

Initial Cytokine Exposure Determines Function of Macrophages and Renders Them Unresponsive to Other Cytokines¹

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The functional properties of infiltrating macrophages (M ϕ) must be tightly regulated to facilitate appropriate responses to complex conditions in an inflammatory focus. This study was designed to ascertain whether uncommitted M ϕ that have been exposed to combinations of cytokines with opposing functions develop properties dictated by one cytokine or by cytokine mixtures. Uncommitted rat bone marrow-derived M ϕ (BMDMs) were incubated with IFN- γ , TNF- α , TGF- β , IL-4, IL-6, and IL-10 alone or sequentially in combinations. After 48 h, function was assessed by nitric oxide (NO) generation, uptake of apoptotic neutrophils, and β -glucuronidase expression. IFN- γ followed 4 h later by TNF-induced NO generation. The pretreatment of BMDMs before IFN- γ priming with TNF, TGF- β , and IL-4 suppressed NO generation by 87%, 92%, and 85%, respectively; IL-10 had no effect. The same cytokines administered at 4 h after IFN priming had no effect on NO generation. The uptake of apoptotic polymorphonuclear leukocytes was augmented by TNF (40% vs 29% controls; $p < 0.05$) and decreased by IFN- γ , IL-10, and IL-4. The TNF response was unaffected by subsequent treatment with IFN- γ , IL-4, or IL-10. Similarly, the decreased polymorphonuclear leukocyte uptake induced by IFN- γ , IL-4, or IL-10 was unaffected by the subsequent addition of TNF. β -glucuronidase expression was increased by TGF- β and decreased by IFN- γ . These responses were not modified by cytokines with the opposing function. Thus, the functional response of BMDMs to complex mixtures of cytokines was determined by the first cytokine to which they were exposed. Once activated, BMDMs become unresponsive to alternative activating signals, a finding which has obvious implications for M ϕ function in vivo. *The Journal of Immunology*, 1998, 161: 1983–1988.

Macrophages (M ϕ)³ influence almost all aspects of immunologic and inflammatory responses and play an essential role in linking innate and acquired immune systems (1). M ϕ ingest and destroy bacteria and other microorganisms (2), kill virally infected and malignant cells (3), and present Ags to T lymphocytes (4); in addition, they not only induce inflammation (5) but also promote its resolution (6), and they have important roles in angiogenesis (7), tissue remodeling, and repair (8). Infiltrating M ϕ are heterogeneous primarily because they adapt to the local microenvironment by developing sets of attributes that enable them to perform a particular function (9). Consequently, an understanding of the basis for M ϕ adaptation and knowledge of what controls (or limits) the range of activities that M ϕ develop is essential for determining how inflammation is regulated.

Injury causes a rapid increase in the number of M ϕ in a tissue; this increase is principally due to infiltration by monocytes that

then differentiate into M ϕ , but it is also a result of local cell division (10, 11). The properties of M ϕ within an injured site vary according to the nature of the injury and the length of its evolution. This principle was exemplified many years ago by North et al. and Mackaness et al. (12, 13), who demonstrated that an injection of thioglycolate into the peritoneal cavity caused an influx of M ϕ that were more adhesive than resting M ϕ , released more lysosomal enzymes, but were not cytotoxic to other cells. They then established that M ϕ infiltrating the peritoneum as part of the T cell-mediated response to i.p. mycobacterial purified protein derivative not only released more enzymes but was cytotoxic as well. These and other similar experiments led to the concepts of elicited M ϕ and M ϕ activation (14) and to the identification of IFN- γ as the principal M ϕ -activating factor (15). Since then, it has become apparent that M ϕ can be "alternatively activated," for example by IL-4 (16), and that other M ϕ activation states exist (9).

The rapid increase in the number of cytokines and growth factors that have been shown to modify M ϕ function has amply demonstrated the complexity of M ϕ activation; for instance, many of the cytokines involved are also produced by M ϕ and are likely to have autocrine effects. Some cytokines, such as IL-2, IL-12, IL-15, and TNF, enhance the proinflammatory properties of M ϕ , whereas others, including IL-4, IL-13, and TGF- β , down-regulate these properties (reviewed in Ref. 17). Even so, the results from many studies are contradictory, either because different concentrations of cytokines have opposing effects (18), or possibly because of the different sources of M ϕ used. The studies that examined the effects of single cytokines emphasize the degree to which M ϕ function is controlled but are relatively uninformative with regard to how cytokines interact to induce coordinated sets of activities that enable them to perform a particular function. This aspect of M ϕ function has been examined by Riches et al. using murine bone marrow-derived M ϕ (BMDMs) as a model (19–21). They showed that

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³ Abbreviations used in this paper: M ϕ , macrophage(s); BMDM, bone marrow-derived macrophage; NO, nitric oxide; IGF-1, insulin-like growth factor-1; PMN, polymorphonuclear leukocyte; SOCS, suppressor of cytokine signaling; JAK, Janus kinase.

Table I. Cytokine-induced functional properties of BMDM

Cytokine (concentration) ^a	Nitrite Concentration (arbitrary units)	Uptake of Apoptotic PMNs	β -Glucuronidase Expression Scores
Control	2.7 \pm 1.8	29.4 \pm 3.5	2.3 \pm 0.2
IFN + TNF (20 U/ml, 10 ng/ml)	22 \pm 3.2 ^b	17.1 \pm 2.4 ^c	1.3 \pm 0.2 ^c
IFN (20 U/ml)	5.2 \pm 2.0 ^b	21.6 \pm 2.0 ^c	—
TNF (10 ng/ml)	2.5 \pm 2.6	40.1 \pm 3.8	1.8 \pm 0.3
IGF (7.5 ng/ml)	1.7 \pm 1.6	26 \pm 2.7	3.5 \pm 0.1 ^c
IL-4 (5 μ l/ml)	1.9 \pm 1.6	13 \pm 1.4 ^b	2.1 \pm 0.2
IL-10 (100 ng/ml)	3.2 \pm 2.5	22.8 \pm 2.7	0.7 \pm 0.3 ^c

^a Concentrations shown are those with maximum effect.

^b $p < 0.05$ relative to unstimulated control

^c $p < 0.05$ relative to unstimulated controls

uncommitted BMDMs can be induced to develop coordinated sets of nonoverlapping and mutually exclusive properties when exposed to IFN- γ , TNF, or TGF- β . Thus, BMDMs primed with IFN- γ and activated with TNF generate large amounts of nitric oxide (NO) and synthesize complement components (such as factor B of the alternative pathway) but do not synthesize insulin-like growth factor-1 (IGF-1) or express the lysosomal hydrolase β -glucuronidase. By contrast, BMDMs activated with TNF without prior exposure to IFN- γ synthesize IGF-1 but do not generate NO and cannot be induced to generate NO even when subsequently exposed to IFN- γ . Consequently, IFN- γ priming profoundly effects the M ϕ response to TNF; in fact, the order of exposure to these two cytokines results in the development of cells with mutually exclusive sets of properties.

The effect of IFN- γ and TNF on NO generation suggests that uncommitted M ϕ might be preconditioned to a particular activity by the first cytokine encountered. The principal purpose of this study was to first test this hypothesis in studies using the "anti-inflammatory" cytokines IL-4, IL-6, IL-10, and TGF- β , because they have been reported to modulate NO generation by IFN- γ -primed M ϕ or M ϕ cell lines, and second by studying the control of two other M ϕ functions, namely the uptake of apoptotic neutrophils and the expression of β -glucuronidase. The results show that all three functions are modulated by the cytokines studied, and that in each case the response is determined by the first cytokine to which the M ϕ are exposed. Thus, IFN- γ priming for NO generation is abrogated by pretreatment with IL-4, TGF- β , or TNF; the TNF-induced uptake of apoptotic polymorphonuclear leukocytes (PMNs) is prevented by pretreatment with IFN- γ , IL-4, and IL-10; and the TGF- β -induced expression of β -glucuronidase is prevented by IFN- γ . None of the treatments had any effect when administered after the primary stimulus; the first exposure inhibits the ability of BMDMs to respond to alternative activating stimuli. These results provide a model for the functional activation of M ϕ infiltrating an inflammatory focus and have obvious implications for the autocrine role of cytokines in M ϕ activation and for cytokine-based therapies.

Materials and Methods

Cytokines and Abs

Human rTNF- α was obtained from Boehringer (Ingelheim, Germany), and human rTGF- β , IL-10, and IL-6 were obtained from Sigma (Dorset, U.K.). Rat rIFN- γ was obtained from Bradsure Biologicals (Loughborough, U.K.). Rat rIL-4 was produced in-house as described previously (22) using a Chinese hamster ovary cell line (22) that was generously donated by Dr. Neil Barclay (Medical Research Council Cellular Immunology Unit, Oxford, U.K.).

Isolation and culture of BMDMs

Rat BMDMs were obtained using a previously described technique (20). Briefly, bone marrow cells were flushed aseptically from the dissected

femurs of male Sprague-Dawley rats with a jet of complete medium directed through a 25-gauge needle to form a single-cell suspension. The cells were cultured in 75-mm tissue culture flasks (Corning Glass, Corning, NY) and adhered to plastic in DMEM containing 2 mM glutamine, 100 U/ml penicillin/100 U/ml streptomycin, 10% heat-inactivated FCS, and 10% L929-conditioned medium as a source of M ϕ -CSF. After 7 days in culture, the cells were carefully removed using 1% trypsin/EDTA, dispensed into 24-well culture plates (Corning) at a concentration of 5×10^5 cells/well, and rested for 24 h in M ϕ -CSF-free medium before they were washed and incubated with the cytokines. When combinations of cytokines were used, the initial cytokine was administered 4 h before the second cytokine; the cytokines were not removed from the medium until M ϕ function was assessed.

Quantitation of NO synthesis

The generation of NO was estimated by assaying culture supernatants for nitrite, which is a stable reaction product of NO. Aliquots of 200 μ l of each cell-free culture supernatant were incubated with 50 μ l of Griess reagent (0.5% sulfanilamide and 0.05% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid) in 96-well flat-bottom tissue culture plates for 10 min at room temperature. ODs of the assay samples were subsequently measured at 540 nm using a solution of phenol red-free DMEM. In most experiments, nitrite was measured after 48 h in culture.

Assay for uptake of apoptotic neutrophils

BMDMs were transferred to 24-well plates at a density of 5×10^5 cells/well and rested for 24 h before the medium was changed and cells were incubated with the cytokines in various combinations. The uptake of apoptotic neutrophils was assessed after 48 h using a microscopically quantified phagocytic assay as described previously (23, 24). Briefly, apoptotic neutrophils were prepared from PMNs that had been isolated from fresh, heparinized, normal human blood by dextran sedimentation and plasma-Percoll centrifugation. The neutrophils were aged in Teflon bags for ~24 h in Iscove's modified Dulbecco's medium with 10% autologous platelet-rich, plasma-derived serum. More than 98% of these cells excluded trypan blue, and apoptosis was verified by oil-immersion light microscopy of May-Giemsa-stained cytospin preparations as described previously (25). The apoptotic cells were washed once in HBSS and resuspended in DMEM at a concentration of 2.5×10^6 /ml. A total of 2×10^6 PMNs were added to each well and allowed to interact with the M ϕ for 30 min at 37°C in a 5% CO₂ atmosphere. The wells were washed in saline at 4°C to remove noningested PMNs, fixed with 2% glutaraldehyde in 0.9% saline, and stained for myeloperoxidase to identify ingested PMNs. Next, the proportion of M ϕ that had ingested neutrophils was counted by inverted light microscopy as described previously (25, 26). The results are expressed (as shown in Table I and Fig. 3) as the percentage of M ϕ in which uptake of at least one apoptotic neutrophil could be visualized.

Quantitation of β -glucuronidase expression

β -glucuronidase was visualized by an enzymatic staining method in which β -glucuronidase catalyzed the reaction of α -naphthol AS-BI β -D-glucuronide into the red soluble-chromogenic naphthol AS-BI-HPR complex (27). Cytospin preparations of M ϕ that had been harvested from the 24-well tissue culture plates were fixed in a glutaraldehyde-acetone solution, and the slides were air-dried. Slides were then stained with β -glucuronidase staining solution and counterstained with methylene blue before being mounted with aqueous medium. Slides were coded and scored according to the following scale: 0, no staining; 1, equivocal positive staining; 2, weak positive staining; 3, moderate positive staining; and 4, strong positive staining. Each cell that was counted was multiplied by a number between 0 and

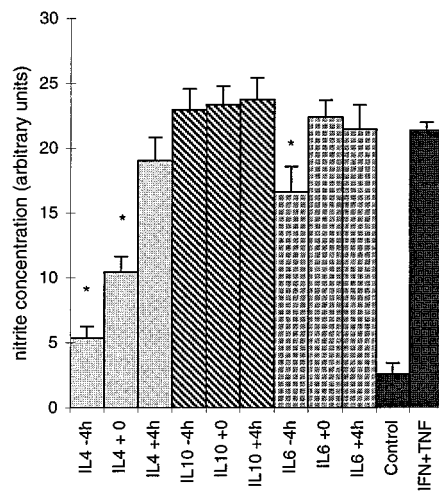


FIGURE 1. Inhibition of IFN priming for NO generation. M ϕ were incubated for 48 h in medium alone (control) or in medium containing IFN- γ (20 U/ml) that was supplemented 4 h later by TNF- α (10 ng/ml). The inhibitory effects of cytokines were assessed by incubating M ϕ with either TGF- β (5 ng/ml) or TNF at 4 h before priming, at the same time as priming, or at 4 h after priming. Mean plus SE; $n = 10$. * $p < 0.01$ vs IFN/TNF-treated cells.

4 according to its score. Numbers were added up, and the total was divided by the number of cells that were counted in each experiment. The score represents an overall change in the density of staining.

Results

IFN priming followed by TNF induces NO generation in rat BMDMs

The effects of IFN- γ and TNF on NO generation by murine BMDMs have been reported previously (20); therefore, it was important to confirm that these cytokines had the same effect on rat M ϕ . Adherent, 7-day BMDMs were transferred into 24-well plates (5×10^5 M ϕ per well), left to adhere for 4 h, washed, and then cultured for 24 h in medium without M ϕ -CSF and other cytokines. The medium was then changed to one containing IFN- γ and TNF alone and in various combinations. BMDMs incubated with 20 U/ml of IFN- γ generated small amounts of NO; TNF (10 ng/ml) had no effect (Table I). However, priming BMDMs with IFN- γ for 4 h before activation with TNF- α resulted in a marked increase of nitrite concentration. This increase was evident after 24 h and persisted for at least 48 h. IFN- γ priming for NO generation was equally affected when BMDMs were primed for various lengths of time between 4 and 24 h before activation, but a minimum of 2 h prestimulation was required for a significant effect.

Prevention of IFN/TNF-induced NO generation by antiinflammatory cytokines

BMDMs incubated with IL-4, IL-10, or TGF- β generated the same amount of NO as unstimulated controls (Table I). However, preincubating BMDMs with either IL-4 or TGF- β for 4 h before IFN- γ priming almost completely abolished NO generation. IL-4 had a partial effect when given simultaneously with IFN- γ priming and caused a 50% decrease in nitrite concentration (Fig. 1). By contrast, TGF- β had no effect when administered at the same time as IFN- γ (Fig. 2), suggesting that IL-4 had a direct effect on IFN priming whereas TGF- β acted indirectly. IL-4, TGF- β , or TNF administered for 4 h followed by incubation of the M ϕ for 48 h in cytokine-free medium did not inhibit IFN/TNF-induced NO generation, indicating that the inhibitory effects of short exposure to

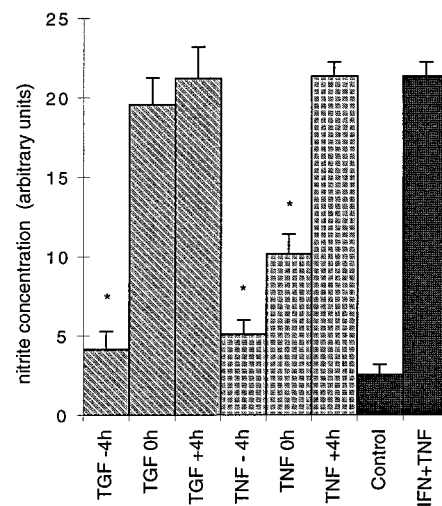


FIGURE 2. Inhibition of IFN priming for NO generation. M ϕ were incubated for 48 h in medium alone (control) or in medium containing IFN- γ (20 U/ml) that had been supplemented 4 h later by TNF- α (10 ng/ml). The inhibitory effects of cytokines were assessed by incubating M ϕ with either IL-4 (5 μ l/ml), IL-6 (10 ng/ml), or IL-10 (100 ng/ml) at 4 h before IFN priming, at the same time as priming, or at 4 h after priming. Mean plus SE; $n = 10$. * $p < 0.01$ vs IFN/TNF-treated cells.

the cytokines are temporary. IL-6 partially inhibited NO generation when BMDMs were treated for 4 h before priming; however, like TGF- β , IL-6 had no effect when given together with IFN- γ priming. Interestingly, IL-10 had no effect on NO generation even when BMDMs were treated 4 h before IFN priming. The differences between the results obtained with IL-4, TGF- β , and IL-10 are not attributable to the doses used, because similar results were obtained over a wide concentration range (e.g., TGF (0.1–10 ng/ml), IL-4-containing medium (0.1–50 μ l/ml), and IL-10 (1–100 ng/ml)). The observation that none of the antiinflammatory cytokines had an effect on IFN/TNF-induced NO generation when administered at 4 h after the M ϕ had been exposed to IFN- γ is partly consistent with some studies but is also in contrast to results that have been reported previously in peritoneal M ϕ and in M ϕ cell lines (28, 29).

Cytokines regulate uptake of apoptotic neutrophils by BMDMs

The uptake of apoptotic neutrophils is thought to be one of the critical steps in the resolution of acute inflammation (30); therefore, it might be expected to be more efficient in M ϕ whose function was reparative rather than to cause tissue destruction. For this reason, we assessed the influence of pro- and antiinflammatory cytokines on the uptake of apoptotic human neutrophils by uncommitted rat BMDMs. TNF significantly increased the proportion of BMDMs that took up apoptotic neutrophils (TNF = 40%, controls = 29%; $p < 0.005$, the Student t test). By contrast, IL-4 reduced the proportion of BMDMs that took up the neutrophils (IL-4-treated cells = 13%; $p < 0.001$, the Student t test). TGF- β had no effect, whereas IFN- γ and IL-10 reduced uptake (Table I). These observations indicate that exposing M ϕ to cytokines before contact with apoptotic neutrophils greatly influences the ability of M ϕ to ingest apoptotic neutrophils. When the number of apoptotic neutrophils ingested by 100 randomly selected M ϕ in each well was counted, the proportionate change mediated by the above cytokines was greater than the change in the percentage of the M ϕ that were taking up apoptotic cells (data not shown).

Experiments in which BMDMs were incubated with TNF, IL-4, IL-10, and TGF- β sequentially show that the first cytokine to

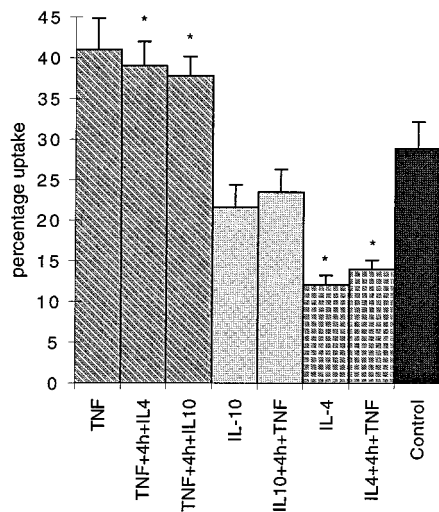


FIGURE 3. Uptake of apoptotic PMNs by BMDMs incubated with cytokines. *M* ϕ were incubated for 48 h in medium alone (control), medium containing a single cytokine, or medium containing a single cytokine that was supplemented 4 h later by a second cytokine. Mean plus SE; $n = 8$. * $p < 0.05$ vs unstimulated controls.

which BMDMs are exposed determines their ability to take up apoptotic neutrophils at 48 h poststimulation (Fig. 3). Thus, an identical proportion of BMDMs incubated for 48 h with TNF alone take up apoptotic neutrophils in the same manner as do BMDMs incubated with TNF followed 4 h later by IFN, IL-4, or IL-10 (Fig. 3). Similar results were obtained with BMDMs that had been incubated with IFN- γ , IL-4, or IL-10 alone or in combination. In each instance, the first cytokine to which the BMDMs were exposed determined the *M* ϕ uptake of apoptotic neutrophils; this program was not modified by subsequent exposure to a cytokine that had the opposite effect on uncommitted *M* ϕ .

β -glucuronidase expression of BMDMs

The experiments to determine both the influence of cytokines on NO generation and the uptake of apoptotic neutrophils suggested that the first exposure to a cytokine rendered *M* ϕ unresponsive to subsequent differentiating signals. Therefore, a third set of experiments was performed to ascertain whether this finding was also the case for the TGF- β -induced expression of the lysosomal hydrolase β -glucuronidase. As reported for murine BMDMs (21), TGF- β induced a marked increase in β -glucuronidase expression compared with unstimulated controls, whereas treatment with IFN/TNF decreased expression. Incubation with IL-10 also reduced β -glucuronidase expression significantly, whereas neither TNF- α nor IL-4 had any effect (Table I).

The treatment of BMDMs with TGF- β followed 4 h later by IFN- γ alone or in combination with TNF resulted in β -glucuronidase expression that was similar to that observed in cells incubated with TGF- β alone. By contrast, TGF- β administered at 4 h after IFN resulted in a level of β -glucuronidase expression that was similar to that seen in cells stimulated with IFN- γ alone. *M* ϕ incubated with IL-4 for 4 h before IFN- γ had similar levels of β -glucuronidase expression compared with cells incubated with IL-4 alone, whereas BMDMs incubated with TNF/IFN- γ for 4 h before IL-4 had β -glucuronidase expression that was similar to that observed in cells incubated with IFN- γ (Fig. 4). These data strongly support the idea that β -glucuronidase expression by BMDMs is dependent upon the first cytokine stimulus used.

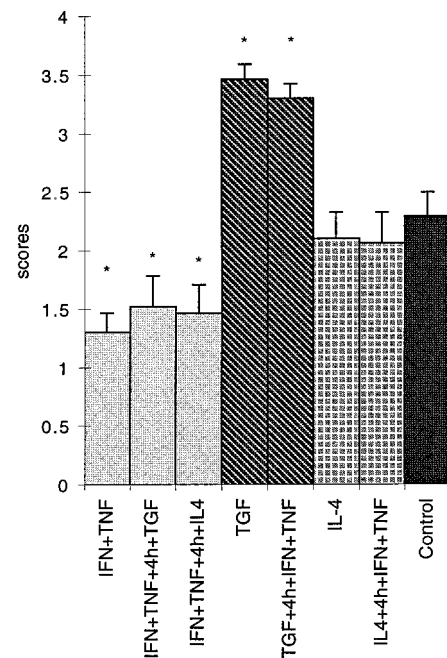


FIGURE 4. β -glucuronidase expression of BMDMs after exposure to cytokines. *M* ϕ were incubated for 48 h in medium alone (control), medium containing a single cytokine, or medium containing a single cytokine that was supplemented 4 h later by a second cytokine. Mean plus SE; $n = 8$. * $p < 0.05$ vs unstimulated controls.

Discussion

M ϕ infiltrating infected or otherwise damaged tissues develop coordinated, and to some extent mutually exclusive, sets of properties, but it is not known how this process is controlled by the local microenvironment. However, the process must be tightly regulated and can be accounted for by two broad models of *M* ϕ functional activation: either *M* ϕ properties could be molded by the sum of all the stimuli to which they are exposed, or else exposure to a specific stimulus (such as a cytokine) could commit the cell to be activated in a particular way and render it unresponsive to alternative activating signals. Here, we studied the responses of uncommitted BMDMs to various cytokines alone and in combination to distinguish between these models, and our results provide unequivocal support for the second model under the experimental conditions used.

M ϕ function was characterized using assays for three complex processes known to be differentially expressed in *M* ϕ . Cytotoxic *M* ϕ generate NO that is thought to be important for the killing of infected cells. NO generation by BMDMs requires priming with IFN- γ followed by activation with TNF or LPS (20) and is reportedly modulated by IL-4, IL-10, and TGF- β in *M* ϕ cell lines and elicited peritoneal *M* ϕ (28, 29, 31, 32). The uptake of apoptotic neutrophils is believed to have an important role in the resolution of inflammation and, as reported here, is up-regulated by TNF and decreased by IFN- γ , IL-4, and IL-10. β -glucuronidase is a lysosomal hydrolase that is strongly expressed and released in bacterial infections or when *M* ϕ ingest particulate matter such as group A streptococcal cell walls, zymosan particles, or β -1,3-glucan; it has been shown that TGF- β primes *M* ϕ to express lysosomal hydrolases in response to particulate stimuli (21). In our experiments, TGF- β markedly increased β -glucuronidase expression by BMDMs compared with the modest amounts found in unstimulated controls (33), and, as shown here, expression is decreased by IFN- γ alone and in combination with TNF. The effect of different

combinations of cytokines on these complex functions enabled us to ascertain whether the exposure of BMDMs to cytokines with opposing functions resulted in a polarized responses or an aggregated one.

In every case, the response of the BMDMs was determined by the first cytokine to which they were exposed. Thus, NO generation was observed only when BMDMs were first exposed to IFN- γ and could be prevented completely if the M ϕ were pretreated with IL-4, TNF, or TGF- β . Similarly, an increased uptake of apoptotic neutrophils was only seen in BMDMs treated first with TNF- α , and the response was not influenced by subsequent treatment with IFN- γ , IL-4, or IL-10, all of which decrease uptake when given alone. Conversely, M ϕ incubated with IFN- γ , IL-10, or IL-4 before exposure to TNF behaved like cells that had been stimulated with these cytokines alone. Preincubation with TGF- β before exposure to IFN/TNF resulted in β -glucuronidase expression that was similar to that observed in cells that were stimulated with TGF- β alone, and incubation with IFN/TNF before exposure to TGF- β resulted in expression that was similar to that observed in cells stimulated with IFN/TNF alone. These results show that the functional activation of uncommitted BMDMs is determined by the first cytokine to which the cells are exposed; once the activation process has started, M ϕ become temporarily unresponsive to alternative stimuli.

The results imply that BMDMs have a general mechanism that prevents them from responding to other, possibly opposing signals, once they are committed to develop a particular attribute. The mechanisms involve at least three signaling pathways: the Janus kinase (JAK)/STAT pathways used by IFN, IL-4, IL-6, and IL-10 (34); the extracellular signal-related kinase and stress-activated protein kinase/JUN families of mitogen-activated protein kinases that are responsible for TNF signaling (35); and the Smad family for TGF- β (36). This mechanism must be selective, in that not all activities of the particular cytokines are suppressed: for example TNF is required for the optimal generation of IFN-primed M ϕ , even though the prior exposure of M ϕ to TNF prevents priming. Similarly, IL-10 has no effect on IFN- γ priming for NO generation but prevents the effect of TNF on the uptake of apoptotic neutrophils. Lastly, the mechanism takes at least 2 h to develop according to our results and those of Riches et al. with regard to the effect of TNF on IFN priming (20) and also our observations and those of others with regard to IL-4 (29).

It is highly unlikely that inhibitory effects can be explained by changes in the surface expression of cytokine receptors, and this possibility was formally excluded in the case of IFN priming because IFNRs are up-regulated by TNF (37) and only modestly decreased by IL-4 and TGF- β (38). The direct inhibition of intracellular signaling pathways provides the most economical explanation, and the recent descriptions of cytokine-inducible families of inhibitors of both the JAK/STAT (reviewed in Ref. 39) and Smad (reviewed in Ref. 36) pathways provide potential mechanisms. The expression of JAK/STAT inhibitors, which include cytokine-inducible Src homology 2-containing protein and suppressor of cytokine signaling (SOCS)-1–3, are induced by a variety of cytokines, including IFN- γ , TNF, and IL-4; such inhibitors suppress signaling by inhibiting the catalytic activity of JAKs (40). The IFN- γ - and IL-6-induced differentiation of murine M1 cells into M ϕ is suppressed by the overexpression of SOCS-1 (41). TGF- β signals through the family of Smad proteins. Two inhibitory Smads (Smad6 and Smad7) have recently been described (42, 43), but less is known about what stimulates their expression. However, Kretzschmar et al. have recently shown that activation of the extracellular signal-related kinase pathway causes the phosphorylation of Smad4, which inhibits its activity by preventing

translocation to the nucleus (44). It is not known whether SOCS family members and inhibitory Smads affect the differentiation of BMDMs, but we are currently conducting experiments to ascertain whether this is the case.

Inhibitory effects could also be caused by the well-described transcriptional controls of cytokine genes and cytokine-activated genes based on positive and negative sequence-specific DNA-binding NFs (reviewed in Ref. 45). Competition for binding different STAT proteins (STAT-binding elements) could explain some of the effects of IL-4 (46). However, the effect would be immediate and could not explain why IL-4 takes 4 h to be fully effective. Clearly, more experiments and much greater knowledge will be needed to determine whether these or other possible explanations are responsible for our observations.

Cytokine effects on M ϕ activation are strongly influenced by the stimulation conditions (cytokine concentration, timing of cytokine addition), the source of M ϕ (bone marrow, peritoneal, monocyte-derived, alveolar), and the state of activation of the M ϕ , even before species differences are considered. Nevertheless, the demonstration of distinct and exclusive pathways for M ϕ maturation in vitro by Riches et al. (9) clearly provides a hypothesis to account for M ϕ heterogeneity and a potential explanation for their multiple postulated roles. This approach has the advantage of examining the effect of a variety of combinations of cytokines at different doses and exposure sequences, and we found that the sequence of cytokine exposure plays a major role in M ϕ differentiation. The situation in vivo is even more complex, because M ϕ would be subject to a wide variety of receptor-mediated events in addition to those that are dependent upon cytokine receptors. In vivo, M ϕ are exposed to chemokines and interact with adhesion molecules even before they are exposed to the cytokine environment in the inflamed tissue in which Ig (47) and complement receptors (48) may be ligated, as well as receptors for extracellular matrix, including integrins and CD44, and receptors such as CD40 that bind other cells. Consequently, we are now conducting studies to examine M ϕ differentiation under conditions that are much closer to the in vivo situation.

Finally, there are a number of implications to our observation that certain cytokines induce M ϕ to develop specific functions while simultaneously rendering the M ϕ temporarily unresponsive to later stimulation with cytokines with opposing effects. Such a mechanism might profoundly affect autoregulation by cytokines and may suggest that the primary effect of the down-regulation of cytokines is to program the next wave of M ϕ to develop a different function.

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