The Phenotype of Inflammatory Macrophages Is Stimulus Dependent: Implications for the Nature of the Inflammatory Response

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The Phenotype of Inflammatory Macrophages Is Stimulus Dependent: Implications for the Nature of the Inflammatory Response

Andrew D. Cook, Emma L. Braine, and John A. Hamilton

Many diseases are characterized by inflammatory reactions involving both the innate and adaptive arms of the immune system. Thioglycolate medium (TM) injection into the peritoneal cavity has long been used as a stimulus for eliciting inflammatory macrophages for study and for determining the importance of a particular mediator in inflammation. However, the response to this irritant may not be relevant to many inflammatory diseases. Therefore, we have developed an Ag-specific peritonitis model using methylated BSA (mBSA) as the stimulus. Priming mice intradermally with mBSA in adjuvant and boosting 14 days later, followed by an i.p. challenge with mBSA after an additional 7 days, led to an inflammatory reaction equivalent in magnitude to that induced with TM as judged by the number of exudate cells. The inflammatory macrophages elicited by the mBSA protocol differed, being smaller and less vacuolated than TM-elicited macrophages. Also, macrophages from 4-day mBSA-induced exudates expressed more MHC class II than TM-induced exudates, were able to stimulate allogeneic T lymphocytes, and upon in vitro stimulation with LPS secreted greater levels of IL-6 and IL-1β. Macrophages from 4-day TM-induced exudates, on the other hand, expressed Ly6C and ER-MP58, immature myeloid markers. The inflammatory response elicited using the Ag mBSA may be more relevant for studying the inflammatory responses in many diseases, such as those of autoimmune origin and those involving an acquired immune response. The Journal of Immunology, 2003, 171: 4816–4823.

M any diseases are characterized by chronic inflammation. To study the development of such chronic inflammation, animal models have become important. However, in mice it is often difficult to obtain sufficient cells from individual tissues on a routine basis to study, for example, the cellular composition of an inflammatory reaction. Thus, there is value in developing a simple inflammatory model that is restricted to a confined location in which population changes can be more readily monitored and regulatory mechanisms identified. The peritoneal cavity in mice represents an ideal location in which to study the development of an inflammatory reaction, since it is a sterile environment in which large numbers of single cells are easily isolated by lavage.

Thioglycolate medium (TM) has long been used to induce an inflammatory response in the peritoneal cavity (1–3). However, the initiating stimuli in this heterogeneous material are unknown, and the host response may not reflect the inflammatory response seen in many diseases. Therefore, we determined whether a convenient Ag-specific inflammation model in the peritoneal cavity could be developed. For this purpose we sensitized mice with an Ag known, for example, to induce arthritis in mice, namely methylated BSA (mBSA) (4), and subsequently challenged them i.p. The resultant peritonitis was compared with that induced by TM. We were able to induce an inflammatory response of equivalent magnitude in terms of cell number to that induced with TM. However, the inflammatory macrophages differed in terms of size, cell surface expression, and functional capabilities. By defining macrophage subpopulations we have been able to dissect out some of these differences, which appear to reflect various stages/states of maturation/activation.

Materials and Methods

Mice
C57BL/6 mice, originally obtained from Central Animal Services, Monash University (Clayton, Australia), were bred in our on-site animal facility. BALB/c mice were obtained from the Walter and Eliza Hall Institute Animal Supplies (Kew, Australia). Mice were fed standard rodent chow and water ad libitum and were housed in sawdust-lined cages in groups of five. Eight- to 12-wk-old mice were used in all experiments, which were approved by the Royal Melbourne Hospital Research Foundation animal ethics committee.

Reagents
mAbs against the following Ags were used in this study: CD11b (Mac-1 α-chain; M1/70-allophycocyanin and -biotin; BD PharMingen, San Diego, CA); F4/80 (CI:A3–1-biotinylated; Caltag Laboratories, Burlingame, CA); Gr-1 (Ly-6G; RB6-8c5-FITC; BD PharMingen), B220 (CD45R; RA3-6B2-PerCP and -biotin; BD PharMingen), and CD3e (145-2C11-PE; BD PharMingen). mAbs derived from the following hybridomas were obtained from the American Type Culture Collection (Manassas, VA); CD11a (LFA-1 α-chain), MHC class II (M5/114.15.2), and ICAM-1 (Y1/1.7.4). mAbs against CD31 (ER-MP12), Ly-6C (ER-MP20), and ER-MP58, used as markers of murine macrophage development (5), were gifts from Dr. P. J. M. Leenen (Erasmus University, Rotterdam, The Netherlands), MUM-4 was a gift from Dr. J. M. Rhodes (Odense University, Denmark) and murine c-Fms (M-CSF receptor; ASF-98) (6) was a gift from Dr. S. I. Nishikawa (Kyoto University, Kyoto, Japan). mAbs against CD19 (1D3-PE-labeled) and Igκ (187.1-biotin-labeled) were gifts from Dr. S. Nutt (Walter and Eliza Hall Institute, Victoria, Australia). Where

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2 Address correspondence and reprint requests to Dr. Andrew Cook, Department of Medicine, Royal Melbourne Hospital, University of Melbourne, Parkville, Victoria 3010, Australia. E-mail address: adcook@unimelb.edu.au

3 Abbreviations used in this paper: TM, thioglycolate medium; i.d., intradermal; mBSA, methylated BSA, PMN, polymorphonuclear neutrophils, RPC, resident peritoneal cavity.

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appropriate. PE-conjugated donkey anti-rat IgG (H+L, F(ab’)2; Jackson ImmunoResearch Laboratory, West Grove, PA) and FITC-conjugated anti-rat IgG (Chemicon, Temecula, CA) were used as secondary Abs. Biotinylated Abs were detected with a streptavidin-PE (BD PharMingen) conjugate. In all cases appropriate isotype controls were used. Either 10% mouse serum or CD16/CD32 (2.4G2) was used as an Fc block where appropriate.

**TM-induced peritonitis**

Peritoneal exudate cells were elicted by i.p. injection of 1 ml of Brewer’s TM (Difco, Detroit, MI) as previously described (7). Cells were harvested at various time points after injection by washing cells from the peritoneal cavity byavage with 5 ml of ice-cold, sterile PBS.

**Methylated BSA-induced peritonitis**

Mice were immunized intradermally (i.d.) in the base of the tail with 100 μg of mBSA (Sigma-Aldrich, St. Louis, MO) emulsified in an equal volume of CFA containing 5 mg/ml heat-killed Mycobacterium tuberculosis (H77Ra; Difco); 14 days later the primary immunization protocol was repeated as a boost, or peritoneal exudate cells were elicited by i.p. injection of 100 μg of mBSA in PBS. In the case where a booster injection was given, 7 days later (i.e., day 21 after the primary immunization) peritoneal exudate cells were elicited by i.p. injection as described above. Cells were harvested at various time points as described above, with day 0 being the day of the i.p. injection eliciting the peritonitis.

**Quantification of peritoneal cavity cell infiltration**

Total leukocyte cell counts from the peritoneal cavity washes were performed using a hemocytometer. Differential cell counts were performed on Diff-Quik (Lab Aids, Narrabeen, Australia)-stained cytospin preparations.

**Flow cytometric analysis**

Cells were washed twice in PBS, counted, and resuspended in FACS buffer (1% BSA in PBS containing 0.01% NaN3). For phenotypic analysis, cells (0.2–1 × 10⁷ cells) were initially incubated with either 10% mouse serum or CD16/CD32 (Fc block) for 20 min at 4°C. Subsequently, cells were incubated with the appropriate unlabeled or biotin-labeled primary Abs followed by appropriate secondary Ab and directly conjugated Abs (for more than one-color staining). All incubations were performed on ice for 20 min and were followed by three washes with FACS buffer. Appropriate isotype controls were used in all cases.

For flow cytometric analysis, a typical forward and side scatter gate was set to exclude dead cells and aggregates; a total of 10⁶ events in the gate were collected and analyzed using a FACS software (BD Biosciences, San Jose, CA).

**Cell sorting**

Cell populations from the peritoneal cavity and T lymphocytes from the spleen were sorted based on their forward/side scatter and cell surface expression using a FACS Vantage SE cell sorter (BD Biosciences). For peritoneal exudates, macrophages found in R1 (see Results for definition) were sorted based on Mac-1 expression; cells in R4 (see Results for definition) were sorted based on Mac-1 expression. T lymphocytes from the spleens of BALB/c mice were sorted based on being negative for B220 (FITC-labeled), Mac-1 (biotin-labeled), and propidium iodide, following purification of the lymphocytes using Lympholyte M (see MLR below).

**Mixed leukocyte reaction**

Lymphocytes were isolated from spleens of BALB/c mice by passage through a nylon cell strainer, followed by passage through Lympholyte M (Cedarlane Laboratories, Hornby, Canada), according to the manufacturer’s instructions. Cells were subsequently stained for B220 (FITC-labeled) and Mac-1 (biotin-labeled followed by streptavidin-PE) and propidium iodide; T lymphocytes were then sorted by negative selection using a FACS Vantage SE cell sorter (see cell sorting above). Note that T cells are negative for these markers. The sorted population was subsequently stained for CD3 and was found to be ≥98% CD3 and negative for B220 and Mac-1. Purified T cells were cultured in 96-well, flat-bottom tissue culture plates (Nunc, Nalge Nunc International, IL) at 2 × 10⁶ cells/well. Macrophages, purified by cell sorting (see cell sorting above), from either 4-day TM-induced or 4-day mBSA-induced C57BL/6 mice, were added to T lymphocytes at 2 × 10⁸ cells/well, such that the final E:T cell ratio was 1:1. Control cultures contained medium or T cells alone. Cultures were established in triplicate and incubated at 37°C in 5% CO₂ for 96 h. Sixteen hours before harvesting, cells were pulsed with 1 μCi of [3H]TdR (Amersham Pharmacia Biotech, Little Chalfont, U.K.). Cells were harvested using an Inotech cell harvester (Lansing, MI), and DNA synthesis was measured by [3H]TdR incorporation using a Beckman beta scintillation counter (Irvine, CA). Results are expressed as counts per minute.

**Phagocytosis assay**

The fluorescent latex Fluospheres (L-5281 carboxylate-modified, 1.0-μm diameter, 2% solids; Molecular Probes, Eugene, OR) were precoated with 1% BSA before 10 μl of the 0.5% BSA-coated bead solution was added to the cells. The cells were incubated with latex beads in the solution for 60 min at 37°C. Control cultures with no addition of latex beads were also included. Cells were then washed five times with cold PBS to eliminate unengested freely suspended particles. Cells were resuspended in PBS before analysis by flow cytometry.

**In vitro LPS stimulation**

Peritoneal cells (5 × 10⁴/ml) from 4-day mBSA exudates or 4-day TM exudates were cultured in 1 μg/ml LPS (Escherichia coli serotype 0127: B8, Sigma-Aldrich) for 0, 4, and 24 h. Following culture, supernatants were collected and stored at −20°C for the detection of cytokines.

**Cytokine ELISAs**

IL-1β and IL-6 levels were measured in peritoneal exudate fluids and LPS-stimulated culture supernatants (see above) by ELISA. The coating and capture Abs were as follows: IL-1β, polyclonal anti-IL-1β Ab and a biotinylated anti-IL-1β mAb (Endogen, Woburn, MA); IL-6 and anti-IL-6 mAb (20F3.11; American Type Culture Collection); and a biotinylated anti-IL-6 mAb (MPS-32C11; BD PharMingen). To detect the cytokines, a streptavidin-HRP conjugate (BD PharMingen), followed by tetramethylbenzidine peroxidase substrate (Kierkegaard & Perry Laboratories, Gaithersburg, MD), was used. A standard curve was constructed using serial dilutions of purified IL-1β or IL-6 starting at a concentration of 2 ng/ml. Each ELISA was sensitive down to 7 pg/ml.

**Results**

**Induction of an Ag-specific inflammatory response in the peritoneal cavity**

The inflammatory reaction in the peritoneal cavity following TM injection was characterized by a typical 5-fold increase in total cell number, peaking on day 4 (Fig. 1A). To attempt to induce an analogous Ag-specific inflammatory reaction, mice were sensitized with mBSA in CFA and 14 days later were challenged i.p. with mBSA. The mBSA-challenged mice showed an increase in cell number in the peritoneal cavity, peaking on day 4, with an ∼3.5-fold increase, followed by a decrease back to normal resident cell numbers by day 14 (Fig. 1A). This increase was due to the mBSA, as saline challenge did not induce a cellular infiltrate. Sensitizing mice with mBSA in CFA without an i.p. challenge or giving mice a single i.p. injection of mBSA into the peritoneal cavity without prior sensitization failed to induce a significant inflammatory response (data not shown).

The magnitude of the Ag-specific exudate was not as large as that induced by TM (Fig. 1A). To induce a possibly larger and more sustained inflammatory response, mice were sensitized with mBSA in CFA as before, but this time in addition were given an i.d. booster injection 14 days later; after an additional 7 days mice were challenged i.p. with mBSA. Once again there was an increase in cell number in the peritoneal cavity, peaking on days 3–4 (Fig. 1B); however, the size of the inflammatory response was significantly larger than that induced following a single priming injection, with cell numbers on day 4 similar to those seen in TM-treated mice (23.8 ± 3.2 vs 23.1 ± 2.6 × 10⁸, respectively). The peritonitis was slightly more chronic than that elicited with TM, with significantly more cells in the peritoneal cavity 14 days post-i.p. challenge with mBSA compared with day 0 (9.9 ± 0.4 vs 4.6 ± 0.6 × 10⁸; p < 0.05; Fig. 1B). For TM-induced inflammation, the number of cells present in the peritoneal cavity had returned to normal levels by this time point (6.4 ± 1.6 vs 5.4 ± 0.9 × 10⁸; Fig. 1A).
The development of an inflammatory response in the peritoneal cavity: total cell number. A, Exudates induced by i.p. injection of TM; i.d. injection of mBSA in CFA, followed 14 days later by an i.p. challenge with mBSA; or saline. B, Exudates induced by two i.d. injections of mBSA in CFA 14 days apart, followed by an i.p. challenge with mBSA or saline 7 days later. For mBSA-induced inflammation, day 0 represents the day of i.p. challenge. Results are expressed as the mean ± SEM for four independent experiments.

**Morphologic characterization of cells following mBSA-induced peritonitis**

The composition of cells present in the peritoneal cavity was analyzed morphologically at each time point following the induction of mBSA-induced peritonitis. On the day of i.p. challenge with mBSA (day 0) in both mBSA-dependent models (one and two i.d. immunizations, respectively), the cell populations present in the peritoneal cavity were similar to those seen in the resident peritoneal cavity (RPC). There were few, if any, polymorphonuclear neutrophils (PMN) or eosinophils present, with macrophages being the predominant cell type (Table I). The macrophages were relatively small in size compared with their exudate counterparts (Fig. 2A; see also below).

Following i.p. challenge with mBSA, the changes in cell composition over time were similar regardless of whether one or two i.d. immunizations were given; however, as discussed above, the number of cells was much greater in mice receiving two i.d. injections before i.p. challenge. On day 1 following i.p. challenge, there was a significant recruitment of PMN (Fig. 2B), with double the number of PMN present in mice given two i.d. injections compared with one i.d. injection. This represents a much greater proportion of PMN in the peritoneal cavities of the former mice (two i.d. injections, 43 ± 6.3%; one i.d. injection, 22 ± 2.6%), as the total cell number was not significantly different between the two protocols on day 1 (see Fig. 1, A and B). There was a subsequent decline in PMN numbers over the following days (Fig. 2B). This increase in PMN was not seen in saline-treated mice (Fig. 2B).

By days 3–4 following i.p. challenge, the predominant cell type was the macrophage (Table I and Fig. 2C). There was a range of macrophage sizes, most being larger than those seen in the RPC (Fig. 2A). However, compared with 4-day TM-induced peritoneal exudates, the macrophages in the 4-day mBSA-induced exudates were smaller and less vacuolated (Fig. 2A).

There was also a small percentage of mononuclear-like ring cells, accounting for up to 3.0% of cells on day 4 in both mBSA or TM-induced exudates (Table I). Similar cells are found in the bone marrow and have a cytoplasmic center that is smaller than the width of the ring (8). The presence of these cells suggests that immature myeloid precursor cells originating in the bone marrow may have been recruited to the peritoneal cavity (8).

Eosinophils were also found to enter the peritoneal cavity from day 1 onward following both TM- and m-BSA-induced inflammation. Peak numbers of eosinophils were found 4 days post-i.p. challenge, with more being found in TM-induced exudates compared with mBSA-induced exudates (Table I).

There was a slight increase in the number of lymphocytes on day 4 following m-BSA-induced inflammation (Table I).

**Characterization of cell populations in the peritonitis models by surface marker analysis**

As previously reported by us, c-Fms (CSF-1 or M-CSF receptor) is a useful marker for defining macrophage lineage cells during an inflammatory reaction (7). Using flow cytometry and surface marker expression, particularly that for Mac-1 and c-Fms, macrophage lineage cells present in the peritoneal cavity at different time points following TM- and m-BSA-induced peritonitis were defined and compared. For mBSA-induced peritonitis, the results shown below are from the two-i.d. immunization protocol.

**Resident peritoneal cavity.** To be able to assess the properties of the exudate macrophage populations, the resident peritoneal macrophages were first defined. In the RPC there was a large population of macrophages, as judged by c-Fms expression. By forward and side scatter, measuring size and granularity, respectively, the macrophages were of medium size and granularity (Fig. 3A). For convenience, the region where these cells are located by forward-side scatter has been defined as R1 (Fig. 3A). Cells in R1 accounted for 38 ± 4% of total cells in the RPC. Their surface marker expression is presented in Table II. The vast majority of these cells were c-Fms+ F4/80+ Mac-1high, with 40% being MUM-4+. MUM-4 is only found on resident peritoneal macrophages (9).

By forward/side scatter, a second region, consisting of cells that were less granular than those present in R1, was defined as R2 (Fig. 3A) and contained 25 ± 1% of the resident peritoneal cells (Table III). Most cells in R2 expressed Mac-1 (87 ± 2%) but at lower levels per cell compared with cells in R1 (data not shown). Some 41 ± 8% of R2 cells were also B220+, suggesting that they were B1 lymphocytes (10). The B220+ Mac-1low and B220+ Mac-1low cells were arbitrarily subdivided further into R2A and R2B regions, respectively (Fig. 4A), and other surface markers were measured (Fig. 4B). As shown in Fig. 4B, the B220+ Mac-1low cells (R2A) were predominantly CD19+ IgK+ c-Fms+ F4/80+ supporting their B1 lymphocyte characterization (Fig. 4B) (10). The

![Table I. Differential cell counts (×10⁶) in the peritoneal cavity](http://www.jimmunol.org/DownloadedFrom)

<table>
<thead>
<tr>
<th></th>
<th>RPC</th>
<th>4d TM</th>
<th>4d mBSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>3.9 (0.3)</td>
<td>21.1 (8.3)</td>
<td>18.4 (4.9)</td>
</tr>
<tr>
<td>PMN</td>
<td>0</td>
<td>0.3 (0.2)</td>
<td>0.3 (0.2)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.1 (0.2)</td>
<td>1.3 (0.2)</td>
<td>2.0 (0.7)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>&lt;0.1</td>
<td>2.6 (0.5)</td>
<td>0.4 (0.2)</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.1 (0.03)</td>
<td>0.2 (0.05)</td>
<td>0.1 (0.1)</td>
</tr>
<tr>
<td>Mononuclear-like ring cells</td>
<td>0</td>
<td>0.2 (0.1)</td>
<td>0.3 (0.1)</td>
</tr>
</tbody>
</table>

*RPC, 4-day TM exudates, and 4-day mBSA exudates (two i.d. immunizations). Data are the mean (SEM) from four experiments.
B220−Mac-1low cells (R2B) were CD19−IgK−, with 54 ± 4 and 53 ± 5% being c-Fmslow and F4/80low, respectively and presumably are of the macrophage lineage (Fig. 4B).

T lymphocytes, as defined by CD3 staining, and the rest of the B lymphocytes (B220+, CD19+, Mac-1−), were smaller and less granular than cells in R2. By forward/side scatter, the region where the lymphocytes are located has been defined as R3 and contained 22 ± 4% of total cells (Fig. 3A). R3 cells were c-Fms−Mac-1−F4/80−MUM-4− (data not shown), suggesting that the B lymphocytes found in this region are B2 lymphocytes (10).

**TM-induced peritonitis.** As previously reported, following an i.p. TM injection there was an initial decline in the numbers of c-Fms+ cells in R1 within the first day post-i.p. challenge, followed by an increase on days 3–4 (7). Mac-1 expression, on the other hand, did not decrease, due to its expression on neutrophils, which were found in large numbers on days 1 and 2 post-i.p. injection (data not shown) (7). By day 4, when the macrophage response was maximal, the c-Fms+ macrophages present in R1 were larger and more granular than those seen in the RPC (Fig. 3B), reflecting the morphologic analysis, and accounted for 71 ± 4% of total cells (Table II). While expressing high levels of c-Fms, F4/80, and Mac-1, these cells were negative for MUM-4, indicating they were inflammatory exudate cells (Table II) (7, 9).

Cells in R2 accounted for only 8% of the total cells on day 4 (Fig. 3B) and consisted of macrophages, as determined by F4/80 (26 ± 8%) and c-Fms (17 ± 6%) staining, and B1 lymphocytes, as determined by B220 (45 ± 4%), CD19 (46 ± 3%), and IgK (45 ± 2%) staining (Table III). There were actually similar numbers of cells in R2 in day 4 TM-induced exudates and in the RPC (1.8 ± 0.2 vs 1.4 ± 0.3 × 10⁶).

**mBSA-induced peritonitis.** A similar decrease in the number of c-Fms+ cells in R1 was seen within the first day following i.p. challenge with mBSA, followed by an increase over days 3–4. However, 4 days post-i.p. challenge with mBSA, the macrophages present in R1, while being larger and more granular than those present in the RPC, were smaller and less granular compared with the macrophages present in 4-day TM-induced exudates (Fig. 3C), in agreement with the morphologic analysis (see Fig. 2A). Furthermore, R1 cells from 4-day mBSA-induced exudates accounted for only 39 ± 2% of the total cells compared with 71 ± 7% from 4-day TM-induced exudates (Table II). However, they expressed the same macrophage markers (c-Fms, F4/80, Mac-1) as TM-induced exudate macrophages and were negative for MUM-4 (Table II).

There was a similar percentage of cells in R2 on day 4 as in the RPC (20 ± 2 vs 25 ± 1%). Cells in R2 on day 4 consisted of macrophages, as determined by F4/80 (25 ± 3%) and c-Fms (35 ± 4%) staining, and B1 lymphocytes, as determined by B220 (24 ± 4%), CD19 (20 ± 3%), and IgK (19 ± 2%) staining (Table III). Thus, there was an increase in the total number of cells in this region compared with the RPC (2.8 ± 0.2 vs 1.4 ± 0.3 × 10⁶).

**Eosinophils.** By forward/side scatter, an additional cell population was evident during the development of both TM- and mBSA-induced peritonitis, which was not found in the RPC. These cells were small in size, but highly granular; the region where these cells lie has been designated R4 (Fig. 3, B and C). Cells in R4 accounted for 10 ± 4 and 7 ± 1% of the total cells from 4-day TM- and mBSA-induced exudates, respectively. These cells expressed high levels of F4/80 and low levels of Mac-1, but were negative for c-Fms (data not shown), suggesting that they were not macrophage lineage cells. Sorting R4 cells from 4-day TM-induced exudates and staining the cells with Diff-Quik showed these cells to be eosinophils (data not shown).

**Other properties of macrophages**

We next compared a number of other properties of macrophages elicited in the two peritonitis models.

**Other lineage markers in the peritonitis models.** Previously, we have defined Ly6C (ER-MP20) and ER-MP58 as being useful markers for defining inflammatory c-Fms+ macrophage-lineage cells in the TM-induced peritonitis model (7). These markers are found on myeloid bone marrow cells and are progressively lost as the cells mature into monocytes (Ly6C− ER-MP58+) and, subsequently, macrophages (Ly6C− ER-MP58−) (5); they have therefore been viewed as being markers for the development of macrophage lineage cells (5). In the RPC, macrophages were negative
injection of mBSA without prior sensitization fails to induce any significant inflammatory response, suggesting memory T cell dependence. Methylated BSA-induced peritonitis in general is T cell dependent (4). Given these findings, the expression of various markers on R1 cells in the two models were measured.

In the RPC the macrophages present in R1 did not express MHC class II to any significant degree (7 ± 3%; Table II). Following TM-induced peritonitis, class II was present on 26 ± 6% of R1 cells from day 4 exudates; however, expression levels per cell were low (Fig. 5B and Table II). By contrast, 80 ± 6% of R1 cells from 4-day mBSA-induced peritonitis were positive for class II (Fig. 5B and Table II).

To determine whether there were any differences or changes in integrin expression, presumably involved in trafficking and adhesion of cells, levels of CD11a (LFA-1) and CD11c were measured on R1 cells. LFA-1 was expressed on 19 ± 2% of R1 cells in the RPC. Four days post-i.p. challenge with TM, the proportion of LFA-1+ R1 cells increased to 26 ± 6%, and for 4-day mBSA exudates, the proportion of LFA-1+ R1 cells increased to 31 ± 9% (Table II). RPC were negative for CD11c, and following stimulation with either TM or mBSA, the macrophages remained negative for CD11c (data not shown). ICAM-1, a ligand for both Mac-1 and LFA-1, was expressed on 63 ± 5% of R1 cells in the RPC. Its expression has been reported to increase on macrophages as they mature (11). There were no significant differences in the proportion of ICAM-1+ R1 cells from 4-day TM or mBSA-induced exudates compared with the RPC (Table II).

For Ly6C and ER-MP58 (Table II), as previously described (7). By day 4 post-TM-induced peritonitis, 80 ± 9% of R1 cells expressed ER-MP58, and 38 ± 5% of R1 cells expressed Ly6C (Table II). However, by contrast, only 15 ± 4% of R1 cells expressed ER-MP58, and 13 ± 8% of R1 cells expressed Ly6C following i.p. challenge with mBSA (two-i.d. immunization protocol; Table II), and where positive, expression levels per cell were significantly lower (shown for ER-MP58; Fig. 5A). These findings suggest that following TM-induced peritonitis there is a greater recruitment of immature macrophages compared with mBSA-induced peritonitis (see Discussion).

Of note, eosinophils present in R4 expressed high levels of Ly6C and particularly high levels of ER-MP58 (data not shown).

**MHC class II expression and integrin expression.** While an i.p. injection of TM induces an inflammatory reaction, an equivalent

**TABLE II.** Peritoneal cell surface marker distribution within region 1 (R1)*

<table>
<thead>
<tr>
<th>Marker</th>
<th>RPC</th>
<th>4d TM</th>
<th>4d mBSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of total cells</td>
<td>38 (4)</td>
<td>71 (4)</td>
<td>39 (2)</td>
</tr>
<tr>
<td>c-Fms</td>
<td>76 (9)</td>
<td>70 (13)</td>
<td>72 (4)</td>
</tr>
<tr>
<td>F4/80</td>
<td>71 (15)</td>
<td>91 (5)</td>
<td>70 (7)</td>
</tr>
<tr>
<td>Mac-1</td>
<td>95 (3)</td>
<td>97 (1)</td>
<td>98 (1)</td>
</tr>
<tr>
<td>MUM-4</td>
<td>40 (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gr-1</td>
<td>0</td>
<td>2 (2)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>ER-MP58</td>
<td>2 (2)</td>
<td>80 (9)</td>
<td>15 (4)</td>
</tr>
<tr>
<td>Ly-6C (ER-MP20)</td>
<td>0</td>
<td>38 (5)</td>
<td>13 (8)</td>
</tr>
<tr>
<td>MHC class II</td>
<td>7 (3)</td>
<td>26 (6)</td>
<td>80 (6)</td>
</tr>
<tr>
<td>LFA-1</td>
<td>19 (2)</td>
<td>26 (6)</td>
<td>31 (9)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>63 (5)</td>
<td>65 (10)</td>
<td>54 (15)</td>
</tr>
<tr>
<td>B220</td>
<td>10 (2)</td>
<td>8 (4)</td>
<td>14 (4)</td>
</tr>
</tbody>
</table>

*Percentage of positive cells for various cell surface markers in R1. R1 is defined by c-Fms expression and forward/side scatter profiles (see Fig. 3). RPC, 4-day TM exudates, and 4-day mBSA exudates (two i.d. immunizations). Data are the mean (SEM) from four experiments.

**TABLE III.** Peritoneal cell surface marker distribution within region 2 (R2)*

<table>
<thead>
<tr>
<th>Marker</th>
<th>RPC</th>
<th>4d TM</th>
<th>4d mBSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of total cells</td>
<td>25 (1)</td>
<td>8 (1)</td>
<td>20 (2)</td>
</tr>
<tr>
<td>c-Fms</td>
<td>16 (2)</td>
<td>17 (6)</td>
<td>34 (4)</td>
</tr>
<tr>
<td>F4/80</td>
<td>20 (3)</td>
<td>26 (8)</td>
<td>24 (3)</td>
</tr>
<tr>
<td>Mac-1</td>
<td>87 (2)</td>
<td>82 (5)</td>
<td>80 (1)</td>
</tr>
<tr>
<td>Gr-1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B220</td>
<td>41 (8)</td>
<td>45 (4)</td>
<td>22 (4)</td>
</tr>
<tr>
<td>CD19</td>
<td>41 (4)</td>
<td>46 (3)</td>
<td>20 (3)</td>
</tr>
<tr>
<td>IgK</td>
<td>30 (3)</td>
<td>45 (2)</td>
<td>19 (2)</td>
</tr>
</tbody>
</table>

*Percentage of positive cells for various cell surface markers in R2. R2 is defined by forward/side scatter profiles and Mac-1 expression (see Fig. 3, A-C). RPC, 4-day TM exudates, and 4-day mBSA exudates (two i.d. immunizations). Data are the mean (SEM) from four experiments.
Mixed leukocyte reaction. Given the preferential expression of MHC class II on macrophages from 4-day mBSA-induced exudates, the ability of R1 cells to stimulate allogeneic naive T cells was tested in an MLR. R1 cells from 4-day mBSA-induced exudates were able to stimulate the allogeneic T cells, whereas R1 cells from 4-day TM-induced exudates were not (Fig. 6A).

Phagocytosis. By definition, macrophages are capable of phagocytosis. Cells in R1, when incubated at 37°C with fluorescently labeled latex beads, were able to ingest them. However, 4-day TM-induced exudates were more efficient at ingesting multiple beads compared with 4-day mBSA-induced exudates (55 ± 3 vs 19 ± 1%, respectively; ingested more than three beads), as shown by flow cytometry (Fig. 6B) and fluorescence microscopy (data not shown), an observation in line with their larger size.

Cytokine production from 4-day TM- and mBSA-induced exudate cells. IL-1β and IL-6 could not be detected in peritoneal exudate lavage fluid following either 4-day TM- or mBSA-induced inflammation (data not shown). However, low levels of both IL-1β and IL-6 could be detected in culture supernatants from 4-day

FIGURE 4. Cell surface marker expression of R2 resident peritoneal cells (see Fig. 3A). A, Mac-1 and B220 expression; B, expression of c-Fms, F4/80, CD19, and IgK on cells found in R2A (B220<sup>+</sup>Mac-1<sup>+</sup>) and on cells found in R2B (B220<sup>+</sup>Mac-1<sup>-</sup>), as defined in A. CD19<sup>+</sup> and IgK<sup>+</sup> cells are predominately found within R2A. c-Fms<sup>+</sup> cells and F4/80<sup>+</sup> cells are predominately found within R2B. The former cells are proposed to be B1 lymphocytes, and the latter cells are presumably of the macrophage lineage.

FIGURE 5. Cell surface marker expression of R1 peritoneal exudate macrophages (see Fig. 3). A, ER-MP58; B, MHC class II. Data are for day 4 TM-induced exudates and for day 4 mBSA-induced exudates (two i.d. immunizations), respectively.

FIGURE 6. Functional capacity of TM- and mBSA-induced macrophages. A, Stimulatory ability of macrophages from day 4 TM-induced exudates and day 4 mBSA-induced exudates (two i.d. immunizations). Sorted macrophage populations (2 × 10<sup>5</sup> cells/well; see Materials and Methods) were incubated with allogeneic splenic T cells (2 × 10<sup>5</sup> cells/well) at a ratio of 1:1 for 96 h at 37°C in 5% CO<sub>2</sub>. Cultures were pulsed with [3H]TdR for the last 16 h (see Materials and Methods). Control cultures contained medium or T cells alone. Results are expressed as the amount of [3H]TdR incorporation (mean ± SEM) in counts per minute. B, Phagocytic capability of peritoneal exudate macrophages. Percentage of cells in R1, from day 4 TM-induced exudates and day 4 mBSA-induced exudates (two i.d. immunizations), phagocytosing latex beads (total), and phagocytosing one, two, three, or more than three beads. Results are expressed as the mean ± SEM from three independent experiments.
mBSA-induced exudates in the absence of LPS (Fig. 7). Following LPS stimulation in vitro (4 and 24 h), there was an increase in the levels of both IL-6 and IL-1β in culture supernatants from 4-day TM- and mBSA-induced exudates compared with cultures containing no LPS stimulation (shown for 24 h; Fig. 7), with cells from mBSA-induced exudates secreting more IL-6 and IL-1β per cell compared with cells from 4-day TM-induced exudates.

**Discussion**

We have successfully developed an immunologically mediated inflammatory reaction in the peritoneal cavity of mice using mBSA as the eliciting Ag, thus allowing examination of the cellular changes taking place during this type of response. Priming mice i.d. with mBSA in adjuvant and challenging i.p. 14 days later led to a relatively weak inflammatory reaction, with approximately half the number of inflammatory cells present in the peritoneal cavity 4 days post-i.p. challenge compared with 4-day TM-induced exudates. The inclusion of a booster injection of mBSA in CFA 14 days after the initial sensitization, followed by an i.p. challenge an additional 7 days later, led to an inflammatory response comparable to that induced by TM in terms of the number of inflammatory cells in the exudates. Regardless of whether mBSA or TM was used as the eliciting stimulus, the course of the inflammatory response following i.p. challenge was characterized by an initial influx of PMN, followed by an influx of macrophages. However, despite there being similar numbers of exudate cells present 4 days post-i.p. challenge with either mBSA or TM, the macrophages differed in size, granularity, cell surface molecule expression, and functional capabilities.

As previously found by us (7), c-Fms proved to be the most suitable marker of macrophage lineage cells. All other cell surface markers present on macrophage lineage cells were also found on other cell types: Mac-1 on PMN, eosinophils, and B1 lymphocytes, albeit with intermediate or low expression per cell; F4/80 on eosinophils; and MUM-4 only on resident peritoneal macrophages. Based on cell surface expression of c-Fms, Mac-1, and F4/80 and the size and granularity of the cells present in the peritoneal cavity, we defined four regions, three of which were found in both the unstimulated and stimulated cavities, the fourth being present in only the stimulated cavity. In R1, macrophages were found. These cells differed greatly in size and granularity depending on whether they were from the RPC or from a stimulated cavity. Furthermore, the nature of the stimulus also affected their size and granularity. R2 consisted of a mixed population of cells, including smaller macrophages and B1 lymphocytes. B1 lymphocytes may represent an early B cell lineage and are reported to develop only in the fetal omentum and the adult peritoneal cavity (12). Several studies have shown that B1 cell lines can differentiate into macrophages, further supporting the contention that B1 cells are a separate primitive lineage (reviewed by Kipps (13)). PMN, when present during the early phase of the inflammatory reaction, were found in both R1 and R2. R3 contained T and B lymphocytes, the latter being the more typical B2 lymphocytes (10). Cells in R4, which were present only during inflammation, were predominately eosinophils.

The cellular response following TM-induced inflammation was previously described by us (7), with most macrophages from 4-day exudates expressing the immature myeloid lineage cell markers Ly-6C and ER-MP58 not normally found on mature macrophages (5). Resident macrophages in a variety of tissues either do not express or express low levels of Ly-6C (14). Ly-6C+ -cultured monocytes and Ly-6C− monocyte-like cell lines, but not fully differentiated macrophages and macrophage-like cell lines, can be induced to express the Ly-6C Ag by IFN-γ (14). ER-MP58, the function of which is unknown, can be found on myeloid cells as late as the monocytic stage, but is not found on mature macrophages (5). Resident macrophages are highly expressed on GM precursors and eosinophils (15). The observed detection of both Ly-6C and ER-MP58 on the TM-induced macrophages suggests that they are not fully differentiated, but are less mature phagocytic macrophage lineage cells, most likely recruited from the circulation. However, it is possible that the expression of these markers represents an activation change.

In our mBSA-induced peritonitis model, prior sensitization with the Ag is required for the development of an inflammatory response, suggesting the need for an Ag-specific T cell response. The macrophages from 4-day mBSA-induced exudates expressed MHC class II, were able to stimulate allogeneic T lymphocytes, and secreted larger amounts of proinflammatory cytokines upon in vitro LPS stimulation. Peritoneal macrophages can be induced to differentiate in vitro into cells exhibiting typical dendritic cell morphology, phenotype, and function (16–18). These dendritic cells express MHC class II and the integrin CD11c, a marker found predominantly, although not exclusively, on dendritic cells in the mouse (17). TM-elicited peritoneal macrophages, on the other hand, have lost the ability to differentiate into dendritic cells (16). In the current study, following elicitation with mBSA, the macrophages appear to take on a dendritic cell-like role, expressing higher levels of class II and being efficient stimulators of allogeneic MLR. However, they do not express CD11c, nor do they exhibit the morphology of dendritic cells, suggesting that they are not fully differentiated dendritic cells.

The mBSA challenge has been used as an in vivo model of a delayed-type sensitivity granuloma (19) and in the air pouch model (20). The latter has been employed as a model of a facsimile synovium by virtue of histologic properties; however, its relevance has been questioned due to the distribution and ultrastructure of small blood vessels in the pouch wall being quite different from the known characteristics of the microcirculation of synovial membrane (21). While our current model is also not a facsimile synovium, it is a very easy, convenient one in which cell types can be studied using differing stimuli. The Ag-induced arthritis model (4)
and the currently described mBSA-induced peritonitis model are established in a similar manner, with a prior sensitization and booster injection, and differ only in the site of challenge. An intra-articular injection is given for the Ag-induced arthritis model, and inflammation develops in the joint (4), while an i.p. injection is given in the current model, and inflammation develops in the peritoneal cavity. Thus, it is likely that the inflammatory responses that ensue will also share similarities.

In summary, TM-induced peritonitis has been the gold standard, providing elicited macrophages for study and for determining the importance of a particular mediator in the development of an inflammatory reaction. The TM-elicited macrophages are large, vacuolated, and highly phagocytic. We suggest, however, that this type of inflammatory response may not be relevant to many inflammatory disease situations, particularly where an acquired immune response is important. Our mBSA-induced peritonitis model described here, which involves such an immune response, may be more relevant for studying the inflammatory responses in many diseases, such as those of autoimmune origin. Our new peritonitis model is a simple protocol for generating Ag-elicited macrophages and is a convenient one for studying the roles of different mediators, such as growth factors and cytokines, in the development of an immune-mediated inflammatory response.

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References