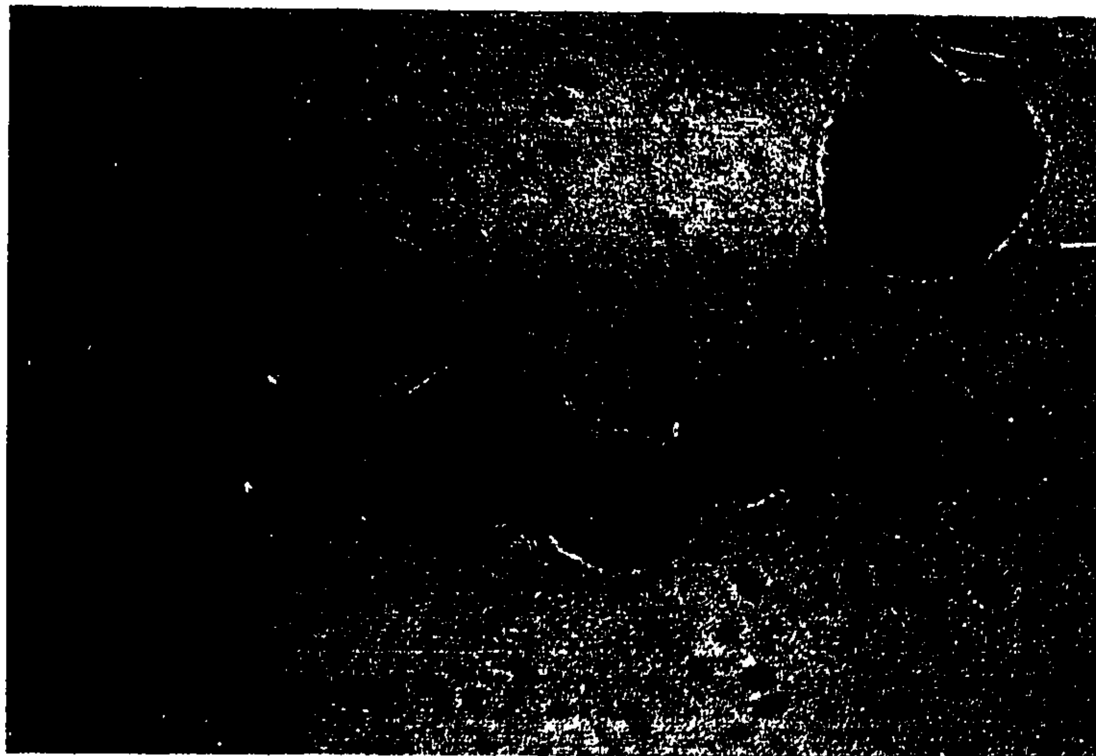
Cytoplasmic expression of PDGF was studied by immunocytochemistry following a 5-day incubation of rat AM in the presence or absence of M-CSF (50 U/ml) or GM-CSF (50 U/ml). Representative PDGF immunocytochemistry staining is shown in Figure 34. Figure 34A represents the negative control staining from a sample stimulated with LPS for 3h and incubated with nonimmune serum. Figures 34B, 34C and 34D show the profiles of PDGF cytoplasmic expression of total AM from control, M-CSF or GM-CSF-treated groups respectively, and Figure 34E shows PDGF expression after 3h LPS stimulation of total AM. Compared to control, proportion of cells expressing PDGF was increased by M-CSF and GM-CSF. When MGC were monitored, some MGC did express PDGF, again suggesting that these MGC may be functionally active. It was also noticed that generally the staining intensity of type 1 MGC was stronger than that of type 2 MGC (Figures 35A and B). Evaluation of the proportion of MGC expressing PDGF was performed and Figure 36 represents the data from 3 experiments. Treatment with M-CSF or GM-CSF increased the number of total AM expressing PDGF compared to controls. When type 1 and type 2 MGC were analyzed, the proportion of type 1 MGC expressing PDGF in unstimulated AM was higher compared to total AM, whereas a lower percentage of type 2 MGC expressed PDGF. M-CSF increased the number of type 1 MGC expressing PDGF ($p<.01$) whereas GM-CSF decreased the number of type 1 expressing PDGF compared to controls

Figure 34. Representative PDGF immunocytochemistry staining of total AM (magnification x 825). AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. PDGF was detected by immunocytochemistry as described in **Materials and Methods**. A, negative control with nonimmune serum from the sample stimulated by LPS for 3h; B, control (unstimulated AM); C, M-CSF-treated AM; D, GM-CSF-treated AM; E, LPS stimulation of AM for 3h.

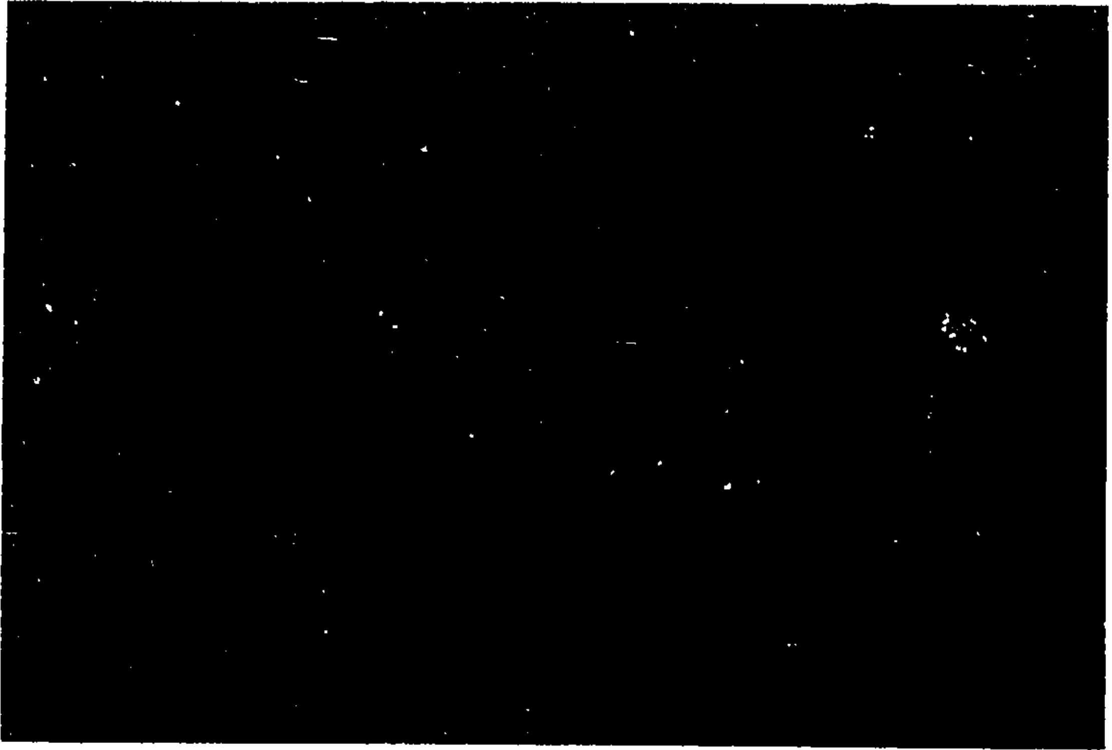
34A



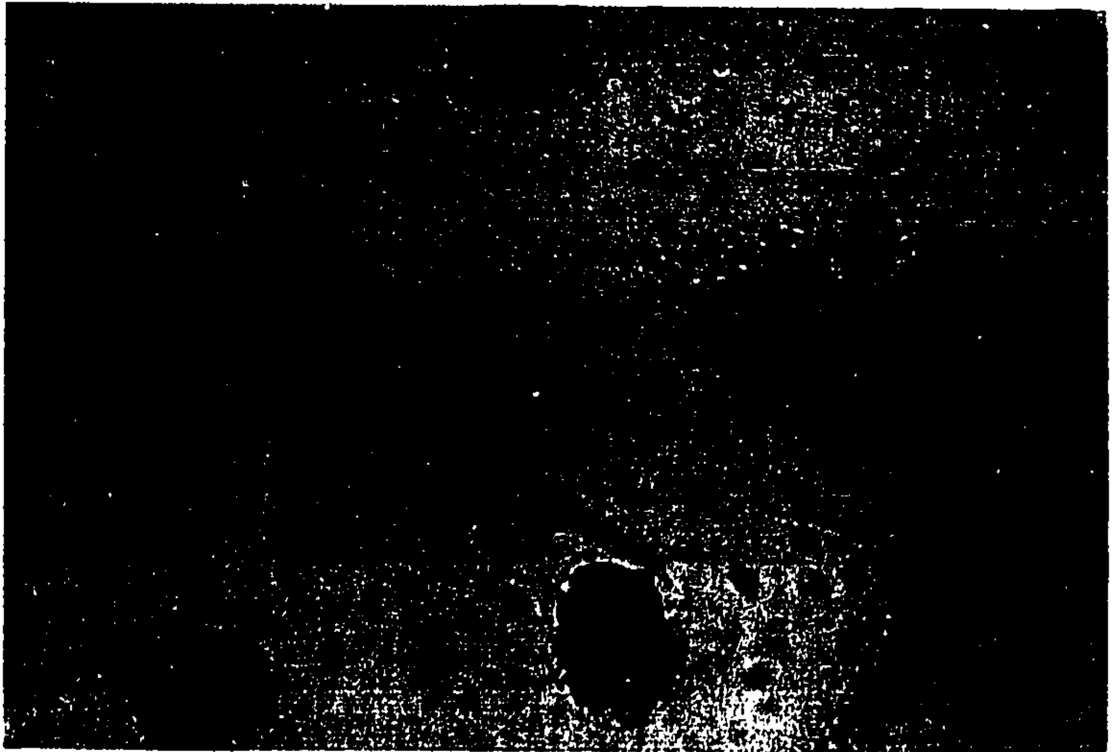
34B



34C



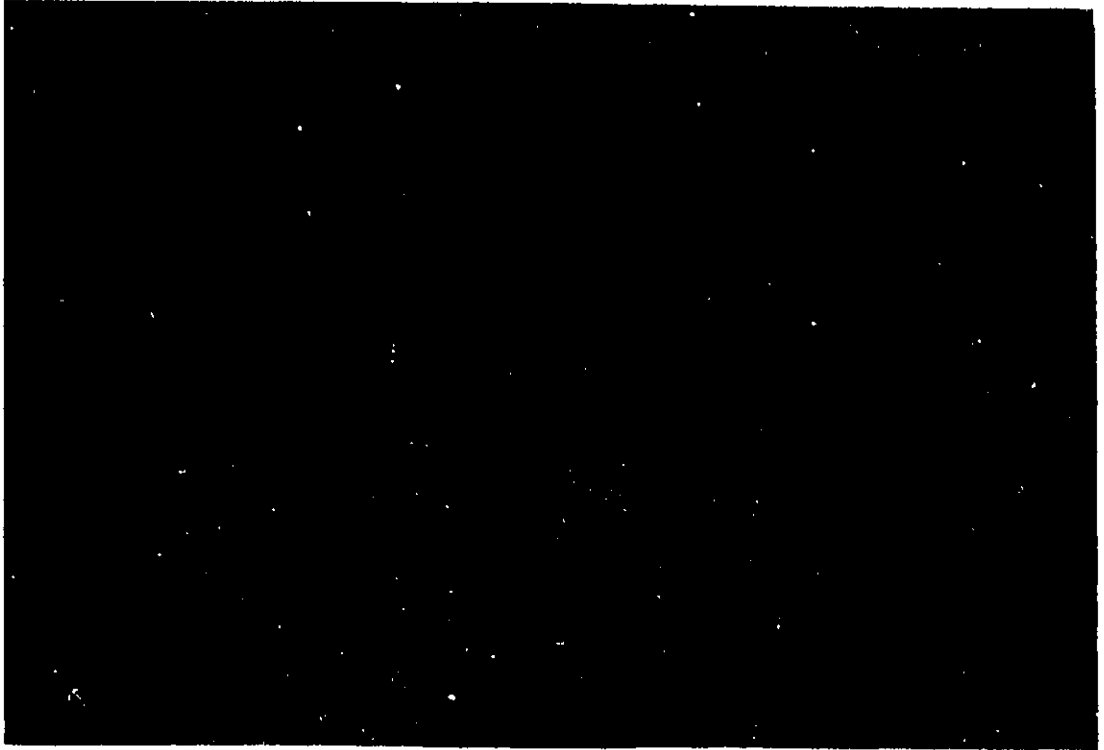
34D



34E

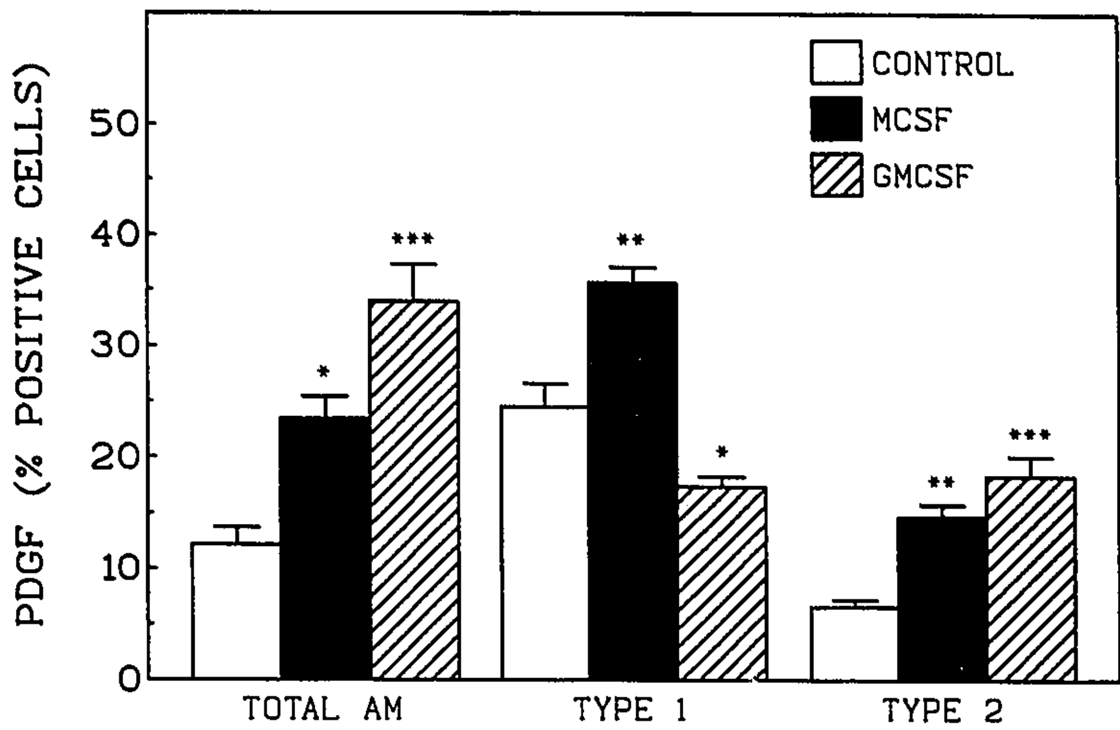
Figure 35. Representative PDGF immunocytochemistry staining of MGC (magnification x 825). AM were incubated in the presence of M-CSF or GM-CSF for 5 days. PDGF was detected by immunocytochemistry as described in **Materials and Methods.** A, type 1 MGC after culture of AM with M-CSF (50 U/ml); B, type 2 MGC after culture of AM with GM-CSF (50 U/ml).

35A



35B

Figure 36. Percentage of total AM, type 1 and type 2 MGC expressing cytoplasmic PDGF. AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. PDGF was detected by immunocytochemistry as described in **Materials and Methods**. More than 200 AM or MGC were counted under microscope (magnification x 825) and percent positive cells calculated. Values represent the mean ± SEM from 3 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*), $p<.01$ (**), or $p<.001$ (***).



CONTROL
MCSF
GMCSF
PDGF (% POSITIVE CELLS)
0
10
20
30
40
50
*
***
**
*
**
***
TOTAL AM
TYPE 1
TYPE 2

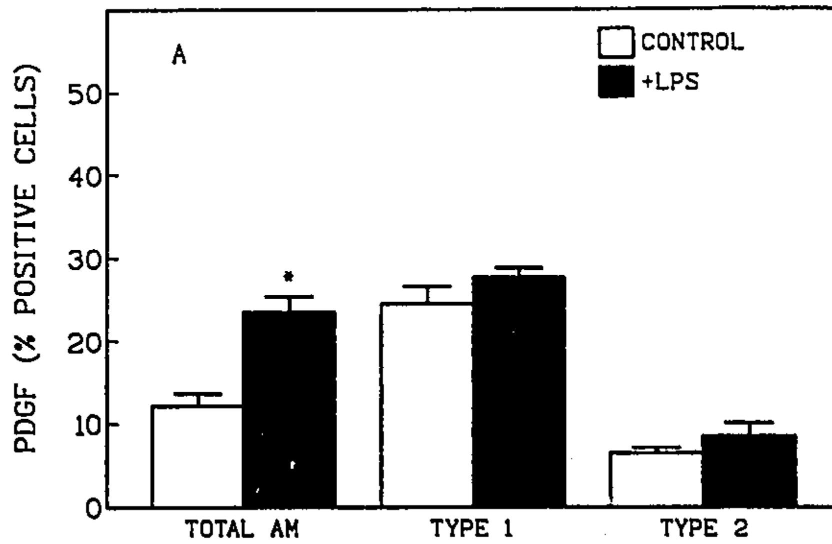
($p<.05$). Both M-CSF and GM-CSF increased the number of type 2 MGC expressing PDGF compared to unstimulated cells ($p<.01$). However, in control and M-CSF treated groups, the percentage of type 2 MGC expressing PDGF was lower than that observed for type 1 MGC whereas in GM-CSF treated group, a similar percentage of type 1 and type 2 MGC expressed PDGF.

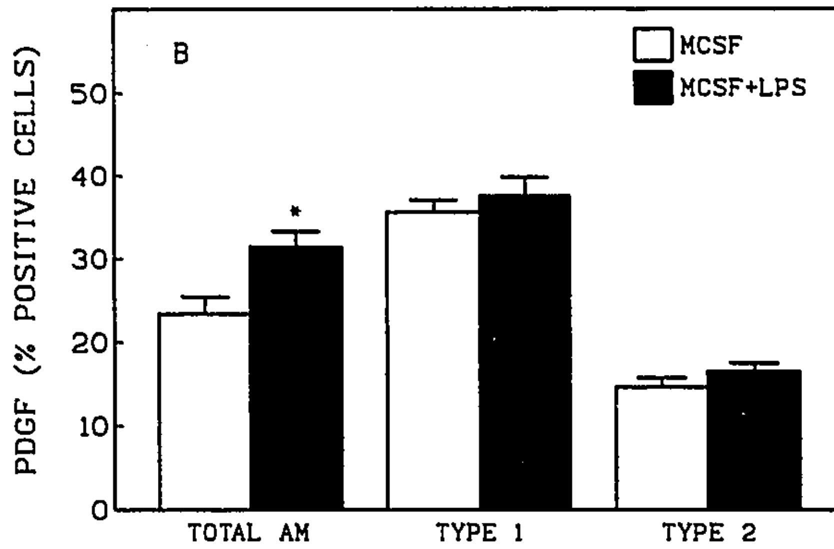
LPS was added to samples 3 hours prior to completion of incubation. Figure 30E shows immunocytochemical staining after LPS stimulation (1 μg/ml) of total AM which were incubated for 5 days. LPS increased the number of cells expressing PDGF compared to unstimulated total AM. The results from 3 experiments after LPS stimulation for PDGF expression are presented in Figure 37. After 5-day incubation of AM in the absence of M-CSF and GM-CSF (Figure 37A), in the presence of M-CSF (50 U/ml) (Figure 37B) or GM-CSF (50 U/ml) (Figure 37C), addition of LPS increased the proportion of total AM expressing PDGF over controls ($p<.05$). However, in type 1 and type 2 MGC, LPS did not increase the percentage of type 1 and type 2 MGC expressing PDGF over controls and M-CSF or GM-CSF alone treated groups.

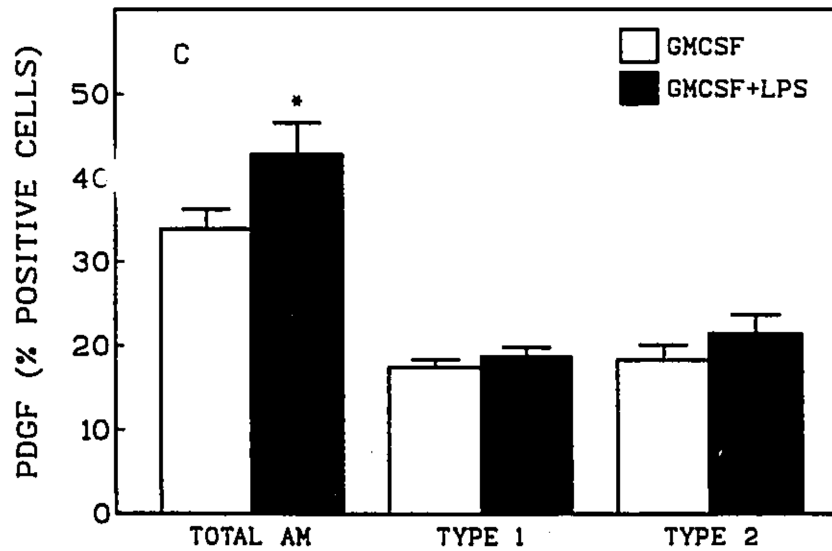
3.4.3. TRANSFORMING GROWTH FACTOR-β (TGF-β)

Immunocytochemistry detection for cytoplasmic expression of TGF-β during AM differentiation was examined. Figure 38A shows the negative control staining from a sample stimulated with LPS for 3h and incubated with nonimmune serum instead of specific first antibody to TGF-β. TGF-β expression of total AM from control, M-CSF or GM-CSF-treated groups is presented in

Figure 37. Percentage of total AM, type 1 and type 2 MGC expressing cytoplasmic PDGF after LPS stimulation. AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. LPS was added to the cultures 3 hours prior to completion of incubation. PDGF was detected by immunocytochemistry as described in Materials and Methods. More than 200 AM or MGC were counted under microscope (magnification x 825) and percent positive cells calculated. Values represent the mean $\pm$ SEM from 3 experiments. A, AM incubated in the absence of M-CSF or GM-CSF and stimulated with LPS; B, AM incubated in the presence of M-CSF and stimulated with LPS; C, AM incubated in the presence of GM-CSF and stimulated with LPS. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*).


A
CONTROL
+LPS
PDGF (% POSITIVE CELLS)
0
10
20
30
40
50
*
TOTAL AM
TYPE 1
TYPE 2


B
MCSF
MCSF+LPS
PDGF (% POSITIVE CELLS)
0
10
20
30
40
50
*
TOTAL AM
TYPE 1
TYPE 2


C
GMCSF
GMCSF+LPS
PDGF (% POSITIVE CELLS)
0
10
20
30
50
*
TOTAL AM
TYPE 1
TYPE 2

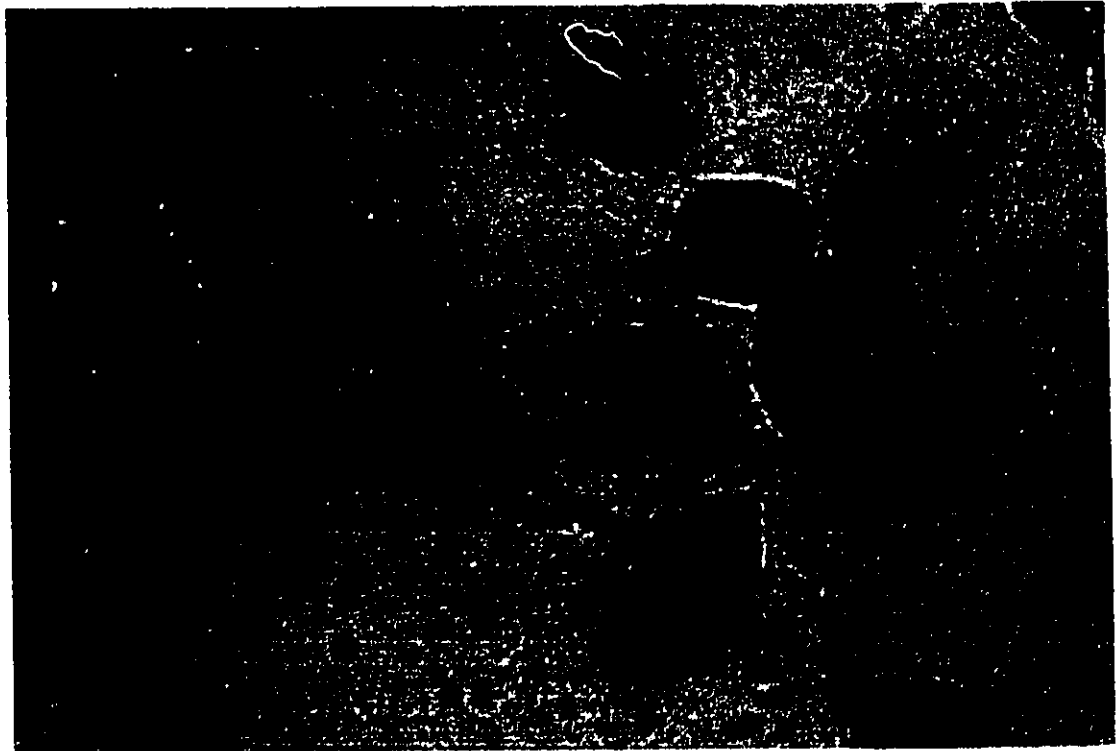
Figures 38B, 38C and 38D. Compared to controls, proportion of cells expressing TGF-β was increased by M-CSF and GM-CSF, which is again consistent with the findings of RT-PCR determination. When MGC were analyzed for their expression of TGF-β, some MGC did express this cytokine. It was also noticed that type 1 MGC showed higher cytoplasmic expression of TGF-β than type 2 MGC (Figures 39A and 39B). This, however, is not quantitated, Evaluation of the proportion of MGC expressing TGF-β was performed and results from 3 experiments are presented in Figure 40. Treatment with M-CSF or GM-CSF significantly increased the number of total AM expressing TGF-β compared to controls ($p<.01$). Type 1 and type 2 MGC were also analyzed for cytoplasmic expression of TGF-β. In unstimulated cells, the percentage of type 1 and type 2 MGC expressing TGF-β was lower compared to total AM. Treatment with M-CSF increased the number of type 1 MGC expressing TGF-β over controls ($p<.01$) whereas GM-CSF did not. In contrast, GM-CSF increased slightly the number of type 2 MGC expressing TGF-β over controls, whereas M-CSF did not. However, in all cases, the percentage of type 2 MGC expressing TGF-β was lower than that seen for type 1 MGC, and for total AM.

LPS (1 μg/ml) was added to samples 3 hours prior to completion of incubation. Figure 41 represents the results of LPS stimulation on TGF-β expression by total AM and MGC. After a 5-day incubation in the absence of M-CSF and GM-CSF (Figure 41A), addition of LPS significantly increased the

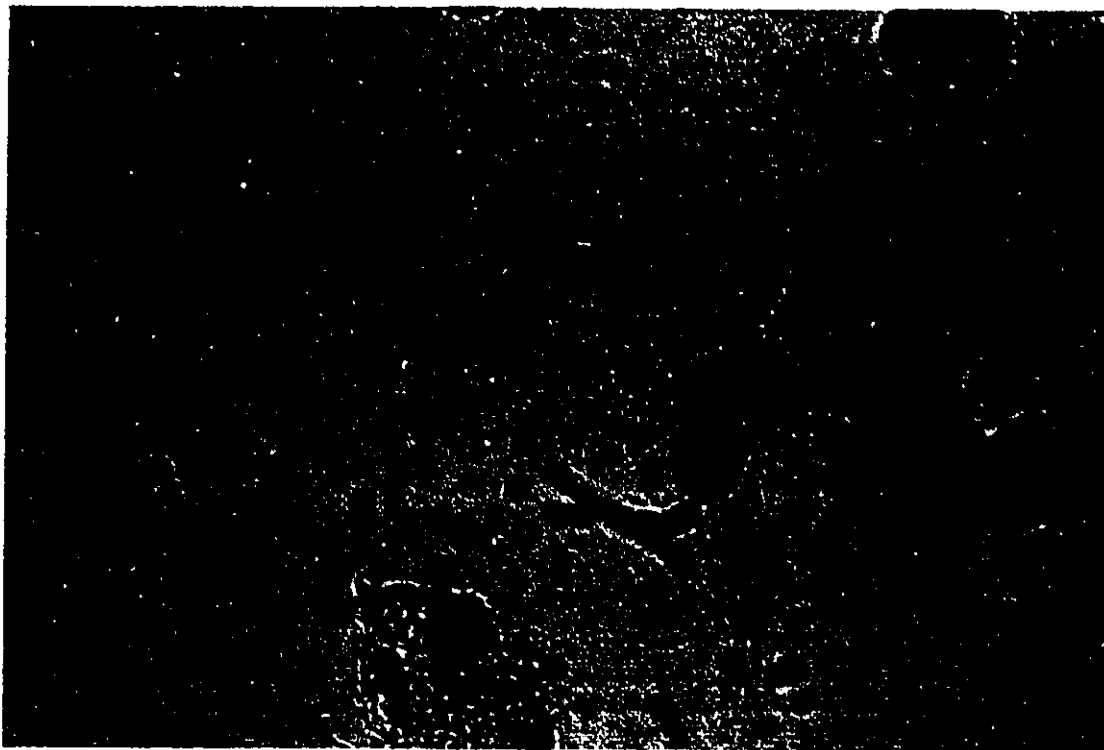
Figure 38. Representative TGF-β immunocytochemistry staining of total AM (magnification x 825). AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. TGF-β was detected by immunocytochemistry as described in **Materials and Methods.** A, negative control with nonimmune serum from the sample stimulated by LPS for 3h; B, control (unstimulated AM); C, M-CSF-treated AM; D, GM-CSF-treated AM.

38A

38B



38C



38D

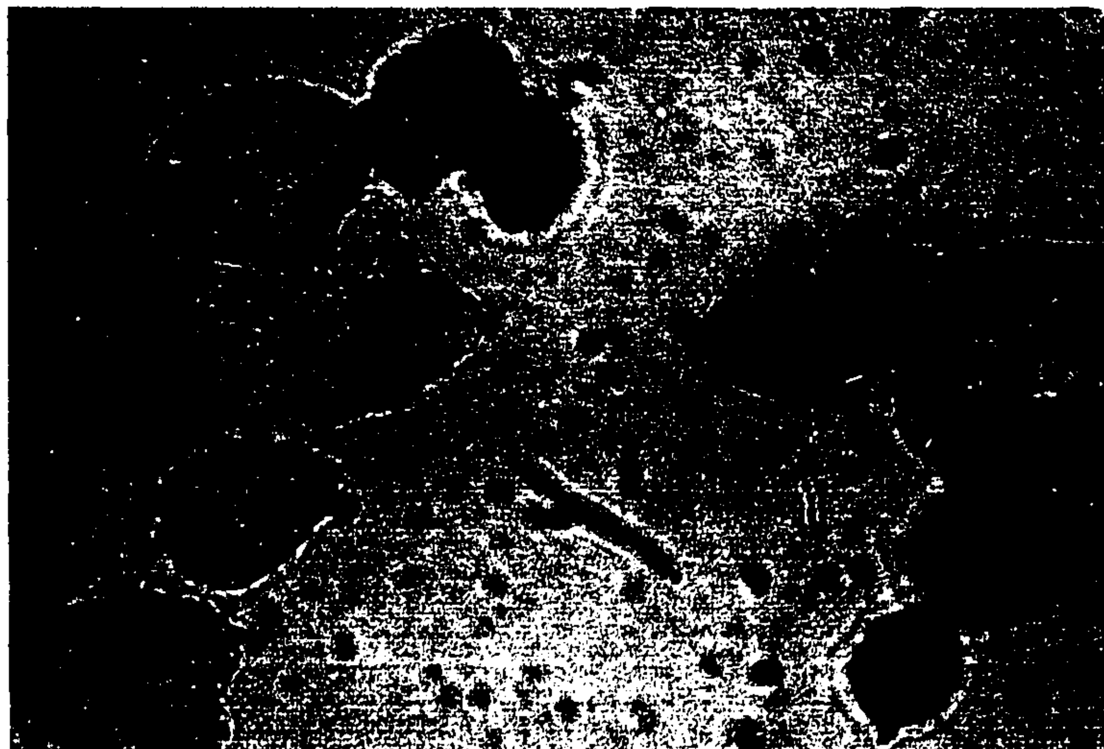


Figure 39. Representative TGF-β immunocytochemistry staining of MGC (magnification x 825). AM were incubated in the presence of M-CSF or GM-CSF for 5 days. TGF-β was detected by immunocytochemistry as described in **Materials and Methods.** A, type 1 MGC after culture of AM with M-CSF (50 U/ml); B, type 2 MGC after culture of AM with GM-CSF (50 U/ml).

39A

39B

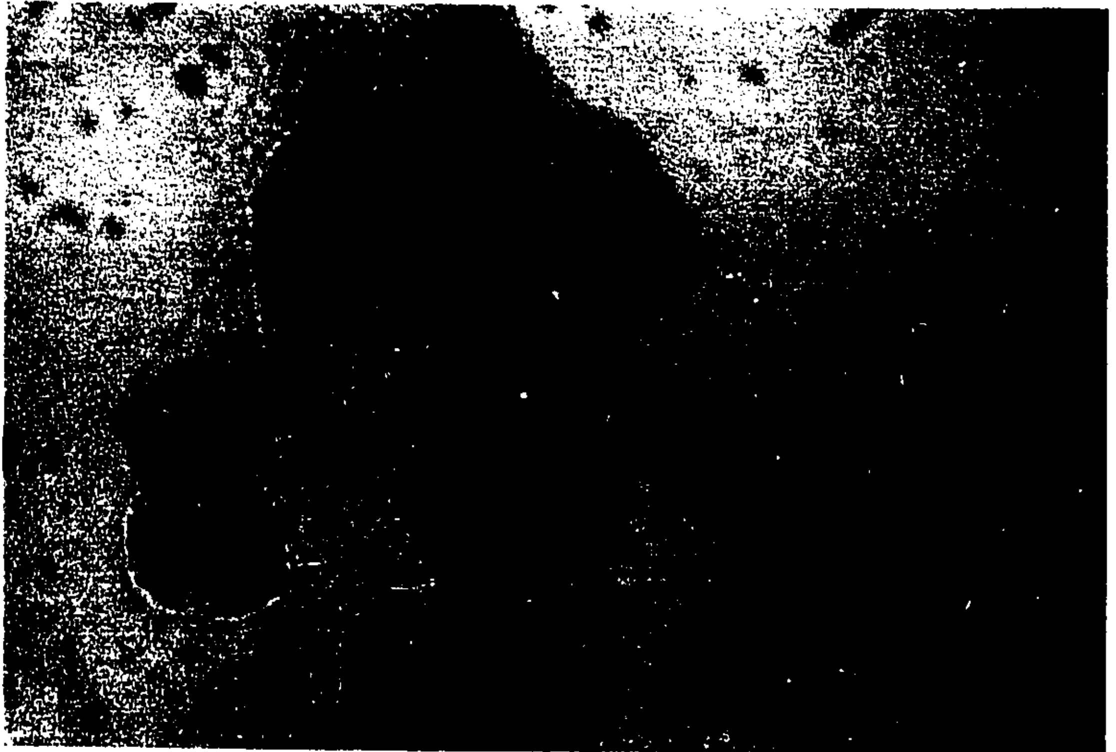
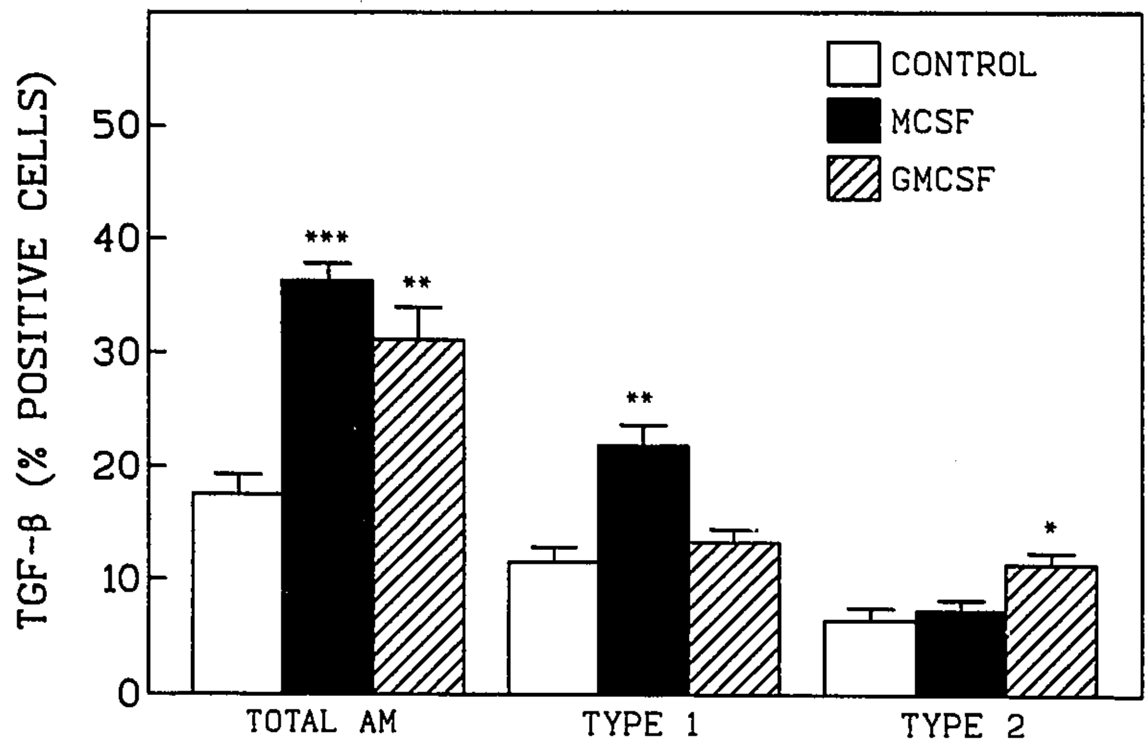


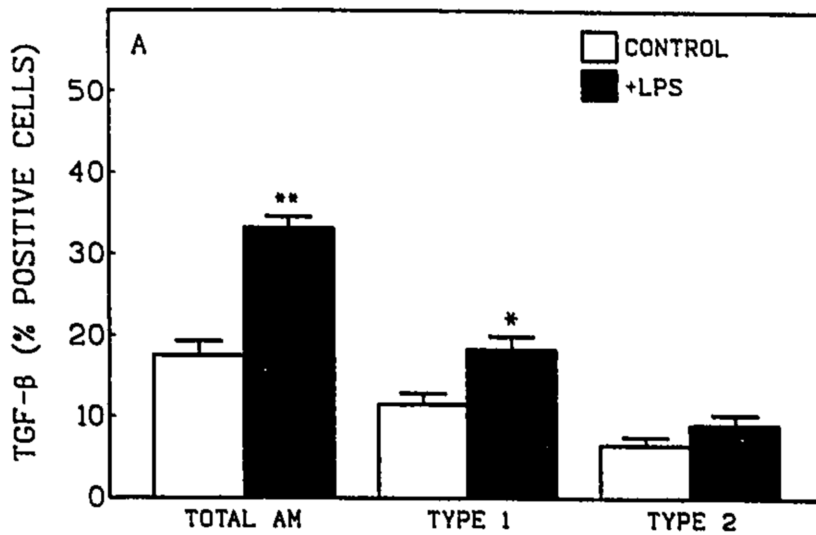
Figure 40. Percentage of total AM, type 1 and type 2 MGC expressing cytoplasmic TGF-β. AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. TGF-β was detected by immunocytochemistry as described in **Materials and Methods.** More than 200 AM or MGC were counted under microscope (magnification x 825) and percent positive cells calculated. Values represent the mean $\pm$ SEM from 3 experiments. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*), $p<.01$ (**), or $p<.001$ (***).

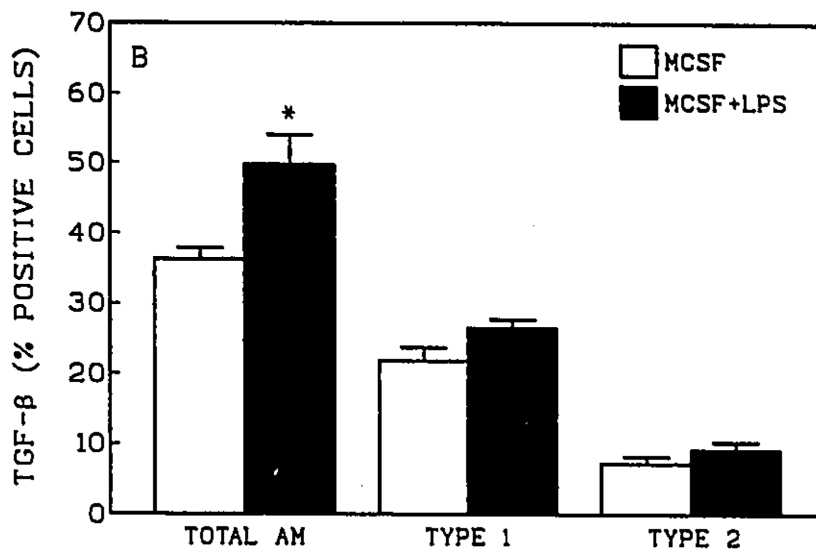


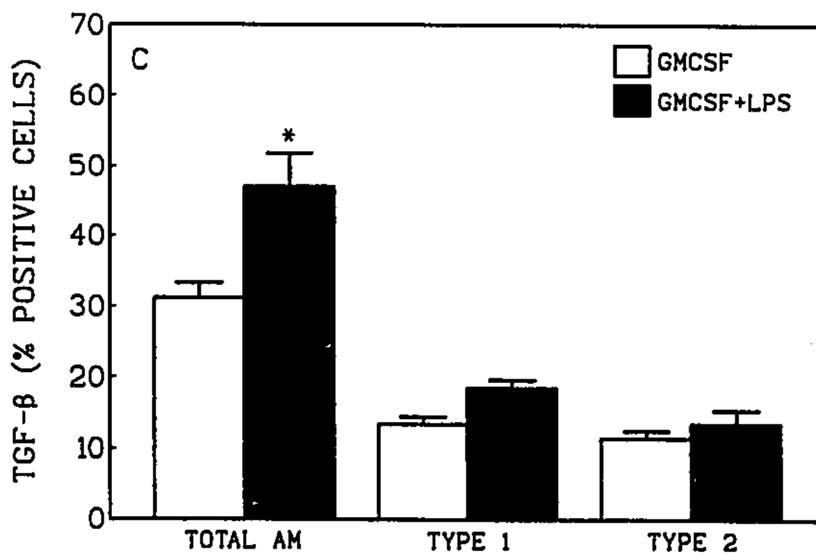
TGF-β (% POSITIVE CELLS)
0
10
20
30
40
50
CONTROL
MCSF
GMCSF
***
**
**
*
TOTAL AM
TYPE 1
TYPE 2

proportion of cells expressing TGF-β in total AM and type 1 MGC ($p<.01$ and $p<.05$ respectively), indicating these cells are still responsive to LPS stimulation under these conditions. However, in the presence of M-CSF (50 U/ml) (Figure 41B) or GM-CSF (50 U/ml) (Figure 41C), addition of LPS only increased the proportion of total AM expressing TGF-β ($p<.05$) compared to M-CSF or GM-CSF alone treatment. The proportion of type 2 MGC expressing TGF-β was not significantly increased by LPS in control, M-CSF or GM-CSF-treated groups.

Figure 41. Percentage of total AM, type 1 and type 2 MGC expressing cytoplasmic TGF-β after LPS stimulation. AM were incubated in the presence or absence of M-CSF or GM-CSF for 5 days. LPS was added to the cultures 3 hours prior to completion of incubation. TGF-β was detected by immunocytochemistry as described in Materials and Methods. More than 200 AM or MGC were counted under microscope (magnification x 825) and percent positive cells calculated. Values represent the mean $\pm$ SEM from 3 experiments. A, AM incubated in the absence of M-CSF or GM-CSF and stimulated with LPS; B, AM incubated in the presence of M-CSF and stimulated with LPS; C, AM incubated in the presence of GM-CSF and stimulated with LPS. Statistical significance of differences between treated and control groups was determined using a one-way analysis of variance and Bonferroni test. Significantly different from control at $p<.05$ (*), $p<.01$ (**).


A
CONTROL
+LPS
TGF-β (% POSITIVE CELLS)
50
40
30
20
10
0
**
*
TOTAL AM
TYPE 1
TYPE 2


B
MCSF
MCSF+LPS
TGF-β (% POSITIVE CELLS)
70
60
50
40
30
20
10
0
*
TOTAL AM
TYPE 1
TYPE 2


C
GMCSF
GMCSF+LPS
TGF-β (% POSITIVE CELLS)
70
60
50
40
30
20
10
0
*
TOTAL AM
TYPE 1
TYPE 2

4. DISCUSSION

4.1. CHARACTERIZATION OF AM DIFFERENTIATION INDUCED BY M-CSF AND GM-CSF

Macrophages are heterogeneous cells with respect to their morphologic, functional and metabolic properties. Such heterogeneity reflects differences in tissue localization, differentiation, maturation, or activation (Falk and Vogel 1990). In the lung for instance, the alveolar macrophage (AM) population is composed of phenotypically different AM based on morphologic, histochemical and functional criteria (Shellito and Kaltreider 1984, Nibbering et al. 1987). Under inflammatory conditions, AM are highly adaptable in responding to foreign agents and it is likely that expression of their extensive repertoire of functions is tightly regulated by activation and differentiation processes (Fels and Cohn 1986). In support of this, changes in AM morphology including a higher incidence of multinucleated giant cells (MGC) were consistently observed during the development of lung chronic inflammatory reaction, both in animal models and in humans (Miller 1978, Lemaire 1985, Lemaire et al. 1989, Takemura et al. 1989). To date, the mechanisms responsible for the generation of these modified forms of AM as well as the physiological implication of AM derivatives are unknown. In particular, the regulation of cytokine production, a key element in the resolution of inflammatory responses may be orchestrated by the sequential appearance of distinct AM populations within inflammatory

lesions.

M-CSF and GM-CSF can directly or indirectly through their receptor modulation regulate proliferation and differentiation of monocyte/macrophage lineage cells (Metcalf 1991, Gliniak et al. 1992). However, most studies have focused on the effect of M-CSF and GM-CSF on pre-mature bone marrow cells and peripheral blood monocytes. The effect of M-CSF and GM-CSF on mature macrophages, especially on alveolar macrophages (AM), is relatively unexplored.

In this study, an in vitro model to investigate the biological effects of colony-stimulating factors, M-CSF and GM-CSF on rat AM was used. M-CSF and GM-CSF act on the overall AM population to induce the formation of MGC that can be readily singled out from other AM populations based on their morphological characteristics.

Although the formation of MGC from AM has been reported in other in vitro systems (Abe et al. 1991, Vignery et al. 1991, Lazarus et al. 1990), this is the first evidence that M-CSF and GM-CSF can induce selectively MGC with two distinct phenotypes. Thus, GM-CSF induces mostly type 2 MGC and has little effect on type 1, whereas M-CSF induces both type 1 and type 2 MGC to a similar extent. Moreover, blockade of M-CSF with specific anti-M-CSF abrogates the formation of type 1 MGC without affecting type 2 MGC, and blockade of GM-CSF with specific anti-GM-CSF abrogates the formation of type 2 MGC without affecting type 1 MGC. These results bring evidence for

different biological roles of M-CSF and GM-CSF on AM differentiation.

Formation of MGC has also been reported with macrophages from other anatomical compartments including human peripheral blood monocytes (Enelow et al. 1992, Kazazi et al. 1994) and bone marrow mononuclear cells (McInnes and Rennick 1988). However, it is not known whether MGC phenotype varies depending on the tissue microenvironment, maturation stages and/or type of inflammatory stimulus. Our study brings some evidence for the latter since we demonstrate that resident AM present within the same pulmonary compartment can be further differentiated in vitro into two types of MGC variants upon the action of different CSFs. Interestingly, MGC generated in vitro exhibit features that are strikingly similar to those displayed in vivo during an inflammatory response to mineral dusts (Lemaire 1985, 1991a). It is noteworthy that serum levels of M-CSF increase in response to inflammation (Nicola 1989) and GM-CSF appears in the serum, indicating that the inflammatory environment may provide suitable conditions for generation of CSF-dependent MGC. These data strongly support the assumption that differentiation of AM plays a role in inflammatory responses, and that modulation and resolution of inflammation are tightly regulated by the types of AM, possibly as well as by MGC present at sites of injury (Lemaire 1995).

It is believed that M-CSF and GM-CSF promote the growth of immature cells of the monocyte/macrophage lineage (Metcalf 1991, Gliniak et al. 1992). However, in this in vitro model examination of cell number did not show

significant differences compared to controls. Theoretically, an increase of MGC induced by M-CSF or GM-CSF may result in a decrease of overall AM cell number. The fact that examination of cell number showed no significant differences compared to controls may result from the compensation of small pool of AM which undergo proliferation under the effect of M-CSF or GM-CSF. In our culture conditions, AM were incubated in 10% FBS and it is possible that other serum factors may contribute to MGC induction. However, in some experiments increase of MGC formation both in unstimulated AM and in response to M-CSF or GM-CSF was also observed in the absence of FBS, providing further evidence for a role of M-CSF and GM-CSF in this process.

AM incubated in culture medium alone still displayed a small number of MGC and the two types of MGC were found in equal proportion. It has been documented that AM constitutively express and secrete M-CSF (Becker et al. 1989, Ogawa et al. 1994), which may explain the formation of type 1 MGC in controls. In contrast, it was reported that GM-CSF is not constitutively produced by resident macrophages (Rose et al. 1991, Agostini et al. 1992). In accordance with this, we demonstrated that GM-CSF is not constitutively expressed in resident AM. Furthermore, we provide evidence that AM in culture or treated with M-CSF express GM-CSF, suggesting that endogenous production of GM-CSF may contribute to type 2 MGC formation in unstimulated AM and in response to M-CSF. These observations also suggest that M-CSF plays a major role in resident AM. Previous work in a rodent system

demonstrated that AM not only express M-CSF receptors (Byrne et al. 1981) and constitutively produce M-CSF (Becker et al. 1989, Ogawa et al. 1994) but also are capable of specific interaction with this factor (Chen and Lin 1982). In view of these considerations, it may be that the presence of M-CSF within the resident AM is necessary to support the functional integrity of macrophages.

Cell binding to the extracellular matrix provides informational signals that greatly influence behaviour patterns of migration, proliferation and differentiation. Among the specific receptors is a family of integral cell surface proteins, termed integrins. Integrins are receptors that both participate in cell-cell adhesion events and permit cells to interact with the extracellular matrix. Each of the integrin receptors comprises an α and β subunit in noncovalent association, and different permutations of α and β chains provide functionally discrete receptors (Bates et al. 1991). It is worthwhile to note that integrin receptors may be differently expressed during AM differentiation. Recent investigation revealed that M-CSF selectively induced the expression of the β_5 chain, whereas GM-CSF specifically induced mRNA and surface expression of the β_3 chain (De Nichilo et al. 1993). As β_5 antibody is not commercially available at the present time, the expression of α_v (CD51) and β_3 (CD61) integrin in response to M-CSF or GM-CSF was investigated by immunocytochemical analysis. We reasoned that expression of β_3 integrin may represent a specific marker for type 2 MGC since type 2 MGC are predominant during AM differentiation induced by GM-CSF. When type 1 MGC were

compared with type 2 MGC for expression of α_v integrin, no difference was seen. However, although β_3 chain was not found to be specifically expressed on type 2 MGC since type 1 MGC also expressed it, we observed that a greater proportion of type 2 MGC expressed β_3 integrin than type 1 MGC, thus bringing additional evidence for differences between type 1 and type 2 MGC. The apparent differential regulation of expression of the β_3 subunit by GM-CSF poses interesting questions in terms of possible mechanisms involved in cell adhesion events potentiating AM migration and differentiation and suggests that this integrin may mediate distinct physiological functions.

4.2. MORPHOLOGICAL ANALYSIS OF AM DIFFERENTIATION INDUCED BY OTHER CYTOKINES

In addition to M-CSF, TGF-β is also constitutively produced by AM and can be upregulated during AM differentiation as is confirmed in our RT-PCR and immunocytochemistry experiments. We provide evidence that TGF-β also plays a role in type 1 and type 2 MGC formation. Experiments done with exogenous TGF-β alone or in combination with M-CSF or GM-CSF indicated that type 1 MGC were increased whereas type 2 MGC were decreased with increasing concentrations of TGF-β. This may result from the down-regulatory effects of TGF-β on AM differentiation (Fan et al. 1992). More recently, TGF-β has been shown to suppress pathogen-induced release of GM-CSF (Hogasen et al. 1995). All these may explain our findings that TGF-β favours type 1 MGC formation.

TNF-*α*, a cytokine primarily synthesized by macrophages, not only causes regression and haemorrhagic necrosis of tumours, but also exerts its antiproliferation effects on and leads to differentiation of monocytes-macrophages (Witsell and Schook 1992, Weber et al. 1993). There are several reports from this laboratory showing the involvement of this cytokine in chronic inflammation, especially granulomatous responses associated with the presence of MGC (Lemaire 1991a, 1995, Ouellet et al. 1993). Evidence suggests that TNF-*α* may be involved in the development of granuloma and MGC formation. In this study, we examined the effect of exogenous and endogenous TNF-*α* on MGC formation during AM in vitro differentiation. Although exogenous TNF-*α* either alone or in combination with M-CSF or GM-CSF had no effect on MGC formation, neutralization of endogenous TNF-*α* inhibited type 2 MGC formation in controls and M-CSF-treated groups, suggesting that TNF-*α* plays a role in modulating AM differentiation and MGC formation. It has been considered that TNF-*α* plays an autocrine and autoamplifying role during macrophage differentiation (Witsell and Schook 1992). Therefore, it is possible that persistent activation may be required for AM differentiation and subsequent type 2 MGC formation, and that antibody to TNF-*α* can inhibit type 2 MGC formation by interrupting the TNF-*α*-mediated autoamplification circuit. Moreover, studies indicated the presence of transmembrane TNF-*α* (Luettig et al. 1989). Thus, another possible explanation is that the transmembrane form of TNF-*α* may be required for AM differentiation.

An interesting aspect of our study is the observation that antibody to TNF-α only partially inhibited type 2 MGC formation in M-CSF-induced or control groups but had no effect on type 2 MGC formation induced by GM-CSF. We have shown by RT-PCR that M-CSF induces GM-CSF expression in our culture system and this coupled with our data from anti-TNF-α antibody experiments, makes us hypothesize that one of the mechanisms by which M-CSF stimulates GM-CSF production may be through TNF-α production. In support of this, a few studies have reported that M-CSF can activate macrophages and induce TNF-α production (Warren and Ralph 1986, Young et al. 1990) which in turn is responsible for stimulation of GM-CSF production (Munker et al. 1986). However, since type 2 MGC formation is only partially suppressed by antibody to TNF-α in M-CSF-treated groups and controls, other possibilities for GM-CSF induction by M-CSF can not be excluded, as M-CSF also promotes the production of other biological factors such as IL-1 which has also been shown to induce GM-CSF production (Marini et al. 1992).

IL-6 is produced by a variety of cells, including AM (Gosset et al. 1991) and has been shown to modulate macrophage differentiation (Chiu and Lee 1989, Oritani et al. 1992, Tanigawa et al. 1995). Our results demonstrated that exogenous IL-6 increased type 2 MGC formation in control and M-CSF-treated AM. Further experiments with anti-IL-6 receptor antibody confirmed the involvement of IL-6 in type 2 MGC formation. Since GM-CSF is associated with type 2 MGC formation in our experimental model, it is possible that GM-CSF

exerts its effects on AM differentiation and MGC formation through stimulation of IL-6 production. Results from our RT-PCR experiments further confirmed that AM in response to GM-CSF showed a significant increase in IL-6 transcription which is correlated with increased IL-6 activity, whereas M-CSF-treated AM did not display an increase of IL-6 expression compared to controls.

4.3. POSSIBLE MECHANISMS AND PATHWAYS OF TYPE 1 AND TYPE 2 MGC FORMATION

MGC have been demonstrated to form as a result of cell fusion. Although the detailed mechanisms leading to MGC formation are currently uncertain, a few in vitro studies indicate that adhesion molecules such as LFA-1 (CD11a and CD18) and ICAM-1 interaction may be involved in MGC formation (Fais et al. 1994, Kazazi et al. 1994, Most et al. 1990). These adhesion molecules can be induced during cell differentiation. It has been observed that both M-CSF and GM-CSF induce expression of CD11a and CD18 in U937 cell line which has served as a model for monocyte/macrophage differentiation in vitro (Bohbot et al. 1993). ICAM-1 was upregulated during monocyte/macrophage maturation and differentiation in response to M-CSF (Goebeler et al. 1993). Moreover, GM-CSF has been reported to be a major macrophage fusion factor present in conditioned medium of concanavalin A-stimulated spleen cell culture (Abe et al. 1991). All of these may contribute to our findings of AM differentiation and MGC formation induced by M-CSF or GM-CSF.

Since two distinct types of MGC were observed and the ratio of these two types of MGC were induced differently in AM in response to M-CSF and GM-CSF, we investigated the pathways involved in their formation. Antibodies were applied to neutralize the effect of M-CSF and GM-CSF respectively to explore whether there was any relationship between type 1 and type 2 MGC formation. Information from cytoplasmic expression of cytokines detected by immunocytochemistry indicates that type 2 MGC represent a relatively more differentiated subpopulation than type 1 MGC. We wonder whether type 2 MGC could be derived from type 1 MGC. In such an event, blockade of type 1 MGC with antibody to M-CSF would result in less or no type 2 MGC formation in samples induced by M-CSF. Our observations that neutralizing antibody to M-CSF mainly caused a decrease in type 1 MGC formation without affecting type 2 MGC formation, suggest that type 2 MGC were not directly derived from type 1 MGC. Conversely, addition of antibody to GM-CSF resulted in a decrease in type 2 MGC formation in all groups and had no effect on type 1 MGC formation. Taken together, our data indicate that M-CSF mainly play a role in type 1 MGC formation while GM-CSF is responsible for type 2 MGC formation. Moreover, it appears that type 1 MGC are not pre-requisite for the formation of type 2 MGC.

It has been shown that M-CSF induces gene expression of GM-CSF. However, when AM were treated with M-CSF and its neutralizing antibody, enhancement of type 2 MGC formation was still observed compared to

controls. This likely results from residual levels of M-CSF not totally neutralized in our experiments. In fact, type 1 MGC was not completely inhibited when anti-M-CSF was added to the cultures in the presence or absence of M-CSF. Alternatively, in addition to M-CSF, other factors such as adherence or unknown bioactive molecules may also be able to induce AM to produce GM-CSF, thus resulting in type 2 MGC formation.

4.4. CYTOKINE MODULATION DURING AM DIFFERENTIATION

A fully differentiated macrophage is the end-result of a stepwise differentiation pathway. During the process of macrophage differentiation, pluripotential hematopoietic stem cells become committed to macrophage precursor cells and eventually differentiate into functionally, morphologically distinct end-stage macrophages. This process is accompanied by the coordinate expression of numerous genes that code proteins necessary for cellular differentiation and specific functions of macrophages. In this study, effects of M-CSF and GM-CSF on the modulation of a variety of regulatory cytokines important in immune, inflammatory and repair processes were investigated by RT-PCR at various time-points of AM differentiation.

TNF-α, IL-1 and IL-6 have been shown to be important in the regulation of inflammatory responses by promoting the migration of inflammatory cells through vascular endothelium, by inducing the production of acute reactive proteins, and by affecting the growth, differentiation, and functions of several

cell types (Arai et al. 1990, Beutler and Cerami 1987, Kishimoto et al. 1992, Witsell and Schook 1992). These cytokines are believed to be produced by macrophages during inflammation in vivo or upon stimulation with LPS in vitro. However, some controversies exist concerning whether these cytokines are constitutively expressed by resident macrophages and whether they are upregulated during macrophage differentiation in the presence of M-CSF or GM-CSF. Some workers reported that TNF-α and IL-1 are constitutively expressed by monocytes/macrophages (Becker et al. 1989, Myers et al. 1989) while others did not observe these phenomena (Haskill et al. 1988, Hamilton et al. 1993, Kelsey et al. 1993). In this study, however, resident rat AM did not display the constitutive expression of TNF-α, IL-1 and IL-6 since PCR products were not detected in freshly obtained AM (0 time). In contrast, AM express constitutively TGF-β as reported by Hoyt and Lazo (1989).

There is obvious disagreement as to whether M-CSF or GM-CSF can, by themselves, induce the synthesis and secretion of TNF-α, IL-1 and IL-6. A few studies revealed that TNF-α, IL-1 and IL-6 activities were stimulated during the differentiation of mouse or human monocytes/macrophages in vitro in the presence of M-CSF or GM-CSF (Fischer et al. 1993, Thomassen et al. 1991, Navarro et al. 1989, Sisson and Dinarello 1988). In contrast, negative observations for TNF-α, IL-1 and IL-6 production by mouse or human monocytes/macrophages in response to M-CSF or GM-CSF have also been documented (Hamilton et al. 1993, Rutherford and Schook 1992). Other

reports demonstrated that GM-CSF induces only TNF-α mRNA expression with no release of this cytokine (Kelsey et al. 1993, Gasson 1991). These discrepancies may result from different experimental conditions, including the purity of the starting cell population, the species and tissue origin of the cells, the sensitivity and specificity of assays and prior in vivo activation of the monocytes/macrophages.

Not much information is available at present concerning the expression and modulation of these cytokines during AM differentiation in vitro. In this study, differentiated rat AM induced by M-CSF or GM-CSF were monitored for the expression of TNF-α, IL-1α, IL-6 and TGF-β and compared with LPS stimulation for these cytokines' production. The question addressed in this study was to determine at which time during AM differentiation the genes encoding for TNF-α, IL-1, IL-6 and TGF-β were expressed. This would help to relate the acquisition of specific AM functions with the expression of the relevant genes. We demonstrated that TNF-α and TGF-β are expressed early during AM differentiation followed by IL-6 which increases with time in culture. Moreover, AM displayed different profiles for the expression of these cytokines in response to M-CSF and GM-CSF.

Although TNF α expression was induced by both M-CSF and GM-CSF, the levels of the expression were different. Compared to GM-CSF, M-CSF-induced AM differentiation was accompanied by earlier and higher expression of TNF-α which was correlated with TNF-α bioassay. Kinetic analysis revealed

that the highest expression of TNF-α occurred at 3h and 24h post-addition of M-CSF, indicating that this cytokine was associated with M-CSF-induced AM differentiation at an early stage.

The mutual interaction between cytokines and macrophages is likely to play a significant role in inflammatory processes, immune reaction and cell differentiation. CSFs, especially M-CSF, induce rapid and high levels of TNF-α expression and production. In turn, TNF-α modulates AM differentiation. In addition to the inflammatory effect, recent studies suggested that TNF-α may also exert its effect on normal macrophage differentiation (Weber et al. 1993). TNF-α treatment of human bone marrow cells led to significant decreases in cell number and increases in differentiated macrophages incubated for 96h. TNF-α gene blocking by antisense oligomers resulted in an increase in proliferation of mouse bone marrow-derived macrophages instead of undergoing terminal differentiation (Witsell and Schook 1992). All these data support the notion that TNF-α plays a role in macrophage differentiation. Although the precise mechanisms by which TNF-α modulates macrophage differentiation remain to be elucidated, based on the findings of RT-PCR, bioassays and neutralization experiments with anti-TNF-α antibody, it is suggested that M-CSF induces TNF-α production which in turn induces the expression of GM-CSF responsible for type 2 MGC formation. More detailed analysis of TNF-α on AM differentiation is under investigation in this laboratory with the techniques of in situ hybridization.

In a separate set of experiments, IL-1α expression by differentiated AM was detected. Similar to TNF-α, IL-1α expression was induced by M-CSF and GM-CSF. Unlike TNF-α, controls did not show IL-1α expression with time in culture raising questions concerning its role in modulating AM differentiation. However, GM-CSF-treated AM expressed relatively higher levels of IL-1α than M-CSF-treated AM. IL-1 production by CSFs has been previously examined. It was reported that mouse microglia and macrophages were induced to produce IL-1 in response to M-CSF (Fischer et al. 1993, Moore et al. 1980). However, most studies showed that GM-CSF was a major inducer of IL-1 (Thomassen et al. 1991, Sisson and Dinarello 1988). Our data are consistent with these results. This study shows that GM-CSF is a main inducer for IL-1 expression, whereas other studies have shown that IL-1 can stimulate production of GM-CSF (Gasson 1991, Marini et al. 1992). This mutual upregulation and modulation may be important in macrophage differentiation and in directing the inflammatory reaction. In addition, IL-1 has been shown to induce the production of IL-6 which exerts its action on macrophage differentiation (Chiu and Lee 1989, Oritani et al. 1992, Tanigawa et al. 1995).

In this regard, this study provides evidence for a role of IL-6 in modulating MGC formation. Thus, IL-6 expression was selectively increased during AM differentiation in the presence of GM-CSF, but not M-CSF. In contrast to TNF-α and IL-1α expression, the expression of IL-6 was persistent and high levels of IL-6 expression were still observed after a 5-day incubation.

These data showed an interesting relationship between GM-CSF-induced AM differentiation and upregulation of IL-6. In addition to its proinflammatory effects, IL-6 acts on macrophage differentiation accompanied by growth arrest (Chiu and Lee 1989). It has been observed that one of the mechanisms by which IL-6 affects macrophage differentiation is through down-regulation of c-fms gene expression resulting in a decrease of M-CSF receptors (Oritani et al. 1992). Based on human monocyte experiments, De Wit et al. (1994) have also shown that there is an inverse relationship between M-CSF and IL-6 mRNA expression. Co-stimulation with calcium ionophore A23187 and phorbol myristate acetate (PMA) resulted in an up-regulation of M-CSF mRNA and a down-regulation of IL-6 mRNA. Conversely, co-stimulation with A23187 plus DBcAMP caused a down-regulation of M-CSF mRNA and an up-regulation of IL-6. These findings are consistent with our results. We did not find any increase in IL-6 mRNA expression during AM differentiation induced by M-CSF compared to controls, whereas GM-CSF-induced AM differentiation was accompanied by an enhancement of IL-6 expression. Since IL-6 has been shown to down-regulate the expression of M-CSF and M-CSF receptors, our hypothesis is that GM-CSF induces predominantly type 2 MGC formation through the regulation of IL-6 expression. Experiments with exogenously added IL-6 and anti-IL-6 receptor further support this hypothesis.

4.5. FUNCTIONAL STATUS OF DIFFERENTIATED AM AND MGC

In order to characterize the biological potential of differentiated AM and AM-derived MGC, cytoplasmic expression of TNF-α, PDGF and TGF-β, three relevant mediators in inflammation and repair processes, were evaluated by using specific antibodies and immunocytochemistry.

In total AM, the number of cells expressing these three cytokines increased in response to M-CSF and GM-CSF which is consistent with our findings of RT-PCR experiments, suggesting that these cytokines may be involved in modulating M-CSF and GM-CSF-induced AM differentiation. Moreover, AM do not lose their ability to respond to LPS stimulation after 5-day incubation in vitro, indicating that differentiated AM are still biologically active.

MGC are observed in a variety of infectious and non-infectious diseases characterized by chronic inflammation (Lemaire 1995). However, many questions are left unanswered including their primary physiological implication and biological functions, and whether they are essential and play a protective role as opposed to a damaging one. Little information is currently available concerning cytoplasmic expression of cytokines by MGC. Results from our experiments reveal that some MGC are functional in expressing TNF-α, PDGF and TGF-β. Moreover, the profiles of TNF-α, PDGF and TGF-β expression by type 1 and type 2 MGC in response to M-CSF, GM-CSF and LPS are different, indicating that type 1 and type 2 MGC have different potential for cytokine expression. Generally, type 1 MGC always show higher levels of cytoplasmic

expression of these three cytokines than type 2 MGC, suggesting that type 1 MGC may represent a relatively more biologically active cell population.

Our observations argue for a functional role of MGC during inflammatory reactions. MGC, particularly type 1 MGC, represent highly stimulated cells of macrophage lineage and may be best considered as a specialized population of AM. It has been suggested that MGC are formed in response to intra-cellular pathogens or particles that are poorly handled by macrophages and experimental evidence exists that their absence, notably in response to opportunistic pathogens may be related to an ineffective host response (Chambers 1978). In the context of mineral-dust-induced lung inflammation, previous studies from this laboratory have shown that experimental lung granuloma and fibrosis are associated with selective changes of AM populations and/or state of differentiation (Lemaire 1991a) and MGC are present in resolving granulomas but absent in fibrosis, which brings additional support for that notion. Although the functional significance of MGC is still unclear, it has been suggested that they may function as antigen-presenting cells (Papadimitriou and Van Bruggen 1986). MGC have been found to exhibit enhanced activity for α-naphthyl acetate esterase, acid phosphatase, and acid phosphatase tartrate-resistant, indicating MGC are still biochemically active (Kreipe et al. 1988). The plasma membrane of MGC was found to be enriched in Na-K-adenosinetriphosphatases (ATPases) and the localization of their Na-K-ATPases was restricted to the nonadherent domain of the plasma membrane

of cells both in vivo and in vitro, thus imposing a functional polarity on their organization (Vignery et al. 1989). In view of these consideration and our findings, the potential role of MGC in protective immunity needs to be considered and warrants further investigation.

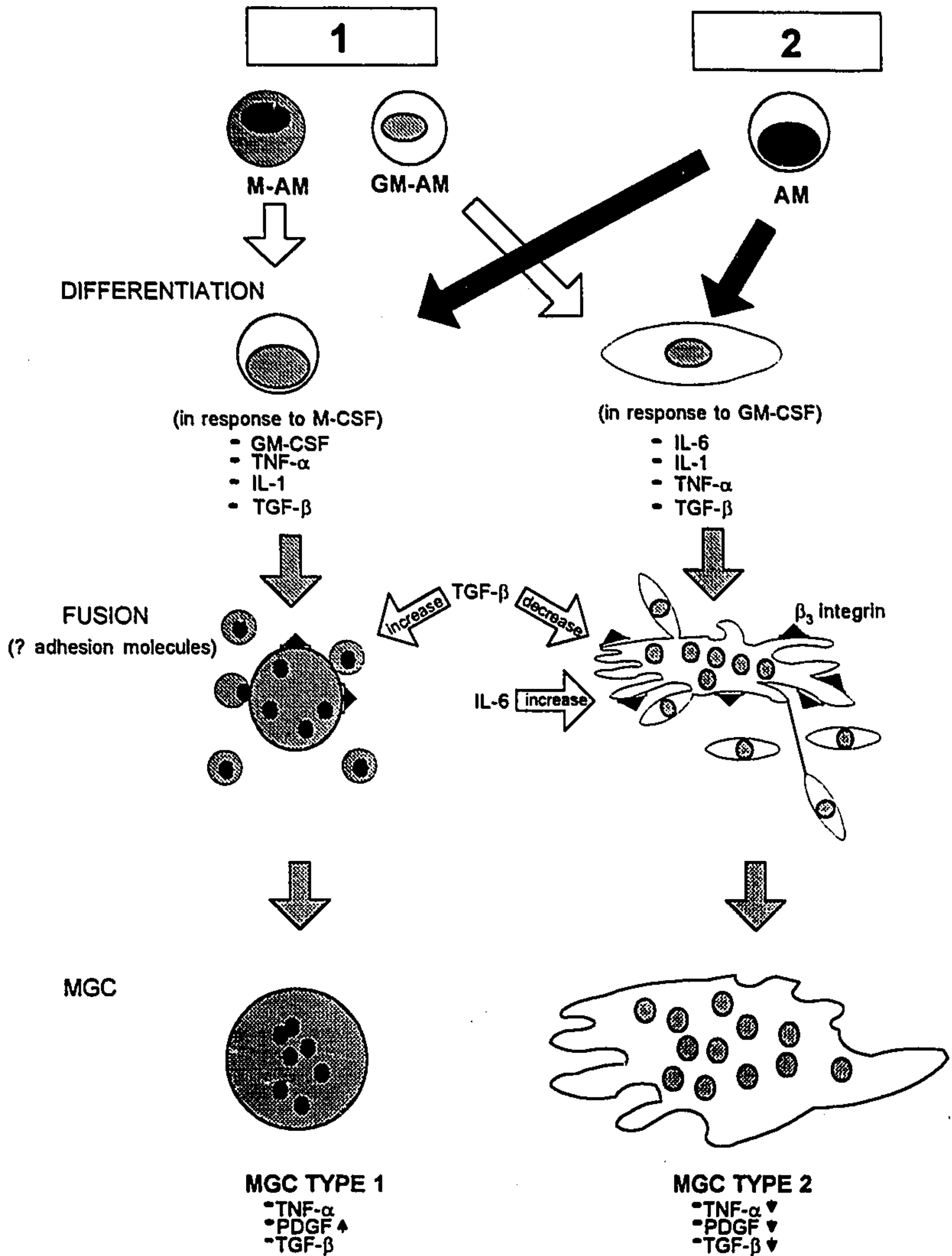
Taken together, our study demonstrates that M-CSF and GM-CSF act on the overall AM population to induce the formation of MGC with two distinct phenotypes evidenced by expression of cell integrin receptors, expression of various cytokines and functional status of MGC. The different actions of M-CSF and GM-CSF on AM differentiation may account for the heterogeneity in AM populations. Such heterogeneity may reflect differences in AM differentiation, maturation and activation, when they respond to cell-derived or environmental signals. M-CSF and GM-CSF have been demonstrated to participate in differentiation of monocyte/macrophage lineage cells (Falk and Vogel 1990, Clark and Kamen 1987, Gasson 1991). The existence of at least two different macrophage populations, M-CSF-derived macrophages and GM-CSF-derived macrophages, was recognized in bone marrow studies (Falk and Vogel 1990, Falk et al. 1991). These two macrophage populations showed marked differences in cell size, expression of Ia antigens, tumoricidal activity (Falk and Vogel 1988, Falk et al. 1988), phagocytosis, and secretion of cytokines and other mediators (Rutherford and Schook 1992a, 1992b). Therefore, it is possible that the two types of MGC observed in this study may originate from distinct subsets of AM that respond selectively to M-CSF or GM-CSF.

Alternatively, type 1 and type 2 MGC may represent different stages in the life of the same cell induced by M-CSF and GM-CSF rather than distinct cell types (Lin et al. 1989). Macrophage progenitors and their maturing progeny simultaneously express specific membrane receptors for all CSFs, an arrangement allowing interactions between the CSFs on individual cells (Nicola 1989). The M-CSF receptor is the product of the proto-oncogene c-fms and is a transmembrane glycoprotein with an intracytoplasmic tyrosine kinase domain (Bazan 1990). Although the receptor for GM-CSF is also a transmembrane glycoprotein, it lacks a tyrosine kinase domain and must elicit signalling by other mechanisms. M-CSF and GM-CSF functionally interact when they influence the behaviour of responding cells (Nicola 1987). These probably result in direct interactions on individual cells, made possible by the co-expression of both CSF receptors. Occupation of one type of CSF receptor by its ligand can down-regulate another CSF receptor (Walker et al. 1985, Gliniak et al. 1990). Thus, through the modulation of CSF receptors, the same cell population may morphologically, functionally and biologically exhibit dissimilarities.

In summary, as illustrated in Figure 42, two distinct subpopulations of AM responding to M-CSF or GM-CSF respectively may exist, or the same cell population may respond to M-CSF and GM-CSF differently depending on the stages of cell maturation and modulation of M-CSF and GM-CSF receptors. In response to M-CSF or GM-CSF, AM undergo further differentiation with distinct morphology. M-CSF-induced AM display large round shape whereas GM-CSF-

induced AM become elongated. Cytokine expression detected by RT-PCR is also different during AM differentiation. Although both M-CSF and GM-CSF induce TNF-α, IL-1 and TGF-β expression compared to controls, the levels of these cytokines' expression are different in response to M-CSF or GM-CSF as determined by quantitative densitometric analysis. Overall, GM-CSF induced AM produce a broader spectrum of cytokines than M-CSF induced AM. One obvious difference is that contrary to M-CSF, GM-CSF induced IL-6 expression. During AM differentiation, cell fusion is observed and compared to controls, a higher proportion of MGC is formed in response to M-CSF or GM-CSF. Morphologically, two types of MGC are observed. M-CSF is related to type 1 MGC whereas GM-CSF induces mostly type 2 MGC and has little effect on type 1 MGC formation. TGF-β favours type 1 MGC over type 2 MGC formation. On the other hand, IL-6 mainly increase type 2 MGC formation. Although the detailed mechanism(s) involved in MGC formation are currently unclear, adhesion molecules may play a role in MGC formation and β_3 integrin may be more related to type 2 MGC. Immunocytochemistry reveals that some type 1 and type 2 MGC express cytoplasmic TNF-α, PDGF and TGF-β with a higher proportion of type 1 MGC expressing these cytokines.

Figure 42. Schematic representation of M-CSF and GM-CSF action on AM. 1. M-CSF and GM-CSF act on different AM subsets. 2. M-CSF and GM-CSF induce selective differentiation stages in AM. Abbreviation: M-AM, M-CSF-derived alveolar macrophage; GM-AM, GM-CSF-derived alveolar macrophage.


1
2
M-AM
GM-AM
AM
DIFFERENTIATION
(in response to M-CSF)
GM-CSF
TNF-α
IL-1
TGF-β
(in response to GM-CSF)
IL-6
IL-1
TNF-α
TGF-β
FUSION
(? adhesion molecules)
increase
TGF-β
decrease
IL-6
increase
β3 integrin
MGC
MGC TYPE 1
TNF-α
PDGF
TGF-β
MGC TYPE 2
TNF-α
PDGF
TGF-β

5. CONCLUSION

1. M-CSF and GM-CSF act on the overall AM population to induce AM differentiation and MGC formation. GM-CSF induces mostly type 2 MGC and has little effect on type 1 MGC, whereas M-CSF is related to type 1 MGC formation. This is the first evidence that M-CSF and GM-CSF can induce selectively MGC with two distinct phenotypes.

2. M-CSF and GM-CSF also display different effects on AM differentiation as evidenced by general AM morphology, expression of cell integrin receptors, and modulation of various cytokines detected by RT-PCR.

3. Experiments demonstrate that MGC are active in expressing TNF-α, PDGF and TGF-β. Type 1 MGC represent a more functional subpopulation in expressing these cytokines compared to type 2 MGC.

4. Increased type 2 MGC formation observed in response to M-CSF results from GM-CSF induction and TNF-α may play a role in such induction. Unstimulated AM produce TGF-β which can be further enhanced by M-CSF and this in turn would favour type 1 MGC formation over type 2 MGC formation. On the other hand, GM-CSF induces production of IL-6 which plays a role in type 2 MGC formation.

5. This study shows that GM-CSF induced AM are more activated than M-CSF induced AM as evidenced by AM morphology and expression of a broader spectrum of inflammatory cytokines. On the other hand, GM-CSF

induced type 2 MGC are less active for TNF-α, PDGF and TGF-β production. Based on these observations, it is postulated that formation of MGC particularly type 2 MGC, may be beneficial during the chronic inflammation and direct the inflammatory reaction towards resolution.

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