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Macrophage heterogeneity occurs through a developmental mechanism

(hematopoiesis/clones/differentiation)

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ABSTRACT **The versatility and importance of macrophages in host defense and homeostasis have long been recognized. Anatomically, macrophages isolated from various tissues manifest extreme differences in shape, in metabolic and functional activities, and in the expression of macrophage-specific markers. To determine the mechanisms responsible for generating macrophage heterogeneity, we have employed the reverse transcription–polymerase chain reaction to molecularly phenotype colonies of bone marrow-derived macrophages during differentiation *in vitro*. By utilizing this method, results have revealed a hierarchal expression of macrophage-associated genes. Tumor necrosis factor α was expressed in all colonies analyzed suggesting an important role for this molecule during macrophage differentiation. Predominant colony phenotypes observed were unique for (*i*) the period of differentiation and (*ii*) the growth factor with which they were derived (either colony-stimulating factor 1 or granulocyte–macrophage colony-stimulating factor). Exogenous stimulation of the cultures with either bacterial lipopolysaccharide or interferon-γ led to predictable phenotypic transitions. These results suggest that macrophage heterogeneity is generated through differentiation-related mechanisms and that generated macrophage phenotypes are then maintained by systemic environmental constraints.**

As macrophages differentiate into mature effector cells, they acquire maturation-associated phenotypes requisite for their diverse functions (1, 2). Until recently, characterization of these cells was based mainly on morphologic, histochemical, and functional criteria. These have included levels and cellular localization of enzyme activities, antibody staining of specific markers, the ability to generate a respiratory burst, and their capacity for antigen presentation to T lymphocytes (3–7). These approaches have been used to discern the heterogeneous nature of macrophage populations; however, these techniques have had limited use in phenotyping studies since only a limited number of markers can simultaneously be studied on individual cells or colonies (4–8).

Abundant data have been reported regarding the functional heterogeneity of resident and inflammatory macrophages found in peripheral tissues of the mouse (9–11). Resident macrophages of various tissues display marked heterogeneity in terms of microbicidal activities, cell–cell interactions, antigen presentation, and involvement in the control of maternal–fetal allograft responses (7, 9, 11–14). Macrophages recruited to sites of inflammation are less mature, retain proliferative capacity, and display phenotypic changes characteristic of activated cells such as an enhanced respiratory burst and an enhanced ability to restrict the growth of intracellular parasites (5, 6, 9, 11).

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Macrophage heterogeneity could conceivably originate through a variety of pathways. Heterogeneity could be generated at the level of the bone marrow precursor cells. In accordance with this model, subsets with preprogrammed diversity would be selectively expanded under different stimuli resulting in populations with functional differences in various systemic environments (11). Alternatively, phenotypically identical cells could lodge in a tissue and be influenced solely by local constraints, thereby generating the same level of diversity through a peripheral mechanism (14). There is also evidence that different growth factors affect macrophage progenitors to influence gene expression and, thus, the behavior of mature cells (15, 16). In addition, it has been proposed that macrophage heterogeneity results 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 (plasminogen activator, inhibitors of fibrinolysis, complement factor C2, and interferon) have all been shown to be expressed maximally at specific stages of differentiation (17–19).

Developmental and activational expression of the macrophage-associated genes, tumor necrosis factor α (TNF-α), interleukin 1α and 1β (IL-1α and IL-1β), α chain of the immune-associated subregion A gene (Aα), and granulocyte–macrophage colony-stimulating factor (GM-CSF) have been examined in whole populations of bone marrow-derived macrophages (BMDMs) by Northern blot analysis (20–22). The advantage of Northern blot analyses over morphologic criteria is that many phenotypic and genetic markers can be monitored simultaneously within the same cell population. However, this method does not permit evaluation of individual cells. Therefore, it has been difficult to determine the extent of heterogeneity within macrophage populations.

Progress in eliciting the basis for macrophage heterogeneity depends on assays that can examine individual macrophage clones derived *in vitro*. Well-established reverse transcription-polymerase chain reaction (RT-PCR) techniques (23–25) have permitted us to molecularly phenotype colonies derived from individual macrophage progenitors and study the changes in gene expression as these cells mature and encounter immunologic stimuli.

Strategy. Our interest is to define the mechanism(s) responsible for the generation of macrophage heterogeneity and understand the role of developmentally programmed versus environmental influences. To approach these ques-

Abbreviations: TNF-α, tumor necrosis factor α; IL-1α and 1β, interleukin 1α/1β, respectively; Aα, α chain of the immune-associated subregion A gene; GM-CSF, granulocyte–macrophage colony-stimulating factor; CSF-1, colony-stimulating factor 1; BMDM, bone marrow-derived macrophage; LPS, lipopolysaccharide; IFN-γ, interferon-γ; RT, reverse transcription; rm, recombinant murine.

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tions, BMDMs were cloned in soft agar by using either colony-stimulating factor 1 (CSF-1) or GM-CSF. Each colony consisted of progeny from an individual progenitor; therefore, examination of a number of these colonies within each experimental group should provide insight to the extent of heterogeneity within the population (26). Experimental groups included (*i*) colonies isolated after 5, 7, and 9 days to determine the maturational difference of the cells, (*ii*) colonies derived in CSF-1 or GM-CSF to determine the effect of hematopoietic stimuli, and (*iii*) stimulation of each of these groups with either lipopolysaccharide (LPS) or interferon-γ (IFN-γ). Individual colonies were isolated and molecularly phenotyped for the presence of TNF-α, IL-1α, IL-1β, GM-CSF, and Aα transcripts (26).

MATERIALS AND METHODS

Cells. Isolation and derivation of cells were performed as described (26). Briefly, bone marrow plugs were harvested from BALB/c femurs in Hanks' balanced salt solution, dispersed into a single-cell suspension, and washed. Cells were plated in soft agar at 1.0×10^5 nucleated cells per ml in six-well polystyrene tissue culture plates. Culture medium consisted of Dulbecco's modified Eagle's medium containing 0.3% Noble agar with 10% (vol/vol) horse serum, 10% (vol/vol) control processed serum replacement 2 (CPSR-2; Sigma), and either 10% (vol/vol) L929 cell-conditioned medium as source of murine CSF-1 (250 units/ml by proliferation assay) or recombinant murine (rm) GM-CSF (Immunex, Seattle; 300 units/ml). Cultures were maintained in a humidified chamber at 37°C in 7% CO_2/93% air for 5, 7, or 9 days. Prior to harvest, cells were treated with either LPS (*Escherichia coli* 055:B5; Difco; 1 μg/ml) for 6–8 hr or rmIFN-γ (100 μg/ml; a gift of Shering through The American Cancer Society) for 18 hr prior to colony isolation.

RNA Isolation. Upon isolation, individual colonies (consisting of 50–200 cells) were mixed with 100 μl of 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% *N*-laurylsarcosine, 0.1 M 2-mercaptoethanol, and 10 μg of carrier *E. coli* tRNA. Immediately 0.1 vol of 2 M sodium acetate (pH 4.0), an equal volume of water-saturated phenol, and 0.4 vol of chloroform/isoamyl alcohol, 49:1 (vol/vol) were sequentially mixed and samples were chilled on ice for 15 min. Phases were separated by centrifugation at 10,000 × *g* for 10 min at 4°C. The aqueous supernatant was extracted with 10 mM Tris·HCl/1 mM EDTA, pH 8.0 (TE)-saturated phenol/chloroform/isoamyl alcohol, 24:24:1 (vol/vol). The aqueous supernatant was aspirated and precipitated with 2.5 vol of absolute ethanol. The RNA pellet was washed with 80% ethanol in TE buffer and dissolved in the RT reaction mixture as described.

RT. Total RNA was reverse transcribed by addition of 500 pM random hexamers (Pharmacia), all four deoxynucleotides (each at 250 μM), 1× Moloney murine leukemia virus (M-MLV) RT buffer, and 200 units of M-MLV RT (BRL; 200 units/μl) in 20 μl. The reaction proceeded for 1 hr at 42°C and was terminated by heating at 90°C for 10 min.

Amplification Primers. Sequences were obtained using DNASTAR and have been published elsewhere (26). Three primer sets were utilized for the amplification of each cDNA sequence. Primer sets consisted of a 5′ primer, an external 3′ primer, and a "nested" internal primer. The nested primers increased the efficiency of amplification in the absence of abundant target sequences.

Amplification Reaction. The initial amplification reaction contained 100 mM Tris·HCl (pH 8.8), 10 mM $MgSO_4$, 25 mM NH_4CL, 15 mM 2-mercaptoethanol, 10 μM EDTA (pH 8.0), all four deoxynucleotides (each at 150 μM), 15% (vol/vol) dimethyl sulfoxide, 1 unit of *Thermus aquaticus* polymerase (Perkin–Elmer), and each primer at 100 nM to a total volume of 50 μl. cDNA (2 μl) from each colony sample was added to five tubes containing primers for each sequence. Twenty PCR cycles were performed, after which the internal 3′ primer was added to each sample for an additional 20 cycles. The PCR profile was as follows: 94°C for 1 min to denature the DNA, 60°C for 1.5 min to allow primer annealing, and 72°C for 1 min for DNA extension.

Detection. Samples (5 μl) of the PCR mixture were electrophoresed in an agarose gel (4%) at 75 V for 90 min. Gels were stained with ethidium bromide for 10 min. Individual products were identified by size since primers were designed to amplify a specific-size gene product for each gene.

RESULTS

Phenotyping of Colonies. Colonies were molecularly phenotyped for the presence of TNF-α, IL-1α, IL-1β, Aα, and GM-CSF transcripts. Gene expression was detected by gel electrophoresis of the RT-PCR products from individual colonies (Fig. 1). By using this method to score gene expression, phenotypic distributions were compiled for each treatment group to demonstrate the influence of differentiation stage, activation, and various hematopoietic stimuli on macrophage gene expression. Phenotypes in every experiment were derived by using each of the five genes described above. Therefore, if macrophage differentiation were totally stochastic, 32 phenotypes would have been detected. Likewise, if a developmentally regulated pathway were responsible for differentiation, a small predictable number of phenotypes would have been expected that would change corresponding to the stage of differentiation of the examined colony.

Preliminary experiments showed that few of the 32 possible phenotypes were present and that phenotypic distributions were highly reproducible for each treatment group. The reproducibility of phenotypes obtained from GM-CSF-derived (300 units/ml) colonies after 7 days of culture is provided (Table 1). In addition, to show the efficacy of the phenotyping technique the cDNA samples from 30 colonies were divided and identical phenotypes were obtained from both samples of the original colony. The predominance of specific phenotypes in each experiment strongly suggested a highly regulated developmental pathway. Similar observations regarding the nonrandom expression of functional macrophage phenotypes have been reported (7, 27). Those experiments utilized splenic macrophage colonies that were subcloned and tested for their ability to present antigen to T-cell hybridomas. Of the proportion of colonies that presented antigen, only the paired subculture could also present antigen, thus suggesting that antigen presentation was not a

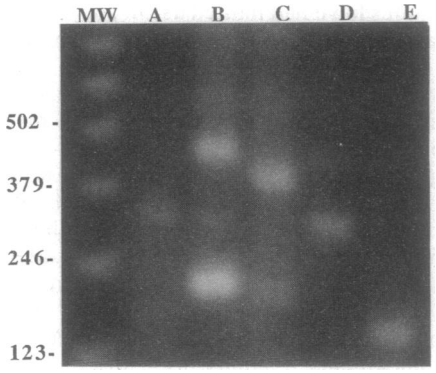

FIG. 1. Amplification products from an individual colony. Two expected-size products are seen in the figure resulting from amplification with three separate primers. Lanes: A, IL-1α; B, Aα; C, TNF-α; D, GM-CSF; E, IL-1β; MW, molecular size markers in base pairs.

Table 1. Phenotypic distribution after stimulation of GM-CSF-derived macrophages

Major phenotypes	Phenotype, % of total phenotypes		
	None	LPS	IFN-γ
TNF-α + IL-1β	19.0 ± 8	21.5 ± 1.5	26.0 ± 7
TNF-α + IL-1β + Aα	26.0 ± 1	10.0 ± 2.5	36.5 ± 0.5
TNF-α + IL-1β + Aα + IL-1α	15.0 ± 3	14.0 ± 1	<10
TNF-α + IL-1β + IL-1α	<10	18.5 ± 1.5	<10
TNF-α + Aα	<10	ND	16.5 ± 4.5

Analyses were performed on BALB/c BMDM soft agar clones cultured in rmGM-CSF (300 units/ml) for 7 days. Before harvest, cells were treated with rmIFN-γ (100 units/ml) for 18 hr or LPS (1 mg/ml) for 6 hr or were not stimulated. Values represent the mean ± SD of two experiments conducted with 19–32 colonies per experiment. Predominant phenotypes from each treatment are shown with percent of colonies expressing each phenotype. Five other phenotypes were found in these experiments but were present at <10% under every treatment. ND, none detected.

random event but was concordantly expressed by both members of the pair (27).

Specific, but different, predominant phenotypes were expressed by CSF-1-derived (250 units/ml) colonies after 5, 7, and 9 days of culture (ref. 28; Table 2). Interestingly, TNF-α transcripts were detected in all colonies monitored regardless of hematopoietic stimuli (CSF-1 or GM-CSF) or treatment with LPS or IFN-γ.† TNF-α has been demonstrated as one of the earliest detectable changes in gene expression associated with monocytic differentiation and is constitutively expressed in BMDM cultures (21, 29). It has also been suggested that TNF-α and IL-1 may participate in a regulatory cascade through autocrine induction (30). The results presented here further indicate the involvement of TNF-α in the developmental process of macrophage differentiation.

Comparison of Hematopoietic Stimuli and Exogenous Stimulation. Additional studies compared the phenotypes of colonies derived after 5, 7, and 9 days of culture. The results revealed a developmentally regulated course of gene expression during differentiation. At day 5 of culture, two major phenotypes comprised 42% of the colonies, only sharing one of the three predominant phenotypes that comprised 59% of the total phenotypes present at day 7 of culture. By day 9 of differentiation, phenotypes were dispersed among seven relatively evenly distributed groups, thus suggesting that a diversity-generating event(s) occurred during the latter stages of the culture period. Interestingly, predominant phenotypes differed for each period analyzed whereas little maturational diversity existed on a given day.

Since the phenotypic distribution of the colonies progressed along a developmental time course (suggesting a regulated pathway due to maturation), we were interested in the contribution of an alternate hematopoietic stimulus to phenotypic differences. Since CSF-1 is found circulating constitutively in the blood and body tissues whereas GM-CSF is detected only during inflammatory responses, the distribution of the phenotypes after culture using these two factors could provide insight into mechanisms regulating the immune response by the generation of specific macrophage phenotypes.

Therefore, IL-1 and Aα gene expression was compared in CSF-1- and GM-CSF-derived colonies (Table 3). IL-1β was

†Amplification efficiencies were tested for primers for every gene by using a plasmid containing the gene and control tissue cDNA samples. No difference in the amplification was found among the genes. Therefore, we concluded that the amplification of TNF-α from every colony was not an amplification artifact, but TNF-α was found in all differentiated macrophage colonies.

Table 2. Predominant phenotypes during CSF-1-induced macrophage differentiation

Major phenotypes	Phenotype, % of total phenotypes		
	5	7	9
TNF-α + IL-1β + IL-1α + Aα	13.0	<5.0	<5.0
TNF-α + IL-1β + IL-1α	29.0	22.0	9.0
TNF-α + IL-1β + Aα	ND	22.0	<5.0
TNF-α + IL-1β + GM-CSF	<5.0	<5.0	18.0
TNF-α + IL-1β	8.0	15.0	9.0
TNF-α + IL-1β + IL-1α + GM-CSF	8.0	7.0	14.0
TNF-α + IL-1β + IL-1α + GM-CSF + Aα	<5.0	7.0	10.0

BALB/c BMDMs were cultured in soft agar containing CSF-1 (250 units/ml) for 5, 7, or 9 days as indicated. Colonies were isolated and phenotyped as in Table 1. Major phenotypes from each day are shown with the percent of total colonies expressing each particular phenotype. Other minor phenotypes were detected at <10% of the totals at each day monitored. ND, none detected.

expressed in 64–85% of the total colonies monitored from days 5 through 9 of culture. This high level of expression was in agreement with Northern blot analysis that showed constitutive expression of IL-1β at each of these time points (21). A higher level of IL-1β than IL-1α transcripts occurred (21, 31) and seemed to be due to the number of cells expressing IL-1β. In individual colonies, IL-1α gene expression was more variable and these transcripts were rarely detected in colonies not expressing IL-1β transcripts (Table 3). For

Table 3. Clonal analysis of IL-1 and Aα gene expression during CSF-1- or GM-CSF-induced macrophage differentiation

Treatment group	Colonies, % of total		
	5 days	7 days	9 days
CSF-1 culture			
Aα			
Untreated	41	46	41
IFN-γ	71	53	58
IL-1β (alone)			
Untreated	13	41	41
LPS	38	23	64
IL-1α (alone)			
Untreated	13	9	<5
LPS	<5	6	ND
IL-1α and IL-1β			
Untreated	58	34	45
LPS	48	63	32
GM-CSF culture			
Aα			
Untreated	27	68	14
IFN-γ	50	66	55
IL-1β (alone)			
Untreated	9	58	57
LPS	23	56	50
IL-1α (alone)			
Untreated	9	ND	ND
LPS	11	<5	13
IL-1α and IL-1β			
Untreated	64	31	7
LPS	58	34	13

IL-1 and Aα gene expression was monitored in 5-, 7-, and 9-day developing macrophage colonies cultured in either CSF-1 or GM-CSF in three or four experiments. For IL-1 studies, colonies were either left untreated or treated with LPS (1 mg/ml) for 6 hr prior to harvest. IL-1β and IL-1α gene expression is shown individually and together representing colonies expressing both transcripts. Aα gene expression was monitored in untreated colonies or colonies treated with rmIFN-γ (100 units/ml) for 18 hr prior to harvest. ND, not determined.

CSF-1-derived macrophage clones, IL-1α was predominantly expressed in untreated samples at day 5 of culture and in LPS-treated colonies at day 7 of culture; whereas, by Northern blot analysis (21), this transcript was only detectable at extremely low levels after LPS stimulation at days 5 and 7 of culture. Radioimmunoassays of human monocytes (32) demonstrated identical levels of expression of IL-1α and IL-1β in LPS-stimulated cells assayed in the presence of 5% (vol/vol) human serum and 1% human plasma. This observation was consistent with the LPS-induced increase of IL-1α transcripts in CSF-1-derived colonies (Table 3).

LPS-induced IL-1α gene expression was the main phenotypic difference between CSF-1- and GM-CSF-derived macrophages. LPS stimulation substantially increased the number of macrophage colonies at day 7 of culture expressing IL-1α in CSF-1-derived cells, whereas GM-CSF-derived cells maintained IL-1β transcripts without shifting the population to express IL-1β plus IL-1α (Table 3).

Expression of Aα plateaued at 50–70% of the colonies expressing the transcript, whether colonies were CSF-1- or GM-CSF-derived, as the phenotypic profiles shifted to Aα positive after IFN-γ stimulation (Tables 1 and 3). A maximum of 50% of CSF-1-derived BMDMs expressing cell surface Aα has been reported (16). Maximal Aα transcripts were expressed in colonies at day 7 of differentiation, and treatment with IFN-γ enhanced expression on each day monitored.

DISCUSSION

The data presented in this report support a developmental mechanism for the generation of macrophage heterogeneity. Although separate subsets of progenitors with unique rates of differentiation cannot be ruled out by our findings, our data provide evidence for developmental regulation. Different specific phenotypes were predominant on each day monitored. Further, these phenotypes were growth factor dependent, and exogenous stimulation of the differentiating cultures resulted in predictable phenotypic transitions. From these data, we propose a hierarchy of gene expression in developing macrophages with TNF-α expressed by all colonies.

IL-1β was expressed in at least 50% of the colonies although the transcript was LPS-inducible in CSF-1- but not GM-CSF-derived cells. IL-1α was rarely expressed without IL-1β and this coordinate gene expression may be indicative of a regulatory cascade. Mature alveolar macrophages have been shown to produce fewer IL-1β transcripts and less protein product than the more immature blood monocytes (31). This suggests the existence of different pathways regulating the developmental potential of cells cultured in different hematopoietic stimuli. Supportive evidence has been provided for the different potential of CSF-1- versus GM-CSF-derived cells on the basis of their proliferative potential as well as on the basis of Aα gene expression (15, 16). Both of these reports attribute differences in the potentials of CSF-1- versus GM-CSF-derived cells to separate subsets of progenitors. Our data support both the activation of separate precursors as well as the differential activation of biresponsive precursors by growth factors.

The data also show clonal differences in differentiating BMDMs with respect to Ia antigen expression that are in agreement with clonal differences found in the antigen processing of splenic macrophage colonies (7). Studies on class II gene expression (20) supported developmentally linked expression of Aα. Pullen *et al.* (20) analyzed populations of adherent CSF-1-derived BMDMs that were controlled for the influence of exogenously produced TNF-α and IFN-γ. This work demonstrated that induction of Aα gene expression was maximal at day 7 of differentiation and was augmented by IFN-γ stimulation (20). Stimulation of Aα gene expression by IFN-γ has been repeatedly reported (16, 20, 33).

This cascade-like hierarchy supports a controlled developmental pathway of differentiation that generates distinct phenotypes. The phenotypes described for each day suggest that the monocyte population, upon release from the marrow, will be a heterogeneous population. Furthermore, the observation that stimulation with LPS or IFN-γ induces phenotypes distinct from those occurring through differentiation suggests that monocytes are capable of responding to various environmental stimuli and thus provides a mechanism for generating tissue-related heterogeneity. Therefore, tissue-related phenotypes could be achieved by maintenance of specific phenotypes after their migration into the tissue.

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