Tumor necrosis factor (TNF) is a protein produced by activated macrophages in response to endotoxin. The effect of recombinant murine TNF (rMuTNF) on the growth of murine tissue-derived macrophage colony-forming units (CFU-M) which are responsive to both macrophage and granulocyte-macrophage colony-stimulating factors (M-CSF and GM-CSF), was studied. TNF alone did not stimulate macrophage proliferation but did prolong their survival in vitro. The proliferative response of CFU-M to M-CSF, however, was greatly enhanced by the presence of TNF. The enhancement effect of TNF is dose-dependent, reaching a maximum at approximately 50 U/mL. In contrast, the proliferative responsiveness of CFU-M to GM-CSF was inhibited by the concurrent addition of rMuTNF. Both effects appear to be caused directly by rMuTNF, rather than by the secondary factor(s) produced by TNF-treated macrophages. TNF treatment also induced a transient downmodulation of M-CSF receptors in cultured macrophages and accelerated their uptake and use of exogenous M-CSF, which may account for, at least in part, the enhanced proliferative activity in response to M-CSF. Short-term treatment (24 hours) was not sufficient to induce either an enhancing or an inhibitory effect upon CFU-M. This study suggests an autoregulatory role for TNF in the production of mature tissue macrophages by selectively enhancing their proliferative response to lineage specific growth factor, M-CSF.

MATERIALS AND METHODS

Mice. Male or female C3H/HeJ mice 8 to 12 weeks of age were obtained from Jackson Laboratory (Bar Harbor, ME). Cells obtained from this strain of mice are refractory to the stimulation of endotoxin in vitro. All mice were fed standard lab chow and water ad libitum.

CSF and reagents. Murine recombinant GM-CSF (rMuGM-CSF) was a generous gift of Dr S. Gillis of Immunex Co (Seattle, WA). Recombinant murine TNF (specific activity, greater than 4 x 10^7 U/mg protein) and recombinant human IL-6 (specific activity, 10^7 U/mg protein) were obtained from Genzyme Co (Boston, MA). TNF activity was assayed on L929 cells in the presence of actinomycin D, as described previously. Recombinant human IL-1 alpha and beta (specific activity, 10^7 U/mg protein) were kindly provided by Biogen Co (Geneva, Switzerland). Highly purified recombinant human M-CSF and IL-2 were generously provided by Cetus Co (Emeryville, CA). Mouse L-cell M-CSF was purified by a five-step procedure described previously. The biologic activity of both GM-CSF and M-CSF was determined from the linear portion of the dose-response curve, assigning 50 U/mL to the concentration and
causing the formation of 50% of maximal colony numbers when 5 x 
10^5 bone marrow cells were cultured in 1 mL of soft agar.4

Cells. Free pulmonary alveolar macrophages (PAM) were ob-
tained by pulmonary lavage using a modified method of Brain and
Frank.14 Bone marrow cells were obtained from femoral shafts by
flushing with 3 mL alpha modified Eagle’s medium (MEM) contain-
ing 10% fetal calf serum (Sterile Systems, Inc, Logan, UT)
(alpha-10). The cell suspensions were passed up and down five times
through an 18 g needle in alpha-10 to disperse cell clumps.
Peritoneal exudate macrophages (PEM) were harvested by peri-
toneal lavage with 5 mL alpha-MEM 3 days after one intraperitoneal
injection of 1 mL of thioglycolate medium.

Assay for colony-forming cells. The assay for bone marrow
CFU-GM and tissue CFU-M was carried out exactly according to
the method described elsewhere.4,5,15

Cell counting. The number of adherent macrophages was deter-
mined by the cetrimide counting technique described previ-
ously.13 Briefly, cultures were depleted of medium by aspiration, washed
once with warm phosphate-buffered saline (PBS), and replenished
with 1 mL warm cetrimide solution (30 g cetrimide, 0.37 g disodium
EDTA, and 8.5 g NaCl in 1,000 mL water, pH 5.0) at 37°C for 3
minutes. This was followed by a thorough rinsing with warm
cetrimide solution (final volume, 10 mL per culture) to completely
remove and lyse the adherent cells. This treatment readily lysed
the adherent cells and liberated intact nuclei, which were then counted
by a Coulter counter (Model ZM; Coulter Electronics, Hialeah,
FL).

Iodination of M-CSF and receptor binding assay. The iodina-
tion of L-cell or recombinant human M-CSF and the details of the
binding assay for M-CSF receptors have been described in detail
previously.15 Nonspecific binding, determined as the amount of
radioactivity bound in the presence of 200-fold excess of unlabeled
M-CSF, was always less than 3% of the total binding.

RESULTS

Effect of rMuTNF on CFU-M proliferation induced by
GM-CSF and M-CSF. We have shown previously that
both GM-CSF and M-CSF alone stimulate the macrophage
proliferation by tissue-derived CFU-M. The proliferative
responses to GM-CSF and M-CSF, however, differ in several
aspects.6,8 As shown in Fig 1, the proliferative rate of
CFU-M in response to GM-CSF was faster with a shorter
lag period than in response to M-CSF. The morphology of
progeny also differs remarkably; macrophage progeny de-

Fig 1. Growth kinetics of C3H/HeJ PEM (500 cells per well) and
PAM (500 cells per well) in response to rMuGM-CSF (1,000
U/mL), M-CSF (1,000 U/mL), and rMuTNF (50 U/mL). At the time
indicated, the number of adherent macrophages per culture was
determined by cetrimide-Coulter counting method. Data are means
of duplicate cultures from one typical experiment.

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determined by cetrimide-Coulter counting method. Data are means
of duplicate cultures from one typical experiment.

markedly derived from GM-CSF stimulated cultures are small and
round, while those derived from M-CSF stimulated cultures are
usually stretched and elongated.6 TNF alone did not
support the long-term growth of CFU-M. However, the cells
in its presence survived longer than those in the presence of
medium alone, in which both PEM and PAM gradually died
within 7 to 10 days. Concurrent addition of rMuTNF in
culture greatly enhanced the proliferative response of tissue
CFU-M to M-CSF in a dose-dependent manner, reaching a
maximal effect at between 50 and 100 U/mL of rMuTNF
(Fig 2). CFU-M also commenced proliferation earlier and
proliferated with a much shortened cell doubling time than
those in the presence of M-CSF alone (Table 1). Conversely,
the proliferative response to rMuGM-CSF by CFU-M was
inhibited in its presence in a dose-dependent manner as well
(Fig 2). Interestingly, the proliferative capacity of CFU-M
in response to GM-CSF was not completely inhibited, even in
the presence of the highest dose of rMuTNF used in this
study (500 U/mL). Cells appeared to proliferate at a much
reduced rate, as evidenced by the prolonged cell doubling
time (Table 1).

Microscopic examination of cultures in the presence of
both M-CSF and rMuTNF revealed giant macrophage
colony formation (Fig 3). The number of colonies from
cultures containing both M-CSF and rMuTNF is essentially
the same as the control culture containing M-CSF alone
(data not shown). On the other hand, the size of colonies
from cultures containing GM-CSF was much reduced in the
presence of rMuTNF. When CFU-M were exposed to
rMuTNF for a short period (1 to 24 hours) followed by
incubation in the presence of either M-CSF or rMuGM-CSF
alone, neither enhancing nor inhibitory effects were ob-

TNF directly regulates macrophage proliferation. We
have shown earlier that GM-CSF, at low concentrations,
potentiates the growth capacity of CFU-M to M-CSF. Since
both TNF and IL-1 are known to stimulate the production of
GM-CSF by a number of target cells,9,13 we investigated
whether the enhancing effect of TNF seen in this study may
be caused indirectly through the production of GM-CSF or
other factors by macrophages. Both PEM and PAM (10^5 per
dish) were cultured in the presence of rMuTNF (10 to 100
U/mL) for various time periods. Thereafter, culture superna-
tants were assayed for both CSF and enhancing activities.
None of these activities were detected in the culture superna-
tants of treated macrophages. Furthermore, the enhancing
effect of TNF appears to be specific; both IL-1 alpha and
beta were unable to cause a synergistic response in PAM (Fig

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EFFECT OF TNF ON MACROPHAGE PROLIFERATION

was calculated by the following formula: 

\[ D = \frac{T \times \log(N_2/N_1)}{72} \]

where \( N_1 \) and \( N_2 \) are the number of cells at day 7 and day 10, respectively, and \( T \) = 72 hours. Data represent means ± SD from four (M-CSF) or three (rMuGM-CSF) identical experiments.

4), despite the many overlapping activities of TNF and IL-1.13

TNF enhances the use of M-CSF. Several lines of evidence suggest that various receptor systems on hematopoietic cells are linked, through which different types of receptors may interact with each other.16 This notion prompted us to examine whether the modulatory effect of TNF may be initiated at the receptor level. The interaction between

Fig 2. Effect of rMuTNF on the growth of alveolar CFU-M and peritoneal exudate CFU-M in response to M-CSF and rMuGM-CSF. C3H/HeJ PAM and PEM (500 cells per well) were cultured in the presence of either M-CSF (1,000 U/mL) or rMuGM-CSF (1,000 U/mL) and various concentrations of rMuTNF as indicated. Data represent means ± SD from three identical experiments.

Table 1. Effect of rMuTNF on the Proliferation of Alveolar CFU-M

<table>
<thead>
<tr>
<th>Addition</th>
<th>Dose (U/mL)</th>
<th>Cell Doubling Time (h)</th>
<th>P Value (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-CSF</td>
<td>1,000</td>
<td>54.3 ± 10.8 (&lt;.01)</td>
<td></td>
</tr>
<tr>
<td>rMuGM-CSF</td>
<td>1,000</td>
<td>24.7 ± 1.9 (&lt;.05)</td>
<td></td>
</tr>
</tbody>
</table>

C3H/HeJ alveolar macrophages (500 cells per well) were cultured in the presence of L-cell M-CSF and rMuGM-CSF with or without 50 U/mL rMuTNF. Cell doubling time (D) from exponentially growing macrophages was calculated by the following formula: 

\[ D = \frac{T \times \log(N_2/N_1)}{72} \]

where \( N_1 \) and \( N_2 \) are the number of cells at day 7 and day 10, respectively, and \( T \) = 72 hours. Data represent means ± SD from four (M-CSF) or three (rMuGM-CSF) identical experiments.

DISCUSSION

The experiments described in this report were undertaken to study the role of TNF in the regulation of macrophage production. Earlier studies have shown that a subpopulation of tissue macrophages, known as CFU-M, can be stimulated by either GM-CSF or M-CSF to undergo long-term proliferation in vitro.44 Nevertheless, macrophage progeny generated in cultures containing GM-CSF are different from those generated in cultures containing M-CSF in several aspects. The biologic significance of such population "heterogeneity" is not fully understood at present. Evidence from previous studies suggests that M-CSF-derived progeny may represent a more mature population directly descending from that of GM-CSF-derived progeny.618 This interpretation is further supported by the present findings that TNF selectively potentiates the proliferative response of CFU-M to M-CSF but, at the same time, inhibits their proliferative response to GM-CSF. As a result, TNF may regulate macrophage production by directing their proliferative responsiveness from a less restricted CSF to a lineage-specific CSF, thus enhancing the generation of more mature macrophages. Consistent with this view, we have recently shown that GM-CSF-derived progeny remain highly proliferative in response to M-CSF, but M-CSF-derived progeny become
poorly responsive to GM-CSF. The enhanced proliferation by TNF-treated CFU-M appears to be a result of shortened cell doubling time as evidenced by the kinetic study and the formation of giant macrophage colonies in cultures containing both M-CSF and rMuTNF (Table I, Fig 3).

Both enhancing and inhibitory activities seen in this study appear to be caused by a direct action of TNF on the target cells, rather than by a secondary factor(s) produced by treated macrophages. We could detect neither CSF activity nor enhancing activity in culture supernatants of TNF-treated macrophages. Nor could we monitor any inhibitory activity responsible for the reduced proliferative response to GM-CSF. Furthermore, IL-1, a monokine known to share several biologic activities with TNF, has no significant effect on the proliferative capacity of CFU-M. Our finding that TNF induces a rapid and transient downregulation of M-CSF receptor also supports this notion and further suggests that the action of TNF takes place at the level of CSF receptors. This hypothesis seems to be corroborated by recent studies that demonstrate that TNF transiently downregulates M-CSF receptors in bone marrow-derived macrophages and PEM and induces a synergistic response in these cell populations to M-CSF. The molecular basis of receptor transmodulation leading to synergistic response is not understood. The fact that TNF mimics the effect of M-CSF in downregulating M-CSF receptors implies a possible mechanism in which the binding of TNF to its receptor leads to the sequential activation of, among others, c-FMS proto-oncogene, which encodes for M-CSF receptors. We have noted an elevated upregulation of M-CSF receptors after prolonged TNF treatment. Moreover, TNF treatment accelerates the receptor-mediated uptake and use of exogenous M-CSF molecules (Table 2), which may represent the initial events leading to an increased generation of proliferative signaling. An oppo-
EFFECT OF TNF ON MACROPHAGE PROLIFERATION

Fig 5. Transmodulation of M-CSF receptors by rMuTNF. (A) PEM (8 x 10^4 per well) were treated with rMuTNF (10 U/mL) for various time periods at 37°C. The receptor-binding assays were performed as described in Materials and Methods. Data are means ± SD (bars) from two experiments. (B) Binding with various concentrations of 125I-M-CSF. After incubation with TNF for 40 hours, cells were washed and binding assays were performed with various concentrations of 125I-M-CSF as indicated for 24 hours at 4°C. All data are corrected for nonspecific binding (greater than 3% of total binding). Data are means of duplicate cultures from one representative experiment.

pointed out that since the binding of M-CSF to its receptor is essentially irreversible, the conventional Scatchard analysis for the determination of receptor number is invalid in this case. Nevertheless, as discussed earlier, the total number of M-CSF receptor sites can be correctly estimated from the binding curves in which the binding assays were carried out with various concentrations of 125I-M-CSF.

The synergistic effect of TNF observed in this study is not unique to CFU-M. Other have shown that TNF directly enhances both the antigen and mitogen-induced T-lymphocyte proliferation. The effect of TNF on T lymphocytes also appears to be mediated at the receptor level by enhancing the expression of functional IL-2 receptors, thereby facilitating IL-2-dependent T-cell growth. It would be interesting to see if TNF also enhances the proliferative capacity in other cell systems.

This study provides the first in vitro evidence that TNF can influence the proliferative responsiveness of macrophage progenitor cells to various CSFs, by suppressing one in favor of the others. This observation indicates that TNF may participate in the steering of macrophage development by directing them to a specific pathway. The fact that TNF is produced primarily by activated monocytes/macrophages also implies an autoregulatory mechanism of TNF in the expansion of local macrophage population at the inflammatory sites. In supporting this view, it has been shown recently that granuloma formation in the liver of mice infected with BCG coincides with local TNF production. Furthermore, injection of anti-TNF antibody into BCG-infected mice prevents the development of granulomas and subsequent mycobacterial elimination. Evidently, TNF plays a key role in the production of macrophages during the process of granuloma formation, presumably by enhancing their proliferative response to locally produced M-CSF.

REFERENCES

5. Chen BD-M, Mueller M, Olencki T: Interleukin-3 stimulates
the clonal growth of pulmonary alveolar macrophage of the mouse: Role of IL-3 in the regulation of macrophage production outside the bone marrow. Blood 72:685, 1988


Recombinant tumor necrosis factor enhances the proliferative responsiveness of murine peripheral macrophages to macrophage colony-stimulating factor but inhibits their proliferative responsiveness to granulocyte-macrophage colony-stimulating factor

BD Chen and M Mueller