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Macrophages Sequentially Change Their Functional Phenotype in Response to Changes in Microenvironmental Influences

Robert D. Stout, Chuancang Jiang, Bharati Matta, Illya Tietzel, Stephanie K. Watkins, and Jill Suttles

Recent studies have described the development of distinct functional subsets of macrophages in association with cancer, autoimmune disease, and chronic infections. Based on the ability of Th1 vs Th2 cytokines to promote opposing activities in macrophages, it has been proposed that macrophages develop into either type 1 inflammatory or type 2 anti-inflammatory subsets. As an alternative to the concept of subset development, we propose that macrophages, in response to changes in their tissue environment, can reversibly and progressively change the pattern of functions that they express. As demonstrated herein, macrophages can reversibly shift their functional phenotype through a multitude of patterns in response to changes in cytokine environment. Macrophages display distinct functional patterns after treatment with IFN-γ, IL-12, IL-4, or IL-10 and additional functional patterns are displayed depending on whether the cytokine is present alone or with other cytokines and whether the cytokines are added before or concomitantly with the activating stimulus (LPS). Sequential treatment of macrophages with multiple cytokines results in a progression through multiple functional phenotypes. This ability to adapt to changing cytokine environments has significant in vivo relevance, as evidenced by the demonstration that macrophage functional phenotypes established in vivo in aged or tumor-bearing mice can be altered by changing their microenvironment. A concept of functional adaptivity is proposed that has important implications for therapeutic targeting of macrophages in chronic diseases that result in the dominance of particular functional phenotypes of macrophages that play a significant role in disease pathology. The Journal of Immunology, 2005, 175: 342–349.

Macrophages are remarkable for the diverse activities in which they engage (1, 2). Many of these activities appear to be opposing in nature: proinflammatory vs anti-inflammatory activities, immunogenic vs tolerogenic activities, and tissue destructive vs tissue restorative activities (1, 3, 4). It has been known for decades that cytokines can alter macrophage functional responses (1, 5, 6). The seminal research of Adams et al. (5, 6) demonstrated that the inflammatory and cytotoxic activities of macrophages were enhanced qualitatively and quantitatively in the presence of IFN-γ, thus establishing the classical two-signal paradigm of activation of macrophage inflammatory function. More recently, Stein et al. (7) reported that IL-4 treatment of macrophages induced a unique phenotype characterized by elevated expression of mannose receptor and a unique pattern of functions that were in sharp contrast to those induced by IFN-γ treatment of macrophages (4). Additional functional phenotypes subsequently were reported to be generated by treatment of macrophages with a variety of agents, including the type 2 cytokines IL-10 and TGFβ (8–11), Although there appeared to be distinctions between the functional phenotypes induced by IL-4, IL-10, and TGFβ (4, 10, 11), several researchers hypothesized that macrophages develop into two major functional subsets that display inflammatory vs anti-inflammatory patterns of function in association with Th1 vs Th2 driven responses, respectively (12–14).

Although macrophages that accumulate in chronic pathologies either display predominantly inflammatory/cytotoxic activities (15–18) or display predominantly anti-inflammatory activities (11, 14, 18, 19), it is not clear that only two functional patterns exist. To the contrary, evidence is accumulating that suggests that macrophages may display several unique functional patterns (8–10, 20). More than one functional subset of macrophages participates in acute inflammatory responses, the early phase of which is dominated by inflammatory and/or cytotoxic activities and the terminal phase of which is dominated by anti-inflammatory/tissue regenerative activities (21–23). How this transition from macrophage inflammatory to anti-inflammatory activities is orchestrated is not known. If the development of functional subsets of macrophages is stable (e.g., involves differentiation), the transition of the innate response from inflammatory/cytotoxic activities to wound resolution would require removal, by apoptosis or emigration, of the inflammatory macrophage subset. Conversely, if the development of functional subsets of macrophages involves differential regulation of signaling cascades (reversible adaptation), the macrophages displaying inflammatory/cytotoxic activities could be induced to change their functional pattern to anti-inflammatory/tissue regenerative activities. To address these issues, we set out to determine (1) whether treatment with IFN-γ, IL-12 (type 1 cytokines), IL-4, or IL-10 (type 2 cytokines) would promote a pattern of functions that was unique for each cytokine rather than promoting qualitatively similar inflammatory vs anti-inflammatory patterns for the type 1 vs type 2 cytokines, respectively, (2) if the cytokine-treated macrophages could continue to adapt such that sequential changes...
FIGURE 1. Modification of cytokine responses of BMMφ by pretreatment with type 1 or type 2 cytokines. BMMφ were cultured for 24 h with the indicated cytokine or ligand, washed thoroughly, and cultured an additional 24 h with LPS. Cytokine production was assayed by ELISA. Mean of triplicate cultures (BMMφ) ± SD are displayed.

in the cytokine environment would progressively alter the pattern of functional capabilities displayed by the macrophages upon activation, and (3) if the anti-inflammatory macrophage subsets generated during chronic pathologies such as cancer or advanced age could be redirected to an inflammatory phenotype.

Materials and Methods

Animals

B6D2F1 and C57BL/6J female mice were obtained from The Jackson Laboratory. CB6F1/nia female mice at 18–24 mo of age were obtained through the National Institute for Aging. All animal protocols received prior approval of the Institutional Animal Care and Use Committee, and all experiments were performed in accordance with relevant guidelines and regulations.

Generation and activation of bone marrow-derived macrophages (BMMφ)

Bone marrow cells were harvested from femur and tibia of 6–10-wk-old B6D2F1/J mice (The Jackson Laboratory). The cells were cultured in RPMI 1640 (HyClone) supplemented with 5% FBS (Atlanta Biologicals), recombinant mouse M-CSF (10 ng/ml; R&D Systems) and L929 conditioned medium (15%) in Nunc cell culture plates. Non-adherent cells were collected after 24 h and were cultured for 7 days in the supplemented medium in Corning/Costar ultralow attachment polystyrene culture plates, changing the medium once on day 4. On day 7, the live cells were purified by centrifugation over Fico/Lite-LM (Atlanta Biologicals). The resulting cell population was >98% CD11b-positive. The cells were resuspended in RPMI 1640 with 5% FBS, dispensed to standard 24- or 48-well cell culture plates with the indicated cytokines before activation by 24 h culture with 0.1 μg/ml LPS (Escherichia coli O111B). For cytokine treatment, the cells were cultured 24 h with IFN-γ (20 U/ml), IL-12 (5 ng/ml), IL-4 (20 ng/ml), or IL-10 (20 ng/ml) (all from R&D Systems). For FcγR ligation studies, the cells were treated with 1 μg anti-FcγRII/III (hybridoma clone 2.4G2) for 30 min on ice, washed and cultured for 4 h before stimulation with LPS. For LPS tolerance, the cells were cultured with 20 ng/ml LPS, washed, and stimulated with 1 μg/ml LPS for 24 h.

Purification of peritoneal macrophages

Peritoneal cells were obtained by peritoneal lavage of untreated mice (resident cells) or of mice injected i.p. 4 days before with 2 ml of thioglycollate broth (elicited cells) (B-D). The macrophages were enriched using MS columns on MinIMACS Separator (Miltenyi Biotec) by depletion with anti-CD19 and anti-CD5 microbeads (Miltenyi Biotec) per the manufacturer’s instructions. The resulting cell populations were >95% CD5+, CD19+, and CD11b+. To maximize purity of macrophage populations in studies comparing young and aged mice or control and tumor-bearing mice, the populations enriched by negative selection were further purified by positive selection with anti-CD11b microbeads, resulting in populations that were >98% CD5+, CD19+, and CD11b+. Peritoneal macrophages were activated as described for BMMφ.

Antibodies, Western blot, and cytokine assays

Western blots were performed on lysates of activated BMMφ using Abs reactive with inducible NO synthase (iNOS),3 arginine, Cox-2 (BD Biosciences Transduction Laboratories) or β-actin (Sigma-Aldrich). The cells were lysed in lysis buffer (25 mM Tris-HCl, 1% Triton X-100, 1% deoxycholate, 500 mM NaCl, and 10 mM EDTA) with a protease inhibitor mixture (Calbiochem). Proteins were quantitated and equal amounts of protein were separated in Criterion pre-cast gels (10% Tris-HCl). Proteins were transferred to nitrocellulose membranes using a Trans-Blot SD SemiDry electrophoretic transfer cell (Bio-Rad). Antibody-bound proteins were detected using an ECL (Amersham Biosciences), and the membranes were exposed to Kodak Biomax XL x-ray film. IL-4, IL-6, TNF-α, IL-12p70, MCP1, IL-1β ELISA were performed on the culture supernatants using kits from BD Pharmingen and R&D Systems. Alternatively, cytokine production was quantitated by the cytometric bead array (CBA) for inflammatory cytokines (BD Pharmingen) per the manufacturer’s instructions using a FACS Calibur flow cytometer (BD Pharmingen).

Tumor-bearing mice

Lewis Lung Carcinoma (3 LLC) was generously provided by Professor Gordon Ross (James Brown Cancer Center, University of Louisville). 3 LLC cells (104) were injected subcutaneously into 6–10-wk-old C57/B6J mice (The Jackson Laboratory). Macrophages were harvested from the mice when the tumor mass approached 1 cm3 (14–16 days).

Results

Different ligands differentially bias BMMφ to display distinct functional patterns upon LPS stimulation

To determine whether treatment of macrophages with different type 1 (IFN-γ, IL-12) or type 2 (IL-4, IL-10) cytokines would promote macrophage production of inflammatory vs anti-inflammatory cytokines, BMMφ were cultured for 24 h in one of the cytokines, thoroughly washed, and stimulated with LPS for 18–24 h. Analysis of the culture supernatant by ELISA revealed that both IL-4 and IFN-γ treatment enhanced the production of the inflammatory cytokines TNF-α and IL-12 and inhibited the production of IL-10 (Fig. 1). However, these two patterns were distinct in that IFN-γ inhibited MCP-1 production whereas IL-4 enhanced MCP-1 production. IL-10 treatment resulted in a more anti-inflammatory phenotype insofar as IL-12 production was profoundly inhibited whereas IL-10 production was enhanced. This increase in IL-10 was not due to “carry-over” of rIL-10 as evidenced by the absence of detectable IL-10 in supernatant fluid from macrophage cultures treated with IL-10 but not activated by LPS (data not shown). To focus on, and more easily visualize, the

3 Abbreviations used in this paper: iNOS, inducible NO synthase; Mφ, macrophage; BMMφ, bone marrow-derived macrophages; CBA, cytokine bead array.
patterns of LPS-induced cytokine production resulting from treatment of BMMφ with type 1 or type 2 cytokines, the cytokine response was normalized to that obtained by LPS stimulation of BMMφ without cytokine treatment. Using this format to view which cytokines were enhanced, unaffected, or reduced by a particular treatment protocol, it becomes apparent that treatment of BMMφ with IFN-γ, IL-12, IL-4, and IL-10 each induced a unique functional phenotype (Fig. 2). FcγR ligation (24) and low dose LPS pretreatment (25–27) have been reported to result in unique patterns of cytokine production upon subsequent stimulation with LPS. To determine whether these patterns of cytokine production were similar to those promoted by the four cytokines presented above, the pattern of LPS-induced cytokine production was determined after treatment of BMMφ with either anti-FcγRII/III or a low dose (20 ng/ml) of LPS. The response pattern to LPS stimulation resulting after FcγR ligation, characterized by reduced TNF-α and IL-12 production and enhanced IL-1β and IL-10 production, and the response pattern to LPS stimulation resulting after LPS pretreatment, characterized by reduced TNF-α, IL-12, and IL-6 production and elevated IL-1β, MCP-1, and IL-10 production, were uniquely distinct from each other and from each of the patterns promoted by the four cytokines reported above (Figs. 1 and 2). Overall, this panel of six ligands biased the macrophages to display one of six different response patterns upon subsequent stimulation with LPS. It should be noted that different macrophage populations (peritoneal vs bone marrow) do not respond identically to LPS (21) and that the same macrophage population from different strains of mice have been reported not to respond identically to LPS (9). Thus, the emphasis here is that six different treatments of B6D2F1/J BMMφ yielded six different patterns of response to LPS.

Another feature proposed for the type1/type 2 macrophage model was that iNOS and arginase expression were counter-regulated such that the ratio of iNOS to arginase should be profoundly elevated in type 1 macrophages and reduced in type 2 macrophages (4, 8, 12, 28). In accord with this hypothesis, LPS stimulation of IFN-γ-treated BMMφ enhanced iNOS and COX-2 expression without inducing detectable arginase expression (Fig. 3). However, LPS stimulation of IL-4-treated BMMφ enhanced iNOS and COX-2 and also induced arginase expression (Fig. 3), thus establishing that all three enzymes could be simultaneously expressed upon treatment with LPS and a type 2 cytokine. Treatment of BMMφ with IL-10 in combination with IL-4-abrogated LPS induction of both iNOS and COX-2 but further amplified expression of arginase (Fig. 3). The impact of IL-4 on BMMφ function thus was influenced by the presence of other cytokines during the treatment period.

The impact of IL-4 on macrophage function has been reported to depend also on whether the treatment occurs before, or concomitantly with, the activating stimulus (LPS) (29). To determine whether this reported temporal impact applied to both type 1 and type 2 cytokines, BMMφ were stimulated with LPS either in the presence of or after a 24-h culture with IFN-γ, IL-4, or IL-10. The impact of IL-4 and IL-10, and to a lesser degree IFN-γ, was dramatically different when the cytokine was added concomitantly with the activating stimulus rather than 24 h before the activating stimulus (Fig. 4). Treatment with IL-4 before LPS stimulation enhanced production of all cytokines except IL-1β and IL-10, which it inhibited. In contrast, costimulation with IL-4 and LPS inhibited production of all cytokines except MCP-1 and IL-10, which it enhanced (Fig. 4). Treatment with IL-10 before LPS inhibited IL-12 and MCP-1 but only moderately inhibited or had no effect on production of the remaining cytokines. In contrast, when present during LPS stimulation, IL-10 profoundly inhibited production of all cytokines tested.

The pattern of macrophage functions continues to shift as the cytokine environment changes from one cytokine to another.

To determine whether the pattern of functional capabilities established by treatment with a cytokine displayed stability indicative of an established subset of macrophages, BMMφ were treated for 24 h with IFN-γ, IL-10, or IL-4, washed free of the cytokine, and cultured for 24 h with an alternate cytokine before washing and stimulation with LPS. IFN-γ-treated BMMφ subsequently cultured in IL-10 before LPS stimulation displayed a pattern of cytokine production clearly distinct from BMMφ stimulated with LPS directly after IFN-γ treatment (Fig. 5). Treating IFN-γ-primed Mφ with IL-10 resulted in a dramatic decrease in IL-12 and TNF-α production and an equally dramatic increase in IL-10 production. Similar results were obtained when IL-10-treated Mφ were cultured with IFN-γ before LPS stimulation or IL-4-treated Mφ were cultured with IFN-γ before LPS stimulation (Fig. 5). In each case, changing the cytokine environment altered the functional pattern established by the first cytokine treatment. In some instances, the functional pattern observed after sequential cytokine treatment was similar, though not identical, to that obtained by treatment only with the second cytokine, as observed with IFN-γ→IL-10 group. In other instances, the functional pattern observed after sequential cytokine treatment was unique, distinct from that observed after treatment with either cytokine alone, as

**FIGURE 2.** Six different cytokine response patterns induced in macrophages. BMMφ were cultured for 24 h with the indicated cytokine or ligand, washed thoroughly, and cultured an additional 24 h with LPS. Cytokine production was assayed by ELISA. The pattern of cytokine response is displayed as the ratio of the response of treated cells to that of untreated cells. The numerical ratio (fold increase or decrease) is displayed above the bars. The asterisk indicates significance of p < 0.05. The experiment is representative of three to five trials.
observed with IL-4→IFN-γ treatment (Fig. 5). The transient nature of the effect of cytokine treatment was also revealed by delaying addition of LPS for 24 h after removal of the cytokine. Such an exposure of the Mφ to an in vitro environment “free” of exogenous cytokine muted the functional changes that were observed upon activation immediately following cytokine treatment, as observed the most dramatically in the IL-10-treated group (Fig. 5).

The results reported above were obtained with BMMφ that have been considered to be relatively immature. This raised the possibility that the observed ability to adapt to changing environments was a trait of a relatively immature macrophage population and would not be observed in mature macrophage populations. To determine whether a similar multistep sequential shift in functional phenotype could be induced in mature macrophages treated ex vivo, thioglycollate-elicited peritoneal macrophages were purified and activated with LPS either immediately ex vivo or after a sequential 24-h culture with IFN-γ (Fig. 6a), IFN-γ followed by IL-10 (Fig. 6b), IFN-γ followed by IL-10 followed by IL-4 (Fig. 6c), or IFN-γ followed by IL-10 followed by IL-12 (Fig. 6d). The macrophages were washed after each cytokine treatment. Compared with peritoneal macrophages activated with LPS without cytokine treatment, treatment with IFN-γ enhanced production of IL-12, TNF-α, and IFN-γ while reducing production of MCP-1 and IL-10 (Fig. 6a). Subsequent treatment with IL-10 altered this response, tempering production of IL-12, TNF-α and IFN-γ and restoring production of MCP-1 and IL-10 (Fig. 6b). Treatment with IL-4 following the sequential treatment with IFN-γ and IL-10 induced a third sequential shift in functional phenotype, yielding a pattern of cytokine production that was distinct from the first two patterns (Fig. 6c). Production of IL-12, TNF-α, IFN-γ, and IL-6 were elevated and production of IL-10 was reduced. Treatment with IL-12 after sequential treatment with IFN-γ and IL-10 resulted in a fourth pattern of cytokine production that bore some
similarities to the pattern originally imprinted by IFN-γ treatment (Fig. 6d). In summary, peritoneal macrophages, like BMMφ, changed their functional phenotype multiple times in response to changes in the cytokine environment.

MN from immunosenescent or tumor-bearing mice retain their ability to functionally adapt to changes in their cytokine environment

The above studies addressed the stability of macrophage functional phenotypes generated by 24-h cytokine treatment. To examine the functional adaptivity of macrophages whose phenotype had been established in vivo for a prolonged period of time, we investigated two pathological states known to dramatically alter macrophage function: cancer and immunosenescence. Animals bearing metastatic tumors have been reported to develop a unique subset of macrophages that accumulate in the tumor mass as well as in distal sites such as the spleen and peritoneal cavity and that suppress inflammatory and immune responses (14, 30, 31). To determine whether these suppressive macrophages represented a stable subset of macrophages that accumulate in the tumor mass as well as in distal sites such as the spleen and peritoneal cavity and that suppress inflammatory and immune responses (14, 30, 31). To determine whether these suppressive macrophages represented a stable subset of macrophages, resident peritoneal cells were removed from mice bearing s.c. 3LL tumor and the macrophages purified by magnetic bead selection. Compared with resident peritoneal cells from normal mice, the macrophages from tumor-bearing mice displayed reduced production of TNF-α, IFN-γ, and IL-6 and enhanced production of MCP-1 and IL-10 upon LPS stimulation (Fig. 7a). Treatment of the macrophages for 24 h with IFN-γ before stimulation with LPS resulted in a shift in the pattern of cytokine production that was similar in Mφ from both normal and tumor-bearing mice (Fig. 7b). Production of TNF-α and IFN-γ was increased and production of MCP-1 and IL-10 was decreased by the IFN-γ treatment.

Macrophages from aged mice (>20 mo) have been reported by many investigators to display reduced inflammatory activity in response to stimuli (32). To determine whether this functional pattern is stable or reversible, thioglycollate-elicited peritoneal macrophages were removed and purified from young (8–12 wk) and aged (>20 mo) CB6F1/nia mice. LPS stimulation of the macrophages from aged mice resulted in 2- to 5-fold lower production of TNF-α, IFN-γ, IL-12, and MCP-1 compared with macrophages from young mice (Fig. 7c). Treatment of the macrophages from aged mice for 24 h with IFN-γ before LPS stimulation in vitro...
enhanced production of TNF-α and IL-12 and reduced production of IFN-γ and MCP-1. The IFN-γ-treated macrophages from aged and young mice displayed nearly identical patterns of cytokine production (Fig. 7d), indicating that both populations of macrophages retained the ability to adapt to a different cytokine environment.

Discussion

It is clear from this and previous studies (4–6, 8, 9, 12, 21, 30) that macrophages display different patterns of function under the influence of different microenvironments. In the current study, macrophages were exposed to a panel of only six agonists and analyzed for production of six cytokines and three enzymes. Nonetheless, the study revealed many different patterns of expression of those proteins. The patterns did not display a strict dichotomy between type 1 and type 2 responses. For example, treatment of macrophages with the type 2 cytokine, IL-4, before LPS stimulation strongly enhanced inflammatory activity (TNF-α production) or after 24 h of culture with IFN-γ (b). The cytokine response was assayed by CBA assay. The ratio of untreated or treated macrophages from tumor-bearing mice to untreated macrophages from normal controls is displayed, as described in the legend for Fig. 2. An experiment representative of four trials is displayed. The elicited peritoneal macrophages of aged or young CB6F1 mice were activated with LPS either immediately ex vivo (c) or after 24 h of culture with IFN-γ (d). The cytokine response was assayed by CBA assay. The ratio of untreated or IFN-γ-treated macrophages from aged mice to untreated macrophages from young mice is displayed, as described in the legend for Fig. 2. The asterisk indicates significance of \( p < 0.05 \). An experiment representative of three trials is displayed.

that treatment of macrophages with different cytokines can differentially alter their functional potential (8, 10, 11). However, none of these studies has addressed whether these macrophages, once functionally polarized, retain the ability to continue to adapt to changes in their environment by progressively changing their functional phenotype. This is an important issue. The reversibility of macrophage functional phenotype would have significant therapeutic value in chronic diseases such as cancer, autoimmune inflammatory disease, or infection with mycobacteria, schistosomes, or toxoplasma, which result in the dominance of particular functional phenotypes of macrophages that play a significant role in disease pathogenesis (11, 14–19). The current data indicate that the functional phenotype of these macrophages can be changed and that macrophages may progress from one functional phenotype to another as the microenvironmental influences change. Therefore, as an alternative to the concept of stable macrophage subsets, we propose a model of functional adaptivity as a basis for understanding macrophage function in physiologically dynamic responses. The model of functional adaptivity predicts not only that macrophages adapt to microenvironmental signals via differential regulation of function, but also that macrophages can progressively change their functional phenotype in response to progressive changes in the environmental signals.

The concept of functional adaptivity provides an alternative perspective on macrophage biology and macrophage function in inflammation and disease. For example, the inflammatory response involves dramatic changes in tissue environment, changes which progress through proinflammatory, destructive, anti-inflammatory and reparative stages. Whether inflammatory macrophages apoptose or emigrate from the site of inflammation when the inflammatory signals subside (38) is still debated. An alternative, though not necessarily exclusive, possibility is that, in response to the changing environment of the successfully debrided wound, the functional phenotype of the macrophages changes to allow participation in wound resolution. Thus the TGFβ-producing anti-inflammatory macrophages in remitting lesions of animal models of multiple sclerosis may have been derived from the TNF-α/NO-producing inflammatory macrophages that populated the active lesions during acute disease (39, 40). There are several reports of...
macrophages or macrophage-derived cells changing their functional phenotype in response to environmental changes. Microglia are monocyte-derived sessile cells in the CNS that display neuroprotective activities such as production of TGFβ and brain-derived neurotrophic factor (41–43). However, inflammatory episodes in the brain cause the microglia to become highly motile “macrophage-like” cells displaying proinflammatory and neurotoxic activities (41–43). Myeloid dendritic cells have been reported to revert to macrophage characteristics (44–46) or, under the influence of Trance, to display the functional and phenotypic characteristics of multinucleated osteoclasts (47). Thus it appears that tissue macrophages and macrophage-derived cells retain their ability to adapt to changes in their environment by altering the pattern of functions they express.

In chronic disease states, where tumors continuously produce immunomodulatory cytokines (30, 31) or a subset of T cells are responsive at least transiently to cytokine therapy (21).

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