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Stimulus-Dependent Requirement for Granulocyte-Macrophage Colony-Stimulating Factor in Inflammation

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

Data from several inflammation/autoimmunity models indicate that GM-CSF can be a key inflammatory mediator. Convenient models in readily accessible tissues are needed to evaluate the GM-CSF-dependent cellular responses to be elaborated. In this study, we show that, in contrast to the response to the commonly used i.p. irritant, thioglycolate medium, an Ag-specific methylated BSA-induced peritonitis in GM-CSF−/− mice was severely compromised. The reduced response in the latter peritonitis model was characterized by fewer neutrophils and macrophages, as well as by deficiencies in the properties of the remaining macrophages, namely size and granularity, phagocytosis, allogeneic T cell triggering, and proinflammatory cytokine production. B1 lymphocytes were more evident in the GM-CSF−/− Ag-specific exudates, indicating perhaps that GM-CSF can act on a common macrophage-B1 lymphocyte precursor in the inflamed peritoneum. We propose that these findings contribute to our understanding of how GM-CSF acts as a proinflammatory cytokine in many chronic inflammatory/autoimmune diseases. Of general significance, the findings also indicate that the nature of the stimulus is quite critical in determining whether a particular inflammatory mediator, such as GM-CSF, plays a role in an ensuing inflammatory reaction.


Materials and Methods

Mice

GM-CSF gene-deficient (GM-CSF−/−) mice, backcrossed onto the C57BL/6 background for 11 generations, were originally provided by the Ludwig Institute for Cancer Research (Parkville, Victoria, Australia). As wild-type controls, C57BL/6 mice, originally obtained from Central Animal Services, Monash University (Clayton, Victoria, Australia), were used. Both strains were bred in our on-site animal facility. BALB/c mice were obtained from the Walter and Eliza Hall Institute Animal Supplies (Kew, Victoria, Australia). Mice, 8–12 wk of age, were used in all experiments.

Reagents

mAbs against the following Ags were used: CD11b (Mac-1 α-chain; M1/70-allophycocyanin and -biotin; BD Pharmingen, San Diego, CA), F4/80 (C1A3-1-biotinylated; Caltag Laboratories, Burlingame, CA), Ly-6G
(1A8-PE; BD Pharmingen), B220 (CD45R; RA3-6B2-PerCP and -FITC; 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-a-chain), MHC class II (M5/114.15.2), and ICAM-1 (Y11/1.7.4). mAbs against the following Ags weregifted by: Ly-2 (MR-MP20) and ER-MPS8 (26, 27) (Dr. P. Leenen, Erasmus University, Rotterdam, The Netherlands); c-fms (M-CSFR; ASF-98) (28) (Dr. S. I. Nishikawa, Kyoto University, Kyoto, Japan); and CD19 (1D3-PE labeled) and IgK (187.1-biotin labeled) (Dr. S. Nutt, Walter and Eliza Hall Institute). PE-conjugated donkey anti-rat IgG (H+L, F(ab')2; Jackson ImmunoResearch Laboratories, West Grove, PA), 2-ME and 5% (v/v) FCS (200 μl/well). Sixteen hours before harvesting, cells were pulsed with 1 μCi [3H]TdR (Amersham Biosciences, Little Chalfont, U.K.). Cells were harvested using an Inotech Cell Harvester, and DNA synthesis was measured by [3H]TdR incorporation using a Beckman beta scintillation counter (Beckman Instruments, Irvine, CA). Results are expressed in counts per minute.

Flow cytometry analysis
Cell staining for flow cytometry analysis, including isotype controls and FcR blocking, was conducted as described previously (25). Briefly, cells were incubated with the primary Ab (unlabeled or biotin labeled), followed by an appropriate secondary Ab and directly conjugated Abs (for more than one color staining). 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 FACSsort (BD Biosciences, San Jose, CA).

Phagocytosis assay
Cells were incubated with fluorescent latex Fluospheres (L-5281 carboxylate-modified, 1.0-μm diameter, 2% solids; Molecular Probes, Eugene, OR) precoated with 1% BSA, for 60 min at 37°C, and analyzed by flow cytometry (25). Control cultures with no addition of latex beads were included.

Cell sorting
Cell populations from the peritoneal cavity and T lymphocytes from the spleen were sorted based on their forward-side scatter and c-Met expression using a FACSVantage SE cell sorter (BD Biosciences) (25). For peritoneal exudates, macrophages found in region 1 (R1; see Results for definition) from C57BL/6 mice and R1a (see Results) from GM-CSF−/− mice were sorted based on c-Fms+4F/80+Mac-1high expression. T lymphocytes from BALB/c mice were sorted by negative selection (25).

Mixed leukocyte reaction
The MLR was performed as previously described (25). Purified T cells (2 × 10⁶ cells/well) and macrophages (2 × 10⁵ cells/well) were cultured at 37°C and 5% CO₂ for 96 h. [3H]TdR labeling, harvesting, and counting were as described for T cell proliferation.

In vitro LPS stimulation
Peritoneal cells or sorted macrophages (see above) (5 × 10⁶/ml) 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, IL-6, IL-10, TNF-α, and MCP-1 levels were measured in peritoneal exudate fluids and LPS-stimulated culture supernatants by ELISA (25). For IL-1β and IL-6, 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, anti-IL-6 mAb (2F03.11; American Type Culture Collection) and a biotinylated anti-IL-6 mAb (MP5-32C11; BD Pharmingen). To detect the cytokines, a streptavidin-HRP conjugate (BD Pharmingen), followed by tetramethylbenzidine-peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD), was used. Standard curve was constructed using serial dilutions of purified IL-1β or IL-6, starting at a concentration of 2 ng/ml. For MCP-1, TNF-α, and IL-10, the appropriate mouse OpEIA Set (BD Pharmingen) was used according to the manufacturer’s instructions. Each ELISA was sensitive down to 7 pg/ml.

Intracellular cytokine staining
Intracellular IL-6 levels were measured in LPS-stimulated mBSA-induced peritoneal cells. Brefeldin A (10 μg/ml; Sigma-Aldrich) was added to the cultures for the final 2 h of LPS stimulation to limit cytokine secretion (29). Following FcR blocking, cells were stained for CD11b (Mac-1-a-chain; M170-allophycocyanin) as above, fixed in 4% paraformaldehyde, and permeabilized using 0.5% saponin and 2% FCS in PBS. Cells were subsequently stained with biotinylated anti-IL-6 mAb (MP5-32C11; BD Pharmingen), and detected using a streptavidin-PE (BD Pharmingen) conjugate. Appropriate isotype controls were used. Samples were analyzed by flow cytometry as described above. Results are presented as the geometric mean fluorescence intensity (GMFI), a measure of the amount of cytokine being produced per cell (30).

Statistics
Data are presented as mean ± SEM for indicated experiments. Differences between GM-CSF−/− mice and C57BL/6 mice were performed using the Student’s two-tailed t test. A value of p ≤ 0.05 was considered statistically significant.

Results
Induction of an Ag-specific peritonitis in GM-CSF−/− mice
We confirmed and extended previous findings showing that the inflammatory reaction in the peritoneal cavity of GM-CSF−/− mice following TM injection was no different from that seen in C57BL/6 mice in terms of numbers and types of cells present (Fig. 1). Both the initial, predominantly PMN, influx and the subsequent macrophage one were similar despite the lack of GM-CSF, although the macrophages were, on average, smaller in GM-CSF−/− mice compared with those in C57BL/6 mice (Fig. 1B).

We next assessed the mBSA-dependent peritonitis in GM-CSF−/− mice. On day 1 post-mBSA challenge, the number of peritoneal exudate cells increased from 4.5 ± 0.3 × 10⁶ on day 0 (the day of mBSA i.p. challenge) to 13.6 ± 1.2 × 10⁶, which was similar to that seen for C57BL/6 mice (4.6 ± 0.6 to 11.6 ± 0.4 (×10⁶)) (Fig. 2A). However, although the number of cells in the peritoneal cavity of C57BL/6 mice continued to increase, peaking 3–4 days post-mBSA challenge, the number of cells in the peritoneal cavity of GM-CSF−/− mice remained relatively constant on days 1–4 post-mBSA challenge (Fig. 2A). The number of peritoneal cells gradually returned to resident levels by day 20 post-mBSA challenge in both C57BL/6 and GM-CSF−/− mice; however, there were still significantly more inflammatory exudate cells in C57BL/6 mice compared with GM-CSF−/− mice on days 7 and 14 post-mBSA challenge (Fig. 2A). Saline challenge failed to induce a cellular infiltrate in either GM-CSF−/− or C57BL/6 mice.
mice (8.7/100 μl CSF number of macrophages present in the peritoneal cavity of GM-CSF−/− mice compared with C57BL/6 mice; the kinetics of the eosinophil response also differed with a more gradual increase over the first 4 days post-mBSA challenge, followed by a more rapid decline in the numbers of eosinophils in GM-CSF−/− mice compared with C57BL/6 mice (data not shown).

**Cellular response to mBSA in GM-CSF−/− mice**

Because GM-CSF is implicated in the development of APC (6, 14) and mBSA is a T cell-dependent Ag (31), the in vitro T cell response to mBSA in GM-CSF−/− mice was examined. Spleen cells were isolated from mice 4 days post i.p. challenge with mBSA, i.e., 25 days post the initial primary immunization. There was no difference in the proliferative response of T cells to mBSA from GM-CSF−/− mice compared with C57BL/6 mice (Fig. 3). These data suggest that GM-CSF is not involved in the Ag-priming phase of mBSA-induced peritonitis.

**Properties of macrophage-lineage cells elicited during mBSA-induced peritonitis in GM-CSF−/− mice**

From the morphologic analysis above, there was a dramatic reduction in the number of macrophages post-mBSA challenge in GM-CSF−/− mice compared with C57BL/6 mice. The effect of a lack of GM-CSF expression on several parameters of the macrophage lineage infiltrate in 4-day mBSA-induced peritoneal exudates was next studied.

**Forward-side scatter profile and lineage markers**

As previously reported by ourselves (25, 27), c-Fms (CSF-1R, M-CSFR) is a useful surface marker for defining macrophage-lineage cells in vivo, including during inflammatory reactions. Using predominantly size and granularity (forward-side scatter by flow cytometry), in combination with c-Fms, F4/80, and Mac-1 expression to detect macrophage-lineage cells, we previously defined four regions to help analyze the different cell populations in resident and stimulated murine peritoneal cavities (25). The four regions for the forward-side scatter profile of a 4-day post-mBSA exudate from C57BL/6 mice are reproduced in Fig. 4A (see also Ref. 25). As before (25), macrophages are found in R1, with smaller macrophages being in R2 with B1 lymphocytes; B and T lymphocytes are predominantly in R3 and eosinophils are in R4.

For GM-CSF−/− mice, using these same parameters, cells from the resident peritoneal cavity could be allocated to the same regions and with a similar distribution to that found for the equivalent cells from C57BL/6 mice (data not shown) (25). However, at 4 days post i.p. challenge with mBSA, there were differences in the exudate populations between GM-CSF−/− and C57BL/6 mice, in line with what was observed with the morphologic analysis. These differences are described below for the myeloid cell-containing regions defined above, namely R1, -2, and -4.

**R1 (macrophage rich).** On day 4 post-mBSA challenge, only 25 ± 2% of the exudate cells from GM-CSF−/− mice were found in R1 compared with 39 ± 2% of exudate cells from C57BL/6 mice ($p < 0.001$) (Fig. 4). The reduced percentage of cells in R1 from GM-CSF−/− mice represented a significant reduction in the number of cells in this region (2.2 ± 0.2 vs 9.4 ± 0.6 × 10⁶; $p < 0.001$; GM-CSF−/− vs C57BL/6 mice, respectively). Also, the majority of the R1 cells from the GM-CSF−/− exudates were smaller and less granular. As discussed above, we have previously shown (25) that the cells in R1 from C57BL/6 mice are predominantly...
macrophages (Mac-1\textsuperscript{high}c-Fms\textsuperscript{hi}F4/80\textsuperscript{hi})(Table I). In GM-CSF mice, the cells in R1 were also Mac-1\textsuperscript{hi}(Table I); however, a lower percentage of cells were positive for c-Fms and F4/80 (Table I). In C57BL/6 mice, R1 cells had high levels of Mac-1 expression (Mac-1\textsuperscript{high}), whereas in GM-CSF mice, only 66±4% of cells were Mac-1\textsuperscript{high}, the rest (30±4%) having lower levels of Mac-1 expression (Mac-1\textsuperscript{low}) and being similar to those seen on cells in R2 (see below) (25). Also, by forward-side scatter, the Mac-1\textsuperscript{low} cells were less granular than the Mac-1\textsuperscript{high} cells. Therefore, to aid further analysis of the R1 population in GM-CSF mice, the Mac-1\textsuperscript{high} and Mac-1\textsuperscript{low} populations were arbitrarily subdivided into R1a and R1b regions, respectively, for these mice (Fig. 4).

Mac-1\textsuperscript{high} (R1a) cells from GM-CSF mice were predominantly c-Fms\textsuperscript{hi}F4/80\textsuperscript{hi} and B220\textsuperscript{lo}CD19\textsuperscript{lo}IgK\textsuperscript{lo}(Table II), consistent with a macrophage phenotype and similar to R1 cells in C57BL/6 mice (see Table I). In contrast, Mac-1\textsuperscript{low} (R1b) cells from GM-CSF mice were predominantly c-Fms\textsuperscript{lo}F4/80\textsuperscript{lo} but B220\textsuperscript{hi}CD19\textsuperscript{hi}IgK\textsuperscript{hi}(Table II), and hence similar to B1 lymphocytes found in R2 (see below) (25). The presence of this latter population accounted for the lower overall percentage of R1 cells positive for c-Fms and F4/80 and higher percentage of R1 cells positive for B220, CD19, and IgK in GM-CSF mice (Table I).

Ly-6C (ER-MP20) and ER-MP58, which are expressed on immature cells of the macrophage lineage (26, 27), were present on a very low proportion of R1 cells for GM-CSF mice, as we reported previously for C57BL/6 mice (data not shown) (25). All R1 cells were negative for the PMN marker, Ly-6G, and the DC marker, CD11c (data not shown) (25).

![FIGURE 2](image_url)

**FIGURE 2.** The development of mBSA-induced peritonitis in GM-CSF\textsuperscript{-/-} vs C57BL/6 mice. A, Total peritoneal cell numbers over time. B, No. PMN (x10\textsuperscript{6}). C, No. macrophages (x10\textsuperscript{6}). D, Diff-Quik stains of 4-day mBSA-induced exudate cells. For A–C, results are expressed as the mean ± SEM for four independent experiments.
In conclusion, the fewer macrophages present in the 4-day post-
mBSA exudate from GM-CSF−/− mice are smaller and less granular; also, a population of B1 lymphocytes appears to be more prominent (see Discussion).

**R2 (macrophage and B1 lymphocyte rich) and R4 (eosinophil rich).** We have previously reported (25) for C57BL/6 mice, on day 4 post-mBSA challenge, that, in R2, both macrophages (c-Fms+ F4/80+ Mac-1+ bw) and B1 lymphocytes (Mac-1+ bw B220+ CD19− IgK−) are present. We now find, at this time, there appeared to be an increase in the percentage of cells in this region for GM-CSF−/− cells compared with C57BL/6 mice (26 ± 1 vs 20 ± 2%, respectively; p = 0.05) (Fig. 4). In GM-CSF−/− mice, there was a lower percentage of c-Fms+ cells in R2 from 4-day mBSA-induced exudates compared with C57BL/6 mice (17 ± 2 vs 34 ± 4%, respectively; p < 0.05) equating to a significant reduction in the number of macrophages in this region (0.4 ± 0.1 vs 1.7 ± 0.2 (×106), GM-CSF−/− vs C57BL/6 mice, respectively; p < 0.001) and accounting for the reduced cell number (2.6 ± 0.1 vs 4.3 ± 0.9 (×106), respectively; p < 0.001). In contrast, in GM-CSF−/− mice, there was a higher percentage of B220+ cells in R2 from 4-day mBSA-induced exudates compared with C57BL/6 mice (48 ± 4 vs 22 ± 4%; p < 0.001), which equates to similar numbers of B1 lymphocytes in this region for GM-CSF−/− and C57BL/6 mice (1.25 ± 0.2 vs 0.9 ± 0.2 (×106), respectively).

By morphology, cells in R4 were previously found to be predominately eosinophils in C57BL/6 mice (25), and this is also the case for GM-CSF−/− mice. They represent 7 ± 1 and 9 ± 1% of the exudate populations from C57BL/6 (Fig. 4A) and GM-CSF−/− mice (Fig. 4B), respectively. However, even though somewhat variable, the eosinophil numbers post-mBSA challenge in GM-CSF−/− mice were overall less than those observed in C57BL/6 mice (data not shown), leading to a higher proportion of macrophages in R4 on day 4 post-mBSA challenge as judged by c-Fms expression (37 ± 4 vs 25 ± 5%; p = 0.05; GM-CSF−/− vs C57BL/6 mice, respectively).

**MHC class II and integrin expression**

There was MHC class II expression on both macrophages and B lymphocytes. There was no significant difference in the percentage of R1 cells expressing class II following mBSA-induced peritonitis in GM-CSF−/− mice compared with C57BL/6 mice (45 ± 10 vs 54 ± 15%, GM-CSF−/− vs C57BL/6, respectively) (Table I). There was a similar percentage of macrophages (R1a) and the B lymphocytes (R1b) from GM-CSF−/− mice positive for MHC class II (Table II), and they expressed similar levels.

Given that the absence of GM-CSF results in fewer macrophages being present in mBSA-induced exudates, we examined integrin expression because of their likely involvement in trafficking/adhesion. In R1, there was a trend for fewer ICAM+ cells in GM-CSF−/− mice (45 ± 10 vs 54 ± 15%, GM-CSF−/− vs C57BL/6, respectively) (Table I). In GM-CSF−/− mice, the ICAM+ cells were present in R1a; cells in R1b were essentially ICAM− (Table II). Similar observations were found in R2 (data not shown). For LFA-1, there was a significant increase in the percentage of R1-positive cells from GM-CSF−/− mice (48 ± 5 vs 31 ± 9%; p < 0.05; GM-CSF−/− vs C57BL/6 mice, respectively) (Table I). This was due to a higher percentage of B1 lymphocytes (R1b cells) expressing LFA-1 compared with macrophages (R1a cells) from GM-CSF−/− mice (Table II). When positive, the level of expression of LFA-1 on R1 macrophages from C57BL/6 mice was higher per cell than for GM-CSF−/− mice (data not shown).

In summary, for these markers, the major difference in R1 seems to be in LFA-1 expression, reflecting the increased proportion of B1 lymphocytes.

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**Table I. Peritoneal cell surface marker distribution within R1**

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>C57BL/6</th>
<th>GM-CSF−/−</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Fms</td>
<td>72 (4)</td>
<td>57 (3)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>F4/80</td>
<td>70 (7)</td>
<td>55 (6)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Mac-1</td>
<td>98 (1)</td>
<td>96 (3)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>B220</td>
<td>14 (4)</td>
<td>22 (2)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>CD19</td>
<td>11 (2)</td>
<td>36 (4)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>IgK</td>
<td>10 (3)</td>
<td>33 (3)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>MHC class II</td>
<td>80 (6)</td>
<td>75 (9)</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>54 (15)</td>
<td>45 (10)</td>
<td></td>
</tr>
<tr>
<td>LFA-1</td>
<td>31 (9)</td>
<td>48 (5)</td>
<td>p &lt; 0.05</td>
</tr>
</tbody>
</table>

*Percentage of positive cells for various cell surface markers in R1 from 4-day mBSA-induced exudates from C57BL/6 and GM-CSF−/− mice. R1 (macrophage-rich region) is defined according to c-Fms, F4/80, and Mac-1 expression, as well as by the forward-side scatter profiles from C57BL/6 mice (see Fig. 4). R1 as a percentage of total peritoneal cells: C57BL/6, 39 ± 2%; GM-CSF−/−, 25 ± 2%. Data are the mean (SEM) from four experiments. p values, C57BL/6 vs GM-CSF−/− mice.
4-day mBSA-induced exudate cells from GM-CSF response to LPS. Following LPS stimulation in vitro (4 and 24 h), MCP-1, and IL-10 production from peritoneal exudate cells in re-
and GM-CSF natants from 4-day mBSA-induced exudate cells from C57BL/6 fi
ced T cells were incubated mBSA-induced peritoneal exudates were also able to stimulate /H11002 Ag-speci
fi
induced exudates were not (25). As shown above (Fig. 3), the geneic T lymphocytes, whereas macrophages from 4-day TM-
mBSA-induced peritoneal exudates were able to stimulate allo-
We previously reported that C57BL/6 macrophages from 4-day mBSA-induced exudates from GM-CSF fi
/HC11002 mice. R1 cells from GM-CSF /H11002 mice (see Table I) were divided into Mac-1
Mac-1low (R1b) of GM-CSF /H11002 macrophages from mBSA-induced GM-CSF /H11002 /H9252 /H11002 /H9251 peritonitis (data not shown). However, there was no signi
fi
macrophages to up-regulate in inflammatory cytokine production in
GM-CSF, by itself, is a poor stimulator of the commonly secreted cytokines; however, it can prime, in vivo and in vitro, monocytes/ macrophages to up-regulate inflammatory cytokine production in response to a stimulus such as LPS (2, 3, 7, 8). Therefore, we measured the GM-CSF dependence of IL-6, IL-1β, TNF-α, MCP-1, and IL-10 production from peritoneal exudate cells in re-
response to LPS. Following LPS stimulation in vitro (4 and 24 h), 4-day mBSA-induced exudate cells from GM-CSF
mice secreted less TNF-α, IL-1β, MCP-1, and IL-6 compared with 4-day mBSA-induced exudate cells from C57BL/6 cells (Fig. 6A). How-
ever, there was no significant difference in the production of the anti-inflammatory cytokine IL-10 (Fig. 6A).

We next determined whether the macrophages were the major source of IL-1β, IL-6, TNF-α, and MCP-1 in the LPS-stimulated exudates. First, 4-day mBSA-induced exudates, following LPS stimulation in vitro for 24 h, were stained for Mac-1 and intracellular IL-6 (Fig. 6A). As shown above (Fig. 3), the Ag-specific T cell response to mBSA developed normally in C57BL/6 mice when APC from the spleen were used. We
TABLE II. Peritoneal cell surface marker distribution within R1a and R1b of GM-CSF /H11002 mice

<table>
<thead>
<tr>
<th>R1a (Mac-1&lt;sup&gt;high&lt;/sup&gt;)</th>
<th>R1b (Mac-1&lt;sup&gt;low&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mac-1&lt;sup&gt;high&lt;/sup&gt;</td>
<td>94 (3)</td>
</tr>
<tr>
<td>Mac-1&lt;sup&gt;low&lt;/sup&gt;</td>
<td>8 (4)</td>
</tr>
<tr>
<td>c-Fms</td>
<td>77 (4)</td>
</tr>
<tr>
<td>F4/80</td>
<td>75 (5)</td>
</tr>
<tr>
<td>B220</td>
<td>6 (2)</td>
</tr>
<tr>
<td>CD19</td>
<td>10 (7)</td>
</tr>
<tr>
<td>IgK</td>
<td>7 (4)</td>
</tr>
<tr>
<td>MHC class II</td>
<td>73 (4)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>48 (6)</td>
</tr>
<tr>
<td>LFA-1</td>
<td>37 (7)</td>
</tr>
</tbody>
</table>

* Percentage of positive cells for various cell surface markers in R1a and R1b from 4-day mBSA-induced exudates from GM-CSF /H11002 mice. R1 cells from GM-CSF /H11002 mice (see Table I) were divided into Mac-1<sup>high</sup> (R1a) and Mac-1<sup>low</sup> (R1b) (see Fig. 4). The percentages of total R1 cells were as follows: R1a, 66 ± 4%; R1b, 30 ± 4%. Data are the mean (SEM) from four experiments.

Phagocytosis

R1a macrophages from mBSA-induced GM-CSF /H11002 exudates were just as capable as the corresponding R1 macrophages from C57BL/6 exudates at phagocytosing fluorescent latex beads when monitored by flow cytometry (Fig. 5A) and fluorescence microscopy (data not shown). However, they were less efficient at ingesting multiple beads (Fig. 5A), an observation in line with their smaller size. R1b cells (B1 lymphocytes) from GM-CSF /H11002 mice were not phagocytic.

Mixed leukocyte reaction

We previously reported that C57BL/6 macrophages from 4-day mBSA-induced peritoneal exudates were able to stimulate allogeneic T lymphocytes, whereas macrophages from 4-day TM-induced exudates were not (25). As shown above (Fig. 3), the Ag-specific T cell response to mBSA developed normally in GM-CSF /H11002 mice when APC from the spleen were used. We next determined whether GM-CSF /H11002 macrophages from 4-day mBSA-induced peritoneal exudates were also able to stimulate allogeneic T lymphocytes. When purified T cells were incubated with equal numbers of GM-CSF /H11002 R1a macrophages from 4-day mBSA-induced exudates, they were able to proliferate (Fig. 5B); however, their proliferative capacity was reduced compared with that of comparable numbers of R1 macrophages from C57BL/6 mice (Fig. 5B). As for C57BL/6 macrophages from 4-day TM-induced exudates, GM-CSF /H11002 R1a macrophages from 4-day TM-induced exudates did not induce T cells to proliferate (Fig. 5B).

Cytokine production from 4-day mBSA-induced exudate cells

TNF-α, IL-1β, IL-6, MCP-1, and IL-10 could not be detected in peritoneal exudate lavage fluid following either 4-day mBSA- or 4-day TM-induced peritonitis (data not shown). However, low levels of IL-1β, IL-6, and MCP-1 could be detected in culture supernatants from 4-day mBSA-induced exudate cells from C57BL/6 and GM-CSF /H11002 mice (Fig. 6A), with IL-1β levels being significantly higher in C57BL/6 vs GM-CSF /H11002 culture supernatants. GM-CSF, by itself, is a poor stimulator of the commonly secreted cytokines; however, it can prime, in vivo and in vitro, monocytes/ macrophages to up-regulate inflammatory cytokine production in response to a stimulus such as LPS (2, 3, 7, 8). Therefore, we measured the GM-CSF dependence of IL-6, IL-1β, TNF-α, MCP-1, and IL-10 production from peritoneal exudate cells in re-
spontaneous LPS. Following LPS stimulation in vitro (4 and 24 h), 4-day mBSA-induced exudate cells from GM-CSF /H11002 mice secreted less TNF-α, IL-1β, MCP-1, and IL-6 compared with 4-day mBSA-induced exudate cells from C57BL/6 cells (Fig. 6A). How-

FIGURE 5. Functional capacity of macrophages from 4-day mBSA- and 4-day TM-induced peritonitis exudates in GM-CSF /H11002 vs C57BL/6 mice. A, Phagocytic capability of R1 (C57BL/6 mice) or R1a (GM-CSF /H11002 mice) cells. Results are expressed as the percentage of cells capable of phagocytosing the latex beads (total), as well as the percentage capable of phagocytosing ≤3 or >3 beads. Results are expressed as the mean ± SEM from three independent experiments. Note that R1b cells from GM-
/C57BL/6 were not phagocytic. B, Lymphocyte stimulatory ability of macrophages. Sorted macrophage populations (R1 for C57BL/6, or R1a for GM-CSF /H11002 mice) (2 × 10<sup>5</sup> cells) (see Materials and Methods) were incubated with allogeneic splenic T cells (2 × 10<sup>5</sup> cells) for 96 h at 37°C and 5% CO<sub>2</sub>. Cultures were pulsed with [<sup>3</sup>H]TdR for the last 16 h. Control cultures contained medium or T cells alone. Results are expressed as the amount of [<sup>3</sup>H]TdR incorporation (mean ± SEM) from eight independent samples from two experiments. *, p < 0.001, GM-CSF /H11002 vs C57BL/6. **, p < 0.01, GM-CSF /H11002 vs C57BL/6.
cytokines, and that the percentage of macrophages present in 4-day mBSA-induced exudates was lower in GM-CSF−/− mice compared with C57BL/6 mice. However, as shown for IL-6 (Fig. 6C), there was less accumulation of this cytokine in the macrophages from GM-CSF−/− mice, as measured by the GMFI, compared with the macrophages from C57BL/6 mice (GMFI, 35 ± 6 vs 16 ± 5; p = 0.03; GM-CSF−/− vs C57BL/6, respectively).

Additionally, to demonstrate that the macrophages from GM-CSF−/− exudates were in fact secreting less cytokines per cell than the macrophages from C57BL/6 exudates, R1 exudate cells from C57BL/6 and GM-CSF−/− mice were sorted before in vitro LPS stimulation. *p < 0.01, GM-CSF−/− vs C57BL/6, **p < 0.05, GM-CSF−/− vs C57BL/6.
shown in Fig. 6D, the macrophages from 4-day mBSA-induced exudates from GM-CSF−/− mice secreted significantly less TNF-α, IL-1β, MCP-1, and IL-6 following LPS stimulation compared with the macrophages from 4-day mBSA-induced exudates from C57BL/6 mice. The non-R1 and non-R1a cells, collected during the sorting for the macrophages, were also stimulated with LPS. Very low levels of cytokines could be detected in the supernatants, most likely arising from some contaminating macrophages (data not shown). Once again, there was no difference in the amount of IL-10 secreted by the macrophages from GM-CSF−/− vs C57BL/6 mice following in vitro LPS stimulation (Fig. 6D).

By way of comparison, following LPS stimulation in vitro (24 h), 4-day TM-induced exudate cells from GM-CSF−/− mice secreted less MCP-1 compared with 4-day mBSA-induced exudate cells from C57BL/6 cells (6.0 ± 0.4 vs 1.1 ± 0.03, C57BL/6 vs GM-CSF−/−, respectively; p < 0.01). However, there were no differences between the levels of IL-6, TNF-α, and IL-1β secreted from the exudate cells from these mice (data not shown).

Discussion
GM-CSF has been shown in mice to be critical for the pathogenesis of a number of Ag-driven autoimmune/inflammatory diseases (18–23). It can also exacerbate this type of condition in humans (32). In inflammation models relying on Ag priming and challenge to initiate the effector phase, the absence of GM-CSF, or reduction in its levels, resulted in a dramatic reduction in the cellular infiltrate (18, 20, 21), GM-CSF activity could be important at either or both of these Ag-dependent stages. Data from these disease models suggest that it is unlikely that inefficient activation of T cells by DC in the periphery would account for the observed resistance to disease in GM-CSF−/− mice (18, 20, 21), and data using timed addition of a neutralizing anti-GM-CSF mAb in both the collagen-induced arthritis (20) and experimental autoimmune encephalomyelitis (21) models were consistent with the key function(s) of GM-CSF being at the later (effector) stage, i.e., following Ag challenge. Such a function(s) is subsequent to T cell activation, and most probably affects mediators of inflammation (e.g., cytokines). Inflammatory mediator levels were lowered at the sites of inflammation when GM-CSF was absent or its levels lowered (20). However, from these earlier studies, the cellular composition, as well as the relative contribution of GM-CSF to cell number and/or activation, could not be easily delineated.

Prior studies involving TM-induced peritonitis in GM-CSF−/− mice failed to find a role for GM-CSF, leading to the conclusion that it is not involved in sterile peritonitis (24); other studies examining the peritoneal cavity response in these mice were complicated by the use of infectious organisms as a stimulus for the peritoneal reaction (33, 34). The above findings indicate that our Ag-induced peritonitis model (25) is a convenient one that allows us to explore the role of GM-CSF in Ag-driven autoimmune/inflammatory diseases at a cellular and molecular level. In contrast to TM injection, the mBSA-induced peritonitis in GM-CSF−/− mice was more different to that found in wild-type mice. Although a normal mBSA-specific T cell response developed in GM-CSF−/− mice following mBSA-induced peritonitis, the inflammatory reaction in the peritoneal cavity was severely compromised, reminiscent of what has been described in a number of murine models of disease (18–23). There was an inability to sustain PMN, macrophage, and eosinophil numbers in GM-CSF−/− mice, resulting in a large decrease in the number of inflammatory cells present in these mice. These reduced myeloid cell numbers are consistent with GM-CSF being involved in myeloid cell survival, or even local proliferation, thus maintaining cells at the site of inflammation (35–37).

In addition to an effect on their numbers, the macrophages present in GM-CSF−/− mice following mBSA-induced peritonitis were smaller and less granular overall, less efficient at phagocytosis and at stimulating an MLR, and secreted less proinflammatory mediators in vitro in response to LPS. They also expressed lower levels of LFA-1, although Mac-1, MHC class II, and ICAM-1 expression levels were similar to wild-type mice. These differences suggest that GM-CSF is also involved in the priming/activation of the inflammatory cells within the inflamed peritoneum. There are a number of reports implicating GM-CSF in priming/activating cells for heightened immune/inflammatory responses (2, 3, 6–10, 35, 38, 39). One such consequence is that they produce more inflammatory cytokines, such as IL-1, IL-6, TNF-α, and MCP-1. Therefore, a lack of GM-CSF results in less of these cytokines being produced and, consequently, reduced numbers of cells at the inflammatory site, as the current results show in the mBSA-induced peritonitis model in GM-CSF−/− mice. These effects would not appear to be due to an increased production of the anti-inflammatory cytokine IL-10 in macrophages from GM-CSF−/− mice following LPS stimulation.

GM-CSF has been implicated in APC development (6, 14), and it has been reported that the source of the APC is important in determining whether an impaired T cell proliferative response is seen to a specific Ag in GM-CSF−/− mice (13). GM-CSF is also the most commonly used cytokine for in vitro differentiation of DC (14, 15); however, GM-CSF−/− mice have only slightly reduced numbers of DC (40), thus raising doubt as to its in vivo role in the generation of DC. In support of this, it has been pointed out in a recent review that in vitro DCs, which are often generated with protocols involving GM-CSF, can be quite different from their in vivo counterparts, suggesting that there may be other cytokines that are important in vivo, e.g., Flt3 ligand (41). Our results above show that GM-CSF is not involved in the development of the APC in the spleen responsible for presentation of mBSA to primed T cells. We also found a similar result with type II collagen as the Ag (20). However, when the mBSA-induced peritoneal macrophages from GM-CSF−/− mice were used in an MLR, they were less efficient at stimulating T cell proliferation compared with the mBSA-induced peritoneal macrophages from C57BL/6 mice. We have previously shown that the mBSA-induced macrophages from C57BL/6 mice appear to take on a DC-like role, expressing higher levels of class II and being efficient stimulators of allogeneic MLR (25); however, they do not appear to be fully differentiated DC based on morphology or phenotype, and they still retain macrophage functions. Thus, although the mBSA-induced peritoneal macrophages from C57BL/6 were capable of stimulating T cell proliferation, those from GM-CSF−/− mice were less effective, in line with the cells being less efficient in a number of other functional assays described above.

Apart from the dramatic decrease in the macrophage response, 4-day mBSA-induced exudates from GM-CSF−/− mice also contained a population of cells that were large but not very granular (R1b cells; Fig. 4B). By cell surface marker analysis, these were deemed to be B1 lymphocytes. They were larger than the B1 lymphocytes found in the unstimulated peritoneal cavity where they are a major population of B cells (42). It has previously been suggested that B1 lymphocytes and certain macrophage populations may have a common progenitor (43). It is possible that, in the absence of GM-CSF, there is a block in macrophage lineage development, thereby favoring B1 lymphocyte progression at the expense of the macrophage. In this connection, s.c. administration of GM-CSF led to an increase in the numbers of CD5+ macrophages in the peritoneal cavity (43). In vitro studies support this concept (43, 44).

In summary, TM-induced peritonitis has been the standard method for providing large numbers of elicited macrophages for...
study and for determining the importance of a particular mediator(s) in the development of an inflammatory reaction. As we previously suggested (25), this type of response may not be relevant to many inflammatory diseases, particularly where an acquired immune response is important. We showed above that, for sterile murine peritonitis, a GM-CSF dependence was observed only for an Ag-specific response, involving priming and challenge. An important general conclusion from our studies here is that any assessment of the inflammatory mediator(s) critical for the maintenance and progression of an inflammatory reaction must take into account the type of stimulus or insult that the host is responding to. We contend that the results presented in this paper contribute to our understanding of the suppressed inflammatory response observed under conditions of GM-CSF deprivation in various inflammatory/autoimmunity models (18–23).

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References


