Kinetics of the Phenotype and Function of Murine Peritoneal Macrophages Following Acute Inflammation

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This study was undertaken to have a better understand for the process and the underlying mechanisms to limit macrophage activation and population of activated macrophages. A comprehensive kinetics of cytokine production was performed in murine peritoneal macrophages recovered from Balb/c mice at various time during the course of an intraperitoneal injection with thioglycollate (TG). The expression of cell surface molecules such as MHC-I, MHC-II, B7-1 and B7-2 of these macrophages were also determined by flow cytometry. The present findings of our research suggested that the population of activated macrophages and the activation of macrophages (including cytokines production and expression of cell surface functional molecules) were strictly controlled during inflammation process. This is one of the important mechanisms to retain the host homeostasis. *Cellular & Molecular Immunology*. 2004;1(1):57-62.

Key Words: macrophage, cytokine, phenotype, self-regulation

Introduction

The cells of the monocyte/macrophage lineage play a central role in the induction and regulation of both innate and adaptive immune responses. Monocytes are the blood-brone precursors of macrophages. In the event of infection, tissue damage or other injury, large numbers of monocytes are recruited from the bloodstream into the site of the insult and differentiate into the appropriate macrophage phenotype (1). The primary role of macrophages in immunity is working as a professional phagocyte. Phagocytosis by macrophages can be through either natural (e.g. *via* mannose receptor and scavenger receptor) or acquired (*via* Fc receptor and complement receptor) immune mechanisms (2).

Macrophages are versatile immunocytes. In addition to roles in phagocytosis, they also take part in inflammatory and other immune processes. The participation of macrophages in these actions requires production of a variety of cytokines and the cell-surface expression of a number of glycoproteins (3, 4). Cytokines play a key role in regulating the immune response in infected hosts. The resistance of mice to infection with *T. cruzi* has been associated with the production of IL-12, which triggers the production of IFN-γ by nature killer (NK) and T cells. IFN-γ produced in turn activates macrophages associated with resistance to infection (5). Proinflammatory acute-phase cytokines such as TNF-α and IL-6 are important in many of the physiological effects accompanied infection with a lot of intracellular bacteria. And a successful immune defense response to such infection may depend on the cooperation of macrophages and cytokines induced for activation of other cells of the immune system, including T cells (6). Expression of molecules encoded by the class I and II region of the major histocompatibility complex (MHC class I and II) on macrophages is essential for antigen presentation to T lymphocytes and T cell activation (7). This interaction is required for normal immune function. Optimal T cell activation involves the cognate interaction of the T cell receptor (TCR) with the MHC complex as well as a second, costimulatory signal. The best characterized costimulatory pathway involves the interaction of B7-1 (CD80) or B7-2 (CD86) on the surface of APCs and the T lymphocyte molecule CD28 (8). The costimulated signal also plays a pivotal role in the prevention of anergy and activation of T lymphocytes (9).

Although activated macrophages are crucial for host defense, they are also dangerous cells because of their production of free radicals and lytic enzymes. These products cause extensive local damage, and are responsible for many of the systemic symptoms associated with acute

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Abbreviations: LPS, lipopolysaccharide; IFN-γ, interferon gamma; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; TNF, tumor necrosis factor; FACS, fluorescent-activated cell sorter; TG, thioglycollate; APCs, antigen presenting cells; NK, natural killer.

Received for publication Nov 21, 2003. Accepted for publication Jan 6, 2004.

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and chronic inflammation (10). If uncontrolled, macrophage activation leads to the clinical syndrome of septic shock and ultimately to the death of the host (11, 12). Furthermore, it has been reported that macrophages function as "natural suppressor" cells that down-regulate lymphocyte-dependent immune events both *in vivo* and *in vitro*, and that activated macrophages appear to be more suppressive than their resident or nonactivated counterparts (13, 14). Therefore, activated macrophages must be subject to strict regulatory control. Unlike lymphocytes, which have an elaborate regulatory network to ensure the dearth of unwanted cells, and granulocytes, which spontaneously undergo apoptosis within hours after entering the tissues, for macrophages little is known of the process and underlying mechanisms to limit their activation and population.

The murine peritoneal cavity is often used as a suitable site to follow the appearance of macrophage-lineage cells during inflammatory (15). In this study, experimental inflammation was induced by injecting a sterile chemical irritant, thioglycollate (TG), into the peritoneal cavities of mice. The injection induced an acute local inflammatory response, accompanied by local emigration of phagocytic cells. In order to observe the changes of macrophages in the whole process, TG was injected on various days to onset the inflammation and peritoneal cells were collected on the same day. We first detected the time-related changes of cytokines produced by the adherent peritoneal cells at various days. Second, time-dependent changes in MHC and B7 molecules expression level of the peritoneal macrophages were studied by flow cytometry.

Materials and Methods

Animals

Inbred female Balb/c mice, 6-8 weeks, were provided by Shanghai Experimental Animal Center of Chinese Academy of Sciences. The animals were housed in pathogen-free conditions with room temperature of 24±2°C, 12h light/dark cycle, and sterile food and water was given ad libitum.

Peritoneal cells

Peritoneal exudate cells were induced in different groups of mice (3-4 mice/group) by an intraperitoneal injection of 0.5 ml of 3% TG (WAKO, Tokyo, Japan) on day 0, 2, 4, 6, 8, 10, 12, 14. On day 16, the resident peritoneal cells or peritoneal exudate cells were washed from the peritoneal cavity of mice by lavage with 10ml of ice-cold, sterile phosphate-buffered saline (PBS) containing 2%FBS. Cells were washed with PBS (containing 2%FBS) and resuspended in RPMI1640 (GIBCO, NY, USA) containing 10%FBS supplemented with penicillin 100U/ml and streptomycin 100μg/ml. The cell viability and concentration were determined by trypan blue exclusion.

Cytokine production and detection

Resident peritoneal cells and murine peritoneal exudate cells were plated in 100mm dishes (Corning, New Jersey, USA) at a density of 2×10^6 cells/ml in RPMI1640 supplemented with 10%FBS. Cells were diluted enough to avoid overlapping of cells in layers during the adherent incubation for 2h at 37°C, 5%CO_2. Non-adherent cells were then discarded, and the adherent cells were collected and culture for another 24h in 24 well plate in 2×10^6/2ml/well with 25U/ml IFN-γ and 1μg/ml LPS (Sigma). At the end of the culture, supernatants were harvested and stored at -20°C. Murine IL-12p70, IFN-γ, IL-10, TNF-α, IL-6 productions were determined by ELISA, using PharMingen capture and detection Abs, as previously described (13).

Analysis of flow cytometry

2×10^5 cells/sample were washed once in ice-cold staining buffer (PBS containing 0.1% (w/v) sodium azide, 1%FBS, pH7.2). Then cells were preincubated with 10μl 5% normal Balb/c mouse serum at 4°C for 30 min to block FcR. The cells were then stained in the dark at 4 °C for 30 min with fluorochrome-labeled anti-mouse mAb (PharMingen), specific for a cell surface antigen such as F4/80, H-$2K^d$ (MHC class I), I-A^d (MHC class II), CD80 (B7-1) and CD86 (B7-2). Cells were subsequently washed twice, resuspended in staining buffer and analyzed by flow cytometry using FACSCalibur (Becton Dickinson, San Jose, CA) and data were illustrated by CELLQuest.

Results

The kinetics of cytokine production from TG-induced peritoneal macrophages

In this study, we investigated the cytokines production from mice resident peritoneal cells and peritoneal exudate macrophages at various days after intraperitoneal injection of TG. IFN-γ and LPS have been identified as the major mediators of the classical macrophage activation pathway (14). In our study, cytokines produced by IFN-γ and LPS activated murine peritoneal macrophages were assessed. As shown in Figure 1, resident peritoneal macrophages only produced high levels of IL-10 and IL-6. After TG injection, IL-12 and TNF-α production were increased rapidly and maximally released on the 2, 4 day of the inflammatory response, respectively. The production of IL-12 and TNF-α were decreased significantly after the maximum and returned to the resident level on the 14-16 day after TG injection. IL-10 production not changed distinctly in the process of the TG induced acute local inflammatory response. This caused that the time course of IL-12/IL-10 was almost the same with that of IL-12. The production of IL-6 was high in resident macrophages and persistently reduced during the inflammatory response.

The changes of important molecular expression on TG-induced peritoneal macrophages

The ability of macrophages to present antigen is crucial to immune function (16). The expression of surface markers such as MHC and B7 is known to be very important to the antigen presenting function of macrophages. Because F4/80 was previously shown to be a specific marker of macrophage (17), in this study we gated $F4/80^+$ macrophages from the peritoneal cells collected at various days after intraperitoneal injection of TG and determined the MHC and B7 molecules expression on these cells. As shown in Figure 2, the percentage of $F4/80^+$ cells was increased remarkably after TG injection. This percentage reached the apex in 4-6 days and then decreased to the resident level on day 12-14, while the level of F4/80 expression was much higher on the resident peritoneal cells than TG induced

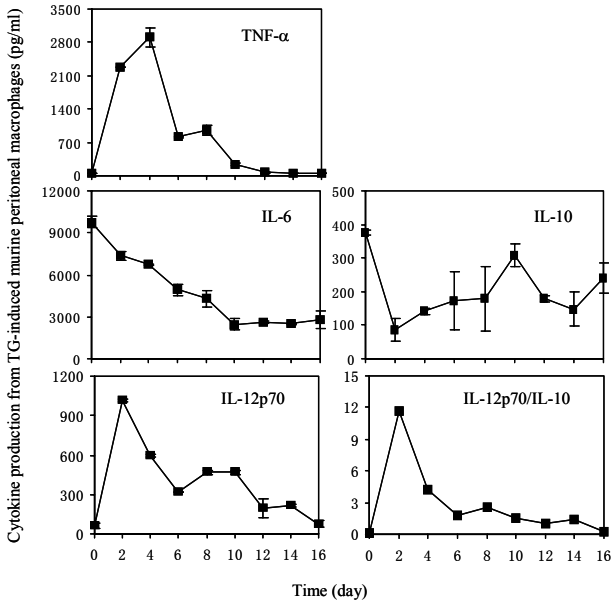

Figure 1. Cytokines production from peritoneal macrophages at various days after an intraperitoneal injection of TG. Murine adherent peritoneal macrophages (2.0×10^6 cells/well) from female Balb/c mice were incubated with LPS (1μg/ml) and IFN-γ (25U/ml) for 24h. Cell-free supernatant was collected and frozen at -20 °C. The productions of TNF-α, IL-6, IL-10 and IL-12p70 were determined by ELISA. Data were expressed as means±SD. When error bars are not presented, the errors are smaller than the size of the symbols. Two experiments were performed and the results shown are from one representative experiment.

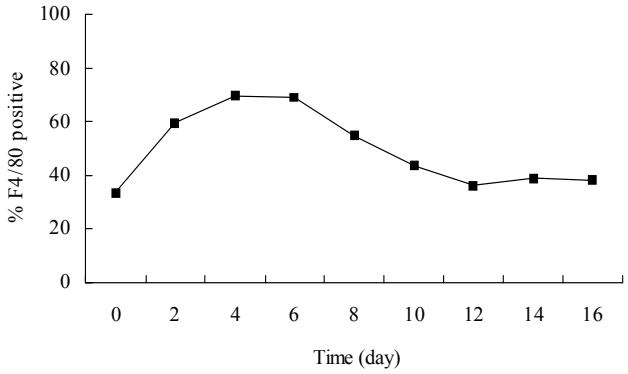

Figure 2. Kinetics of appearance of $F4/80^+$ peritoneal cells. Whole peritoneal cells at various time after TG injection were incubated with anti-F4/80-FITC antibody at 4°C for 30 min. After the incubation, peritoneal cells were monitored for F4/80 expression by flow cytometry. Data were expressed as a percentage of the number of cells of F4/80 positive. Two experiments were performed and the results shown are from one representative experiment.

peritoneal cells (Figure 3). F4/80 expression did not come back to the residential level within 16 days and would reach residential high level about on day 30 (data not shown). The level of $H\text{-}2K^d$ (MHC class I) expression rose modestly and did not change significantly in the inchoative of TG injection, but increased rapidly on day 10 and reached maximum on day 12. Then the expression decreased speedily and reached to the residential level on day 16. The expression of $I\text{-}A^d$ (MHC class II) increased continuously in 2-12 days after TG injection. Like MHC class I, it also reached the peak on day 12. The expression of CD80 (B7-1) and CD86 (B7-2) did not change as much as that of the MHC molecules. They both enhanced after TG injection and got to a high level on day 6. The maximum came on day 14 and then declined on day 16 (Figure 3).

Discussion

Macrophages in various tissues during the steady state and the sites of inflammation are heterogeneous with respect to phenotype as well as function (18). The murine peritoneum is often used as a convenient sterile site to explore the characteristics and the involvement of macrophage-lineage cells in the development of an inflammatory reaction. During the steady state, resident peritoneal macrophages can be derived locally in the peritoneum (19, 20). Soon after injection of an irritant, such as TG, resident peritoneal macrophages disappear by adhering to the peritoneal lining wall, with a concomitant influx of neutrophils and a subsequent appearance of macrophages (21).

In the process of clearing TG, both innate phagocytic defending mechanisms and specific lymphocyte-mediated responses are involved. Cytokines play a critical role in determining the effector functions of both phagocytic cells and lymphocytes (22). Analysis of inflammatory cytokines, which are usually induced early during an *in vivo* infection, shows that there is a significant expression of TNF-α mRNA in murine peritoneal cells only at 24h while persists at a high level until 36h (22). We confirmed these observations in our study. The production of TNF-α determined by ELISA increased markedly on day 2 and had kept a high level until day 4. TNF-α is a pleotrophic cytokine which is involved in the modification of both inflammation and immune reactions and plays a critical role in normal host resistance (23). However over-production of TNF-α may also do harm to the body. It was originally described as the primary mediator of endotoxin shock and cachexia. So TNF-α production must be subject to strict regulatory control. Our results also proved this control by observing the rapid decrease of TNF-α production after the apex. As for IL-12, its production also rose quickly to a high level and the IL-12/IL-10 reached the peak value on day 2. This selected cytokine pattern could provide appropriate conditions for the development of a Th1 response. It was known that increasing of Th1 cytokine production is correlated with enhancement of T lymphocyte proliferation in adaptive immunity and LAK and NK cell differentiation in innate immunity (24), resulting cell-mediated immunity. The over-production of Th1 cytokines will destroy the balance of Th1/Th2. Therefore the production of IL-12, a Th1 cytokine, must also undergo the same control as TNF-α. The production of IL-10 and IL-6 were low in the climax of the inflammation response when TNF-α release was high. IL-6 and IL-10 have been shown to have anti-inflammatory activity and to protect

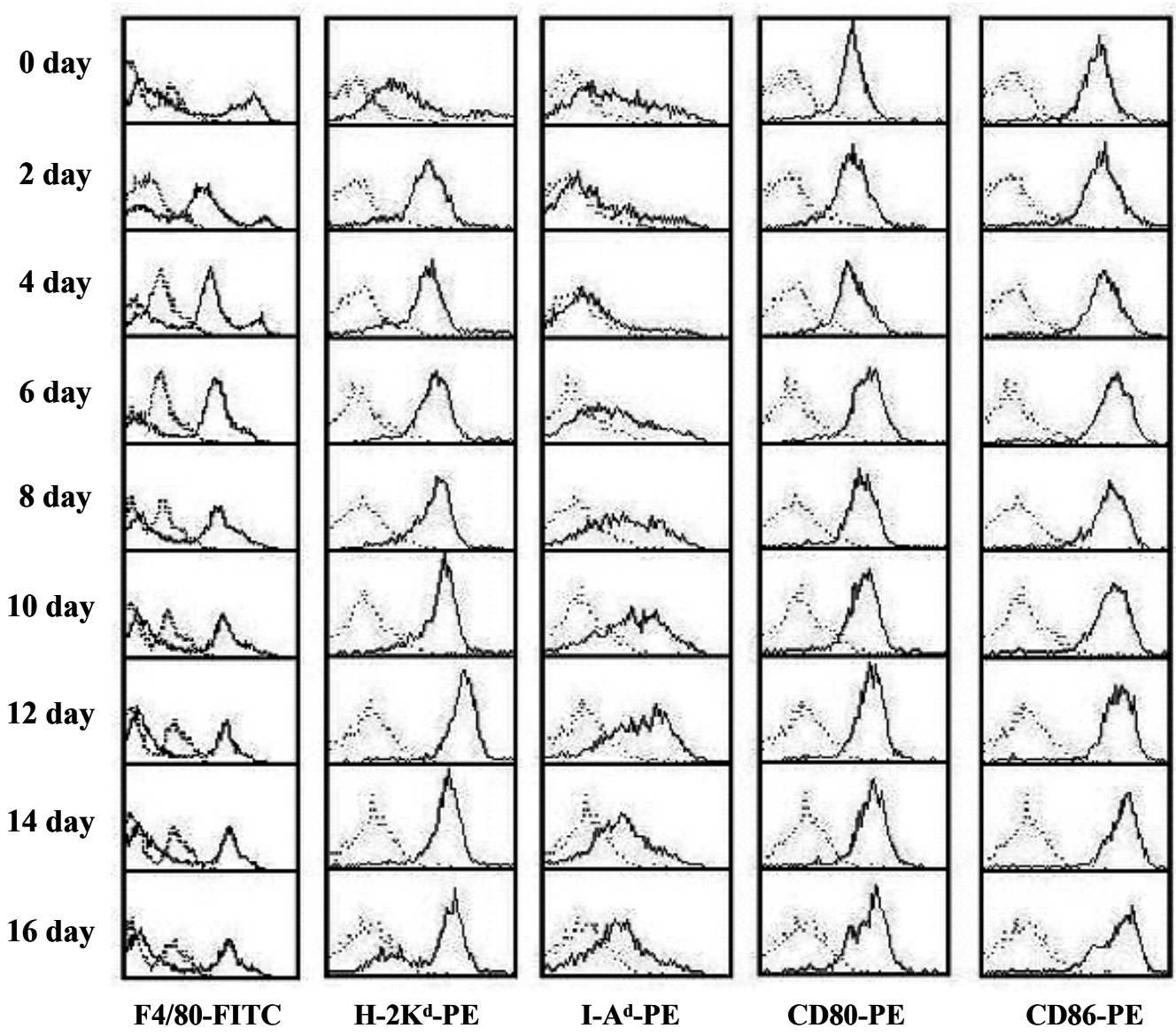

Figure 3. The expression changes of cell surface molecules after TG injection. Peritoneal cells at various time after TG injection were immunofluorescently stained with F4/80 alone or double-stained with F4/80 and other surface molecules (H-2K^d, I-A^d, CD80 or CD86). The expression of MHC (H-2K^d, I-A^d), and B7 (CD80, CD86) were determined by back-gating F4/80 positive cell populations in histogram plots using CellQuest (Becton Dickinson). Dotted line represented cells incubated with isotype-match control antibobies and solid line represented cells incubated with specific antibobies. Two experiments were performed and the results shown are from one representative experiment.

mice from lethal endotoxemia at least partially by inhibiting TNF-α release (25, 26). This may explain why IL-10 and IL-6 release is low during the inflammatory response.

The innate immune response is the first line of defense to protect a host from invading pathogens (27). This first line of defense against an infectious agent involves both the recruitment and activation of leukocytes at the infectious focus, allowing these cells to successfully localize, kill, and clear the pathogen (28). In this study, as a chemical irritant, TG induced localization and activation of macrophages in mice peritoneum cavity. We demonstrated that the percentage of F4/80 positive cells (macrophages) was increased remarkably after TG injection. And these macrophages were the activated ones that produced large amount of functional factors such as TNF-α and IL-12. It seems, therefore, the increase number of the activated macrophages is the benefit of the host for rapid elimination of the invading agents after TG injection. The F4/80 positive cells arrived at the apex in 4-6 days and then decreased. These suggested that there was an inherent mechanism to control the number of macrophages and to keep the homeostasis in the host.

Phagocytosis of pathogens by macrophages initiates the innate immune response, which in turn orchestrates the adaptive response (28). Antigen presentation by macrophages is required in generation of specific T lymphocyte clones capable of recognizing and eradicating infectious microorganisms, providing long-term immunity (29). The

antigen processing process involves several steps, including: 1) internalization of microorganisms *via* phagocytosis, pinocytosis, or Fc; 2) phagosomal fusion with acidic lysosomes containing a spectrum of proteases responsible for processing foreign proteins into antigenic peptides; 3) major histocompatibility complex (MHC) binding of immunogenic peptides; and 4) translocation of the MHC-immunogenic peptide complexes to the APCs surface where they interact with T lymphocytes (16). The expression of B7 molecules is also necessary for the antigen presenting process. In our results, the increasing of MHC and B7 expression was later than the macrophages assemble and cytokine production. These suggested that after TG injection, the cells of the monocyte/macrophage lineage congregated to peritoneum and were activated by the cytokines produced by macrophages. These activated macrophages internalized TG granules *via* phagocytosis, pinocytosis or Fc. After internalizing and processing the antigen, macrophages began to express the molecules related to antigen presenting on their surface. It was reported that the peritoneal macrophages collected from 4 days after TG injection did not express MHC class II and very low levels of CD80 and CD86 were concomitantly recorded (30). Similar conclusions were reached by our study, in which the expression of MHC and B7 was not high on day 4 after TG injection. But these molecules were expressed very high in the later days. Because they are prerequisitely necessary for T cell activation, the high expression of these molecules showed that macrophages in later days had abilities to present antigen to T cells. Besides taking part in the antigen presenting process, B7 also binds with the CTLA-4 (CD152) in T cells to transmit a negative signal and inhibit the activation of T cells (31). The high expression of B7 in the later days (day 14-16) maybe related to this negative signal and prevents the occurrence of excessive immune response.

Recently, Rezzani reported that resting peritoneal cells in the mouse represent an immature population (32). It is reported that immature dendritic cells (DCs) upregulate several cytokines genes and costimulatory molecules, indicating transition to a “mature” phenotype (33). As for macrophages, the costimulatory molecules of resident peritoneal macrophages are also very low, and these would increase markedly after TG injection. And resident peritoneal macrophages only produced high level of IL-10 and IL-6 (both Th2 cytokine) indicating that they were inactive cells. It was proposed that resident macrophages in the peritoneal compartment were likely to go through a series of sequential activation signals during the macrophage disappearance reaction (MDR) in acute inflammatory reaction that resulted in the generation of inflammatory and fully activated macrophages. Sequential steps of macrophage activation have been clearly delineated during activation of macrophage *in vitro* (21). Our research clarified the sequence of macrophage activation *in vivo*. It seems that the TG injection made the macrophages activated and then matured from an immature phagocyte.

In summary, the present discovery suggested that the population of activated macrophages and the activation of macrophages (including cytokine production and expression of cell surface molecules related to antigen presenting) were strictly controlled during inflammation process. These indicate that there is an underline mechanism to avoid the over-activation of macrophages. It is one of the crucial mechanisms to retain the homeostasis of immune system.

Acknowledgment

This research work was supported by the National Natural Science Foundation of China, No A30171086 and the Knowledge Innovation Program of Chinese Academy of Sciences and the number is KSCX2-SW-202.

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